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**Original article**

# CTLA-4 polymorphisms and anti-malarial antibodies in a hyper-endemic population of Papua New Guinea

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**Abstract**

In malaria endemic areas, people naturally acquire an age-related immunity to malaria. Part of this immunity involves anti-malarial specific antibodies. Acquisition of these malaria-specific antibodies depends not only on exposure to malaria parasites but also on the human genetic predisposition. CTLA-4 is a costimulatory molecule that delivers an inhibitory signal to suppress T-cell as well as B-cell responses. We investigated associations between malaria-specific antibody levels and CTLA-4 polymorphisms in 189 subjects living in a hyper-endemic area of Papua New Guinea (PNG), where both *P. falciparum* and *P. vivax* are prevalent. We determined *P. falciparum*/ *P. vivax* specific IgG/IgE levels (Pf-IgG, Pv-IgG, Pf-IgE, Pv-IgE) and polymorphisms in the CTLA-4 gene at position -1661 promoter region (A/G), the +49 exon 1 non-synonymous mutation (A/G), and the +6230 3'-UTR (A/G). All quantified antibody levels were significantly higher in subjects > 5 years (n = 150) than in subjects ≤ 5 years of age (n = 39). In children ≤ 5 years old, significant associations were detected between CTLA-4 +49 (GG/AG vs. AA) and Pv-IgG (median 18.7 vs. 13.7 μg/ml, P = 0.017) and Pv-IgE (266.6 vs. 146.5 pg/ml, *P* = 0.046). No significant difference was observed in subjects > 5 years old. These results suggest that the CTLA-4+49 polymorphism influenced Pv-IgG and Pv-IgE levels among children less than five years old in the studied population, which may regulate the age- and species-specific clinical outcomes of malaria infection.

**Key words:** CTLA-4, IgG, IgE, malaria, polymorphism

## BACKGROUND

Malaria caused by protozoa of the genus *Plasmodium* remains one of the most crucial problems in the global agenda on human health. *P. falciparum*, the most pathogenic species, causes over 1.1 million deaths each year [1], while *P. vivax*, the most widely distributed species, is generally benign but causes anemia and occasionally proves fatal [2].

In malaria endemic areas, immunity to malaria disease and infection is slowly acquired with age and requires regular contact with parasites to persist, antibodies playing a pivotal role in this process [3]. Cohen et al. [4] previously showed that passive transfer of immunoglobulin fractions from immune adults reduced parasitemia in children with acute *P. falciparum* malaria, strongly suggesting the role of malaria specific IgG in immune protection. A recent longitudinal study also documented an association between *P. vivax*-specific IgG level and reduced risk of infection [5]. In contrast, the roles of malaria specific IgE are contradictory. Elevated levels of total and anti-*P. falciparum* IgE have been associated with severe malaria due to overproduction of proinflammatory TNF-α produced by IgE-receptor-bearing effector cells [6], while anti-*P. falciparum* IgE has recently been reported to be associated with protection from subsequent malaria infections in a longitudinal study [7].

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The slow acquisition has been considered to be due in part to the diverse antigenic repertoire of the malaria parasites [8], and it is also suggested to be linked to key features of the immune system that change during normal development and ageing [9].

Recent data [10] showed that the Fulani tribe, despite similar exposure to malaria, is better protected against the disease than other sympatric tribes and that this is associated with elevated anti-malarial antibodies. This suggests the involvement of host immunogenetic factors, such as IL-4 cytokine, in the protection against malaria [11].

Cytotoxic T lymphocyte antigen 4 (CTLA-4) expressed on activated T and B cells [12] is an inducible costimulatory molecule that ligates with CD80 and CD86 on antigen-presenting cells (APC) and delivers an inhibitory signal [13]. The CTLA-4 gene is located on chromosome 2 q33. In experimental studies, engagement of CTLA-4 on B cells has been shown to down-regulate IL-4-driven class switching, leading to diminished IgG1 and IgE production in both humans [14] and mice [15]. Genome-wide screening for genes regulating IgE levels in Dutch patients with asthma produced evidence for the involvement of several genes including CTLA-4 [16].

Some of the Single Nucleotide Polymorphisms (SNPs) in the CTLA-4 gene are known to cause functional alterations. The A allele at +49 A/G non-synonymous mutation (threonine/alanine) in exon 1 has been associated with elevated total-IgE levels [17], with increased expression of CTLA-4 on T cells [18, 19] and with decreased T-cell proliferation upon T cell activation [20]. The G allele at position +6230 A/G in 3' untranslated region (UTR) has been reported to correlate with lower mRNA levels of soluble alternative splicing forms of CTLA-4, leading to the augmentation of T-cell responses [21]. The SNP at -1661 A/G in the promoter region was suspected to affect CTLA-4 gene expression due to the association with type 1 diabetes [22], although no significant association between the -1661 mutation and luciferase transcriptional activity was found [23]. Research on the role of CTLA-4 in the pathogenesis of autoimmune diseases has been conducted quite intensively because of its suppressive effect on cell-mediated immune responses. However, the functional relevance of CTLA-4 on the humoral immune responses, especially in response to infectious agents, has not been well characterized. We hypothesize that CTLA-4 participate in the cell interaction and counter-regulate the class switching, thereby affecting the humoral immune responses in malaria infection.

Immunity to malaria is considered to be species specific [24]. Our study area offers an opportunity to investigate specific immune responses to both *P. falciparum* and *P. vivax*. In this study, we investigated possible associations between the reported functional mutations in the CTLA-4 genes with IgG and IgE antibodies specific for *P. falciparum* and *P. vivax* in individuals living in an area of PNG.

## MATERIALS AND METHODS

### *Study area and subjects*

During the dry season in August 2001, a cross sectional study was conducted in Kiniambu village, East Sepik Province, PNG, where stable transmission of *P. falciparum* and *P. vivax* occurs throughout the year. PNG is estimated to have over 700 languages [25]. Our study population belongs to the Boiken language group, 229 subjects being enrolled in this association study. Among these 229, 40 parasitized individuals (20 each of *P. falciparum* and *P. vivax* cases) were excluded in order to avoid any influence of ongoing infection on the antibody levels. The remaining 189 (male: 89, female: 100) subjects without parasitemia, consisting of 39 (age; 0-5), 48 (6-15), 52 (16-30), and 50 (over 30), were investigated. Informed consent was obtained from all individuals or parents in the case of small children with the assistance of village chiefs, school teachers, and community health workers.

Parasitological diagnosis of malaria was made by microscopy on thin blood smears. Finger-prick blood were drawn into 75 μl heparinized capillary tubes (Drummond Scientific Company, PA, USA) and transferred onto chromatography filter paper (ET31CHR; Whatman, Kent, UK). The dried filter paper was stored at -20℃ until laboratory analysis. Approval for the study was obtained from the National Department of Health Medical Research Advisory Committee of PNG and Tokyo Women's Medical University Ethical Committee.

### *IgG and IgE antibody measurements*

Sera were extracted from a quarter of filter paper blood (19 μl) in phosphate buffered saline containing 0.05% Tween and 0.5% bovine serum albumin by incubating for 2 hours at room temperature on a shaker. The extracts correspond to a serum dilution of 1:100. The extracted sera were diluted 1:1000 (× 10) and 1:100 (× 1) for malaria-specific IgG and IgE detection respectively. *P. falciparum* and *P. vivax*-specific IgG and IgE antibodies were determined by ELISA as previously described [26] using the *P. falciparum* and *P. vivax* crude antigens prepared from the erythrocytic stages of *in vitro* cultured laboratory strain (FC32) [27] and ex-vivo short-term cultured *P. vivax* parasites [28] respectively.

### *DNA extraction and PCR*

DNA was extracted from a quarter of filter paper blood

(19 μl) using the QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN, CA, USA). PCR amplification of CTLA-4 gene fragments containing each target SNPs at -1661 A/G (rs4553808) in promoter region, +49 A/G (rs231775) in exon 1, and +6230 A/G (rs3087243) in 3' UTR was carried out using the following primers yielding 200, 422 and 190 bp fragments respectively: forward 5'-TCAATTCCAGCATTGATCTCAC-3' and reverse 5'-CGCCAACAAGCAATAACAAC-3' for -1661 A/G, forward 5'-TCCAAGTCTCCACTTAGTTATCCA-3' and reverse 5'-GGAGAGTGCAGGGCCAGGT-3' for +49 A/G, and forward 5'-CTTTGCACCAGCCATTACCT-3' and reverse 5'-AGGGGAGGTGAAGAACCTGT-3' for +6230 A/G. These primers were designed using primer 3 software available online. The forward primers for -1661 A/G and +49 A/G and reverse primer for +6230 A/G were biotin labeled. PCR reactions were performed in a 30 μl reaction mixture containing 10 - 20 ng genomic DNA, 1.5 mM MgCl2, 2.5 mM each of dNTP, 5 U of Taq polymerase (TaKaRa Ex Taq HS; TAKARA BIO, Shiga, Japan) with appropriate buffer, and 100 pM each of primer. The PCR cycling conditions were as follows: initial denaturing at 95℃ for 10 minutes, 39 cycles of denaturation at 95℃ for 30 seconds, annealing at 60℃ for 30 seconds, and extension at 72℃ for 1 minute, followed by final extension at 72℃ for 1 minute.

The amplified and digested PCR products were loaded on 1-2% agarose gels for electrophoresis, stained with ethidium bromide and visualized under UV light for verification.

***Pyrosequencing***

Genotyping for CTLA-4 SNPs was conducted by Pyrosequencing (Biotage, Uppsala, Sweden) according to the manufacturer's protocol. Briefly, 25 μl of each PCR product in a 96-well plate was immobilized to 4 μl streptavidin-coated Sepharose beads (GE Healthcare, NJ, USA) with 29 μl of binding buffer (10 mM Tris-HCl, 1mM EDTA, 2M NaCl, 0.1% Tween 20) by agitating at 1400 rpm for 10 minutes at room temperature and transferred to a 96-well filter plate (Millipore, MA, USA). The beads bound to single-stranded DNA labeled with biotin after removing the binding buffer were incubated with 50 μl denaturation solution (0.2 M NaOH) for 1 minute and washed twice with 150 μl washing buffer (10 mM Tris-HCl). The beads were resuspended in 50 μl annealing buffer (20 mM Tris-acetate, 2 mM Mg-Acetate) and transferred to a 96-well PSQ plate (Biotage, Uppsala, Sweden). 5 μl of 2 pM sequencing primers were then added to the plate and allowed to anneal at 85℃ for 2 minutes. The sequencing primers were as follows: 5'-GGCAACAGAGGTTTTT-3' for -1661 A/G, 5'-GTGCAGGGCCAGGTC-3' for +49 A/G, and 5'-TCACCACTATTTGGGATA-3' for +6230 A/G. Pyrosequencing was performed using the PSQ 96 MA System (Biotage, Uppsala, Sweden).

***Statistical analyses***

Genotype frequencies were evaluated for Hardy-Weinberg equilibrium by the chi-square test. Antibody levels were reported as median and interquartile range. Comparison of antibody levels among each group was done by the Mann-Whitney test. A *P*-value less than 0.05 was considered significant. Statistical analyses were performed using JMP IN version 5 (SAS Institute, NC, USA).

## RESULTS

***SNP genotype in CTLA-4***

We genotyped the CTLA-4 polymorphisms at position -1661 A/G, +49 A/G, and +6230 A/G in the 189 subjects. The genotype distributions (A/A, A/G, and G/G) of each SNP in CTLA-4 are as follows: 97, 68 and 24 at position -1661; 107, 63 and 19 at position +49; 37, 85 and 67 at position +6230, respectively. The observed genotype distributions corresponded with Hardy-Weinberg equilibrium ($P > 0.05$).

***Antibody levels and their associations with age***

We quantified *P. falciparum-/P. vivax*-specific IgG/IgE (Pf-IgG, Pv-IgG, Pf-IgE, Pv-IgE) among the 189 non-

**Table 1** Age-specific distribution of antibody levels

| Age groups (years old) | N | Pf[a]-specific IgG (μg/ml) | Pv[b]-specific IgG (μg/ml) | Pf-specific IgE (pg/ml) | Pv-specific IgE (pg/ml) |
|---|---|---|---|---|---|
| 0-5 | 39 | 35.6 (24.7-81.2) | 14.8 (10.2-19.5) | 559.2 (304.5-1170.0) | 187.9 (82.9-331.9) |
| 6-15 | 48 | 129.4 (95.7-155.9) | 21.5 (16.2-28.2) | 986.0 (526.0-1708.0) | 960.0 (426.0-2775.0) |
| 16-30 | 52 | 174.5 (136.5-190.3) | 25.1 (19.3-31.4) | 1542.5 (647.6-1881.3) | 1222.0 (544.0-2582.0) |
| > 30 | 50 | 167.4 (139.3-208.0) | 30.1 (20.9-35.6) | 959.0 (690.0-2158.0) | 1210.0 (424.0-2247.0) |
| total | 189 | 139.5 (98.4-177.0) | 21.8 (16.1-30.1) | 952.0 (559.0-1811.0) | 864.0 (268.0-2212.0) |

Antibody levels are expressed as median and interquartile range.
[a]Pf, *P. falciparum*; [b]Pv, *P. vivax.*

**Table 2a** Associations between CTLA-4 polymorphisms and antibody levels in the younger age group (0-5 years old)

| | N | Pf[a]-specific IgG (μg/ml) | Pv[b]-specific IgG (μg/ml) | Pf-specific IgE (pg/ml) | Pv-specific IgE (pg/ml) |
|---|---|---|---|---|---|
| ***CTLA-4 SNPs*** | | | | | |
| -1661A/G | | | | | |
| G/G or A/G | 19 | 32.7 (24.7-58.0) | 14.7 (10.2-19.5) | 787.7 (308.0-1192.0) | 187.9 (82.9-603.1) |
| A/A | 20 | 61.1 (23.1-93.5) | 15.5 (9.8-20.3) | 462.9 (265.6-854.4) | 183.9 (90.3-271.2) |
| *P* value | | 0.347 | 0.989 | 0.164 | 0.779 |
| ***+49A/G*** | | | | | |
| G/G or A/G | 13 | 58.0 (26.8-113.6) | 18.7 (14.7-23.3) | 559.2 (180.8-1247.0) | 266.6 (153.6-1138.5) |
| A/A | 26 | 32.8 (24.5-67.2) | 13.7 (9.3-17.4) | 544.7 (307.5-968.9) | 146.5 (74.5-296.8) |
| *P* value | | 0.206 | 0.017* | 0.644 | 0.046* |
| ***+6230A/G*** | | | | | |
| G/G or A/G | 29 | 35.6 (25.2-80.7) | 15.7 (11.0-20.8) | 582.5 (276.3-1035.9) | 187.9 (90.1-467.5) |
| A/A | 10 | 44.1 (22.3-83.9) | 12.4 (9.3-15.7) | 482.1 (308.0-1203.0) | 183.9 (72.8-314.8) |
| *P* value | | 0.809 | 0.119 | 0.760 | 0.711 |

Antibody levels are expressed as median and interquartile range.
[a]Pf, *P. falciparum*; [b]Pv, *P. vivax.*
**P* value less than 0.05

**Table 2b** Associations between CTLA-4 polymorphisms and antibody levels in the older age group (over 6 years old)

| | N | Pf[a]-specific IgG (μg/ml) | Pv[b]-specific IgG (μg/ml) | Pf-specific IgE (pg/ml) | Pv-specific IgE (pg/ml) |
|---|---|---|---|---|---|
| ***CTLA-4 SNPs*** | | | | | |
| ***-1661A/G*** | | | | | |
| G/G or A/G | 73 | 154.4 (122.8-180.1) | 25.6 (18.2-31.6) | 964.6 (678.1-1707.0) | 1104.0 (332.1-2592.5) |
| A/A | 77 | 154.8 (126.5-187.4) | 24.8 (19.4-32.3) | 1311.0 (646.3-2244.5) | 1197.0 (571.4-2266.0) |
| *P* value | | 0.708 | 0.774 | 0.165 | 0.540 |
| ***+49A/G*** | | | | | |
| G/G or A/G | 69 | 156.4 (132.5-177.9) | 25.4 (19.5-30.2) | 1004.0 (590.7-1980.5) | 1169.0 (626.7-2623.5) |
| A/A | 81 | 153.2 (116.7-192.3) | 24.1 (17.6-34.2) | 1109.0 (688.3-1847.0) | 1104.0 (352.6-2459.5) |
| *P* value | | 0.736 | 0.818 | 0.943 | 0.369 |
| ***+6230A/G*** | | | | | |
| G/G or A/G | 123 | 154.2 (126.3-178.2) | 24.8 (18.4-30.8) | 1004.0 (637.6-1867.0) | 1169.0 (440.9-2711.0) |
| A/A | 27 | 176.8 (117.7-214.5) | 24.9 (17.3-36.6) | 1311.0 (739.6-2284.0) | 1037.0 (440.9-1690.0) |
| *P* value | | 0.242 | 0.324 | 0.437 | 0.509 |

Antibody levels are expressed as median and interquartile range.
[a]Pf, *P. falciparum*; [b]Pv, *P. vivax.*

parasitized subjects enrolled in this study. Each antibody level showed an age-associated increase and remained high in adults (Table 1).

Since all the antibody levels were significantly higher in subjects 6 years of age or older than in those 5 years of age or younger ($P < 0.05$), we stratified the subjects into the two age groups for further genetic analyses.

***The associations between CTLA-4 polymorphisms and antibody levels***

At CTLA-4 +49 SNP, the G allele carriers (GG or AG) had significantly higher levels of Pv-IgE ($P = 0.046$) and Pv -IgG ($P = 0.017$) than the non-G carriers (A/A) in the younger age group (Table 2a). However, these associations were not found in the older age group (Table 2b).

At CTLA-4 -1661 A/G and +6230 A/G SNPs, no significant association with specific antibody levels was observed in either the younger or older age group.

## DISCUSSION

Among study subjects living in the malaria endemic area of PNG, malaria-specific antibody levels were significantly higher in subjects 6 years of age or older than in those 5 years of age or younger. This was consistent with previous findings that the level of antibodies against malaria increases with age, reflecting the level of cumulative exposure to the parasite, and remains consistently high in adults [29, 30]. The acquisition of natural immunity is considered to be influenced by changes in the immune system during ageing, a phenomenon evidenced in non-immune Javanese transmigrants who moved to hyper endemic Irian Jaya, In-

donesia [9]. After 1-2 years of residence, the adult migrants had less frequent and less intense parasitemia than their children.

In this study, we detected the association between the G allele at +49 SNP and elevated levels of *P. vivax* specific IgG/IgE, which was skewed toward the younger age group of 5 and under. These age-related associations in this population could be a reflection of a different pattern of immune responses between children and adults. Indeed, Baird et al. [9] suggested that the different clinical outcome of malaria infection between children and adults in endemic areas is attributable to the age-related change in the immune system, that is, a shift from Th1-like immune response in children to a Th2-like response in adults. In the present study, CTLA-4, which has been shown to regulate Th1 cell responses [31], was associated with the younger age group, a finding consistent with the above hypothesis.

Our results showed species-specific patterns, i.e. the immunogenetic association of CTLA-4 with anti-*P. vivax* antibodies, but not *P. falciparum.* Since the precise mechanisms for the regulation of malaria-specific antibody production after *P. falciparum* and *P. vivax* infections are yet to be elucidated, it is possible that CTLA-4 is implicated in *P. vivax*-specific antibody production, but not *P. falciparum.* Furthermore, in endemic populations on Melanesian islands, it has been reported that *P. vivax* infection usually establishes earlier after birth and is more predominant than *P. falciparum* in the younger age group [32]. Our results suggest the involvement of immunogenetic mechanisms in the age-specific and species-specific regulation of parasitemia. In this study, however, no correction was made for the number of tests done, so there is a certain risk of overestimated significance.

It has been reported that CTLA-4 expression during malaria infection is restricted to T cells [33]. Thus, anti-*P. vivax* antibody production might be modulated by CTLA-4 on T cells that are needed to provide help rather than the direct control by CTLA-4 on B cells.

Costimulatory signals are required for immunoglobulin class-switching to IgG and IgE. CTLA-4 is not the only stimuli driving this switching. The implication of other costimulatory molecules is another potential explanation for the lack of associations between Pf-specific antibodies and the polymorphism in the CTLA-4 gene. The inducible costimulator (ICOS) gene, which is located downstream from the CTLA-4 gene, is one candidate. ICOS together with CD40L have been shown to be implicated in the crosstalk between $CD4^+$ helper T and B cells, which result in class switching of T-cell-dependent antibody responses [34, 35].

Our data are inconsistent with previous studies that have reported an association between elevated IgE levels and the A allele at CTLA-4 +49 A/G SNP [17]. This discrepancy may suggest that these allelic polymorphisms are in linkage disequilibrium with other functional mutations in other parts within or outside the genes.

We could not find any significant association between CTLA-4 -1661 A/G and +6230 A/G SNP and the specific antibody levels in either the younger or older age group. However, a larger sample size is necessary to confirm these observations.

Walther et al. [36] recently suggested that the susceptibility to infectious diseases is influenced by regulatory T cells, whose suppressive action is mediated partly by CTLA -4 [37]. This raises a hypothesis that CTLA-4 may play an important role in the development of severe malaria through the modification of not only humoral but also cell-mediated immunity. Indeed, a challenge with *P. berghei* following CTLA-4 blockade was shown to cause neurological signs reminiscent of murine cerebral malaria [38]. In this study, we found small genotypic variations at CTLA-4 -319 promoter polymorphism (94.2% CC homozygote; data not shown). Recent functional investigations of this locus have found that the T allele at position -319 (rs5742909; referred to as -318 in some reports, but located at -319 from ATG start codon according to the base sequence obtained at NCBI database) conferred higher promoter activity [23, 39] and increased cell-surface expression of CTLA-4 on T cells [18]. Whereas it is speculated that the C allele has some biological advantages in this area, the fact that the T allele is maintained at a low percentage may result from the balancing selection exerted by malaria, considering that the excessive proinflammatory response is associated with severe malaria and thus that the T allele is theoretically expected to favor survival against malaria. The implication of CTLA-4 in the pathogenesis of severe malaria needs to be further elucidated.

The allelic repertoire selected by malaria, if any, could be highly area-specific. In Melanesia, hemoglobin abnormalities, such as α-thalassemia [40] and glucose-6-phosphate dehydrogenase (G6PD) deficiency [41], have been reported to be selected as a consequence of balancing selection. In Africa, meanwhile, it has been reported that TNF2 allele, which constitutes some percentage of the TNF -α promoter polymorphic variants in the population, is associated with cerebral malaria [42]. The malaria situation in PNG is characterized by the high endemicity of both species: the endemism of *P. falciparum*, which is locally comparable to the level in Africa, and the concurrent endemism of *P. vivax*, which is basically absent in Africa [43]. In view of these unequal epidemiological conditions, the results obtained in this study may not be true in other areas. It

will be interesting to determine whether or not the genetic associations of CTLA-4 can be observed in other malaria endemic areas.

In conclusion, we detected significant associations between the G allele at CTLA-4 +49 SNP and elevated Pv-IgG and Pv-IgE levels in the younger age group of an endemic population in PNG. Our results demonstrate for the first time the age-specific and species-specific immunogenetic regulation of malaria-specific antibody levels, which might influence clinical outcomes of malaria infection in the endemic population.

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