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Vitamin D levels in Indian systemic lupus erythematosus patients: association with disease activity index and interferon alpha

Manamita Mandal[1†], Rina Tripathy[2†], Aditya K Panda[3], Sarit S Pattanaik[1], Simanchal Dakua[1], Anjan Kumar Pradhan[4], Soumen Chakraborty[4], Balachandran Ravindran[3*] and Bidyut K Das[1*]

Abstract

Introduction: Low levels of vitamin D have been associated with several autoimmune disorders including multiple sclerosis, rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus (SLE). The major source of vitamin D is sunlight but exposure of SLE patients to UV rays has been shown to exacerbate disease pathology. Studies in various populations have shown an association between low vitamin D levels and higher SLE disease activity.

Methods: We enrolled 129 patients who fulfilled American College of Rheumatology criteria in the study. There were 79 treatment-naïve cases and 50 patients who were under treatment for underlying SLE. There were 100 healthy subjects from similar geographical areas included as controls. Plasma 25-OH vitamin D_3 and interferon (IFN)-α levels were quantified by enzyme-linked immunosorbent assay (ELISA). The gene expression level of IFN-α was determined by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Results: Plasma 25-OH vitamin D_3 significantly correlated in an inverse manner with systemic lupus erythematosus disease activity index (SLEDAI) scores (P <0.0001, r = −0.42), anti-dsDNA (P <0.0001, r = −0.39), plasma IFN-α (P <0.0001, r = −0.43) and levels of IFN-α gene expression (P = 0.0009, r = −0.45). Further, plasma levels of IFN-α positively correlated with gene expression of IFN-α (P <0.0001, r = 0.84). Treatment-naïve SLE patients displayed significantly higher plasma levels of IFN-α compared to patients under treatment (P <0.001) and controls (P <0.001).

Conclusions: These results suggest an important role of vitamin D in regulating disease activity in SLE patients and the need to supplement vitamin D in their treatment.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder which appears in a group of individuals and which is related to several factors, including environmental and host genetics that contribute to the development of the disease [1]. Patients with SLE develop an immune response against numerous, mostly intracellular self-antigens. This results in formation of immune complexes that get deposited in vascular beds in most organs of the body. Immune complex deposition causes local inflammation and tissue damage that probably amplify the autoimmune response [2]. This has serious consequences on the outcome of the disease.

The importance of vitamin D in various autoimmune disorders has been reported. Vitamin D deficiency has been associated with multiple sclerosis (MS), rheumatoid arthritis (RA), type 1 diabetes mellitus, inflammatory bowel disease (IBD), mixed connective tissue disease, autoimmune thyroid disease, scleroderma and SLE [3-5]. Vitamin D supplementation improves disease outcome in various animal models of MS [6], RA [7], type 1 diabetes mellitus [8], IBD [9], autoimmune encephalomyelitis [10] and SLE [11]. The role of vitamin D in murine models of SLE has been investigated to a limited degree. Administration of vitamin D and its synthetic analogs to

* Correspondence: ravindran8@gmail.com; bidyutdas@hotmail.com
†Equal contributors
[3]Infectious Disease Biology Group, Institute of Life Sciences, Nalco Nagar Road, Bhubaneswar, OD 751023, India
[1]Department of Medicine, SCB Medical College, Medical College Road, Cuttack, OD 753007, India
Full list of author information is available at the end of the article

murine models has resulted in improved dermatological manifestations [11], reduced proteinuria [12] and increased survival [12,13]. An earlier report highlighted vitamin D_3 insufficiency in two-thirds, and deficiency (<10 ng/ml) in approximately one-fifth of SLE patients [14]. In addition, serum vitamin D_3 (25-OH) levels have been found to correlate inversely with SLE disease activity index (SLEDAI) scores [15-17].

The major source of vitamin D is the conversion of 7-dehydrocholesterol to previtamin D_3 in the skin when exposed to solar ultraviolet radiation [18]. Previtamin D_3 then gets converted to vitamin D_3 (cholecalciferol) through a heat-mediated process in the skin [18]. A lesser amount of vitamin D_3 (25-OH) is obtained from foods that supply less than 20% of the body's requirements. Vitamin D_3 undergoes two hydroxylations to achieve its functional form. The first hydroxylation occurs in the liver resulting in 25-hydroxyvitamin D ($25(OH)D_3$) or calcidiol, which is normally quantified for evaluating vitamin D status, and the second hydroxylation takes place in the kidney to its active form 1,25-dihydroxyvitamin D3 (1, $25(OH)_2D$) [18]. In addition to the liver and kidney, hydroxylation of vitamin D_3 also occurs in the lymph nodes and skin [19].

Several studies worldwide have investigated the role of vitamin D_3 in the pathogenesis of SLE. However, to date, there have been no reports from an Indian population. Although the prevalence of SLE in India is rare (3 per 100,000) [20], the survival rates of these patients (5-year: 70%; 10-year: 50%) are low compared to Western cohorts [21,22]. Interestingly, vitamin D_3 insufficiency or deficiency appears to be widespread in the Indian subcontinent [23], which makes it important to analyze its role in the background of SLE from an Indian cohort. We have addressed this issue in a tertiary-care, hospital-based, case-control study, to assess the role of vitamin D_3 in SLE in a cohort from eastern India.

Methods

Subjects

The patients recruited for the study were all inpatients, admitted to the Department of Medicine, under the Clinical Immunology and Rheumatology unit of SCB Medical College, Cuttack, Odisha. As described earlier [24-26], diagnosis of SLE was based on the revised American College of Rheumatology (ACR) classification criteria [27]. After a detailed clinical examination and laboratory investigation, the clinical manifestations were categorized. The clinical profiles of 129 SLE patients are summarized in Table 1. Since, SLE affects women primarily [28], 50 age-matched healthy females (medical students: HCA) and 50 healthy subjects from similar geographical areas (HCB) were included as healthy controls (HC). None of the controls reported any history of autoimmune disorder. About 5 ml blood in EDTA was collected from each participant. The study was approved by the institutional ethics committee of SCB Medical College, Cuttack. Informed consent was obtained from each patient and healthy control.

25-OH vitamin D quantification in plasma

The plasma levels of 25-OH Vitamin D were quantified by enzyme-linked immunosorbent assay (ELISA) kit (CPC, Euroimmun, Lübeck, Germany) according to the manufacturer's instructions. Vitamin D deficiency was defined as plasma levels of 25-OH vitamin D <10 ng/ml and insufficiency as 10 to 30 ng/ml [18].

Quantification of plasma interferon alpha

Plasma levels of interferon (IFN)-α were measured by ELISA kit (Bender MedSystems Inc., Burlingame, CA, USA) according to the manufacturer's protocol.

RNA extraction and reverse transcription

According to the manufacturer's instructions, total RNA was isolated from 250 μl of whole blood by TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined by spectrophotometry using an Implen NanoPhotometer (Implen, Munich, Germany). To remove any traces of genomic DNA, 1 μg of total RNA was then treated with 2U DNase (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C. DNase-treated RNA was reverse transcribed with a hexamer primer using a First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Once the cDNA was synthesized, its fidelity was tested by PCR and stored at −70°C.

Real-time PCR assay

Real-time PCR assay of IFN-α was carried out as described earlier [29]. Briefly, reactions were set up in a total volume of 20 μl using 2 μl of cDNA, 10 μl of MESA GREEN qPCR MasterMix Plus (Eurogentec, Seraing, Belgium) and 10 picomole each of gene-specific primer (IFN-α (sense: 5′-TTCCTCCTGYYTGAWGGACAGA-3; antisense: 5′-GATCTCATGATTTCTGCTCTGACA-3′), glyceraldehyde-3 phosphate dehydrogenase (G3PDH) was taken as control (sense: 5′-GGTATCGTGGAAGGACT CATGAC-3′; antisense: 5′-ATGCCAGTGAGCTTCCC GTTCAGC-3′)) and performed in the MJ Research DNA Engine Opticon Real-Time Thermal Cycler (MJ Research, Waltham, MA, USA).The cycling conditions were: 95°C for 4 min; 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s with a single fluorescence measurement; a final elongation step was carried out at 72°C for 10 min. Specificity of the PCR products was confirmed by analysis of the dissociation curve. The melting curve program consisted of temperatures between 55 and 95°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement.

Table 1 Clinical characteristics of SLE patients and healthy controls

Clinical profiles	SLE (n = 129)	Healthy controls (n = 100)
Sex (male/female)	4/125	26/74
Age in years (mean ± SD)	28.14 ± 8.43	31.18 ± 5.32
Duration of disease years (mean ± SD)	2.90 ± 2.66	-
SLEDAI scores (mean ± SD)	18.36 ± 6.73	-
Photosensitivity rash	34 (26)	-
Malar rash	73 (57)	-
Discoid rash	14 (11)	-
Oral ulcer	76 (59)	-
Arthritis	77 (60)	-
NPSLE	11 (9)	-
Myocarditis	3 (2)	-
Serositis	7 (5)	-
Nephritis	46 (37)	-
Vasculitis	17 (13)	-
Treatment details of patients under therapy at the time of recruitment to the study (n = 50)		
	Medicine	Quantity	Number of patient treated (%)
	Prednisolone; mean (range)	18.99 mg (5-50 mg)	50 (100)
	Hydroxychloroquine	6.5 mg/kg body weight	50 (100)
	Calcium	1 g/day	50 (100)
	Vitamin D_3	250-500 IU/day	50 (100)
	Azathioprine	50-100 mg/day	4 (8)
	Mycophenolate mofetil	2 gm/day	4 (8)

Note: data are number (%) of participants unless otherwise specified. SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; NPSLE, neuropsychiatric systemic lupus erythematosus.

Additionally, the amplicons' expected size and the absence of nonspecific products were confirmed by analysis of the real-time PCR products in 1% agarose gel in 1 × TBE, stained with ethidium bromide and visualized under ultraviolet light (expected product size of IFN-α: 375 bp and G3PDH: 187 bp). IFN-α gene expression in each sample was calculated by the $2^{-\Delta Ct}$ method (ΔCt = Ct of IFN-α – Ct of GAPDH) [30].

Statistical analysis

All statistical analysis was performed by GraphPad prism 5.01 (GraphPad Software, San Diego, CA, USA). Distribution of plasma 25-OH vitamin D_3 and IFN-α in treatment-naïve SLE patients, controls and treated patients were assessed by D'Agostino-Pearson omnibus normality test. Based on the results of the normality test, the association of 25-OH vitamin D_3 and IFN-α with clinical disease was analyzed by analysis of variance (ANOVA) or Kruskal-Wallis test followed by an appropriate post test. Correlation of 25-OH vitamin D_3 with double-stranded (ds)DNA, SLEDAI scores and IFN-α was analyzed by Spearman's correlation test. Further correlation of IFN-α gene expression with plasma IFN-α and 25-OH vitamin D_3 levels was analyzed by Spearman's correlation test. A P value <0.05 was considered as significant.

Results

Clinical characteristics of SLE patients

One hundred and twenty-nine patients were enrolled in the current study. Baseline characteristics are shown in Table 1. There were 125 (97%) females and 4 (3%) males with a mean age (standard deviation) of 28.14 (8.43) years. The mean duration of disease (standard deviation) was 2.90 years (2.66). Out of the 129 SLE patients, 50 patients included in the study were already on treatment for SLE and were also receiving supplements of oral calcium and vitamin D_3 at the time of blood collection (Table 1). The other 79 patients were treatment-naive cases, undiagnosed earlier and the details of the treatment received for their complaints before hospitalization were not known since the patients had not maintained any records. The clinical profiles of patients were as follows: photosensitivity rash (26%), malar rash (57%), discoid rash (11%), oral ulcer (59%), arthritis (60%), neuropsychiatric disease (9%), myocarditis (2%), serositis (5%), nephritis (37%) and vasculitis (13%) (Table 1).

Plasma 25-OH vitamin D_3 levels in SLE patients and healthy controls

Plasma levels of 25-OH vitamin D_3 in SLE patients and healthy controls were quantified by ELISA and the results are shown in Figure 1. Patients under vitamin D_3 supplementation (treated cases) displayed significantly higher levels of 25-OH vitamin D_3 compared to treatment-naïve patients (P <0.001) and healthy controls (P <0.001). The levels of plasma 25-OH vitamin D_3 in treatment-naïve SLE patients and healthy controls were comparable.

Vitamin D_3 levels negatively correlated with SLEDAI scores and anti-dsDNA

Analysis of data in SLE patients revealed a significant negative correlation between plasma 25-OH vitamin D_3 levels with SLEDAI scores (P <0.0001, r = −0.42) (Figure 2A) and anti-dsDNA (P <0.0001, r = −0.39) (Figure 2B). Further, SLE patients were categorized into two groups: treatment-naïve and those under treatment. As shown in Figure 2C and D, irrespective of treatment status, the plasma levels of 25-OH vitamin D_3 negatively correlated with SLEDAI scores. In addition, a further analysis of the relationship between 25-OH vitamin D_3 and modified SLEDAI scores (eliminating the anti-dsDNA positive score of 2 from SLEDAI), revealed identical results in both the groups (Figures 2E and F). These findings indicate a significant association between 25-OH vitamin D_3 and disease activity in SLE.

Correlation between 25-OH vitamin D_3 and IFN-α

The role of IFN-α in SLE has been clearly documented and its significant correlation with SLEDAI scores has been demonstrated in patients from different populations [31,32]. As shown in Figure 3A, a significant negative correlation was observed between 25-OH vitamin D_3 levels and plasma IFN-α (P <0.0001, r = −0.43). Treatment-naïve patients displayed higher levels of plasma IFN-α compared to SLE patients on treatment (P <0.001) and healthy controls (P <0.001) (Figure 3B) suggesting an important role of IFN-α in modulating disease activity.

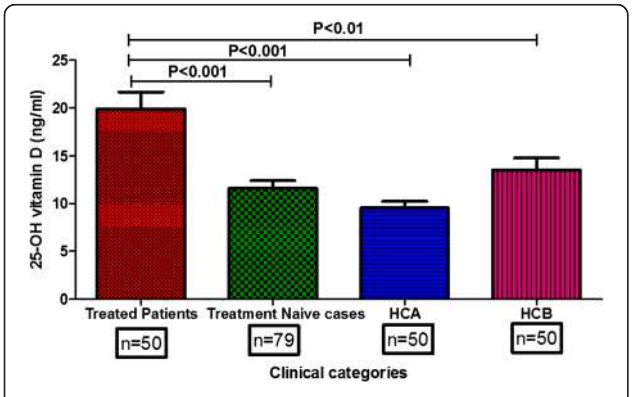

Figure 1 25-OH vitamin D_3 levels in SLE patients and healthy controls. Plasma samples from SLE patients (treatment-naïve case (n = 79); treated patients (n = 50)) and healthy controls (HCA: medical students (n = 50); HCB: controls from same locality (n = 50)) were quantified by ELISA according to the manufacturer's instructions. Treated SLE patients displayed significantly higher concentrations of plasma 25-OH vitamin D_3 levels compared to treatment-naïve patients (P <0.001) and controls (HCA: (P <0.001); HCB: (P <0.01)). SLE, systemic lupus erythematosus.

Correlation of IFN-α gene expression with plasma IFN-α and 25-OH vitamin D_3 levels

To validate the robustness of IFN-α data, considering that it is an evanescent cytokine, we quantified IFN-α gene expression by RT-PCR in SLE patients (n = 49) and correlated the values with plasma levels of IFN-α and 25-OH vitamin D_3. As shown in Figure 4A, a strong positive correlation was observed between IFN-α plasma levels and its gene expression (P <0.0001, r = 84). In addition, IFN-α gene expression negatively correlated with plasma 25-OH vitamin D_3 (P = 0.0009, r = −45) (Figure 4B).

Association of plasma IFN-α with SLE disease severity

Role of IFN-α in the pathogenesis of SLE is an important issue that is being investigated [32]. We analyzed the association of IFN-α with disease severity. As shown in Figure 5A, plasma levels of IFN-α positively correlated with SLEDAI scores (r = 0.26, P = 0.002) and patients with severe phenotype displayed significantly higher levels of IFN-α compared to those with mild disease manifestations (P = 0.01) (Figure 5B). However, duration of disease did not correlate with plasma IFN-α levels (data not shown). We also observed a significant inverse correlation between plasma 25-OH vitamin D_3 and IFN-α levels. This correlation held true while analyzing the association between 25-OH vitamin D_3 levels and IFN-α gene expression.

Discussion

The role of vitamin D_3 in autoimmune disorders has been the subject of several studies with regard to its importance as an immune regulator [33]. This is the first study from India to demonstrate an association between vitamin D_3 and SLE, highlighting its significant inverse correlation with SLEDAI scores, anti-dsDNA and IFN-α. These are markers of disease activity and IFN-α is closely associated with disease pathogenesis.

Low levels of vitamin D_3 in SLE patients have been reported compared to healthy controls in different populations [34]. Interestingly, mean plasma levels of 25-OH vitamin D3 were not significantly different among treatment-naïve SLE cases (11.61 ng/ml), healthy medical students (9.55 ng/ml) and other healthy controls from same locality (13.36 ng/ml). Vitamin D_3 insufficiency has been

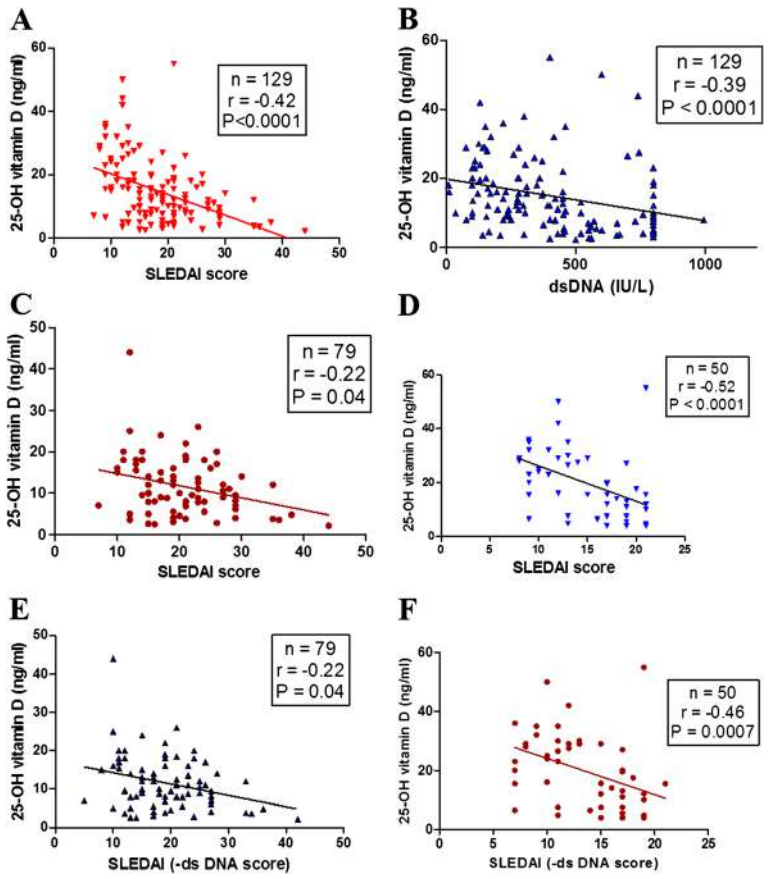

Figure 2 Correlation of 25-OH vitamin D_3 with SLEDAI scores and anti-dsDNA levels. Plasma 25-OH vitamin D_3 levels of SLE patients correlated negatively with SLEDAI scores **(A)** and anti-dsDNA **(B)**. SLE patients were categorized into two groups: treatment-naïve cases and patients under treatment. In both groups plasma levels of 25-OH vitamin D_3 negatively correlated with SLEDAI scores **(C** and **D)**. Modified SLEDAI scores (eliminating the anti-dsDNA score of 2) and its correlation with plasma levels of 25-OH vitamin D_3 were analyzed. Similar to earlier observations, SLEDAI scores (−anti-dsDNA) negatively correlated with 25-OH vitamin D_3 levels in both treatment-naïve cases **(E)** and treated patients **(F)**. Dots represent individual samples. Correlation analysis was performed by Spearman's correlation coefficient. A *P* value less than 0.05 was considered as significant. SLEDAI, systemic lupus erythematosus disease activity index; SLE, systemic lupus erythematosus; dsDNA, double-stranded DNA.

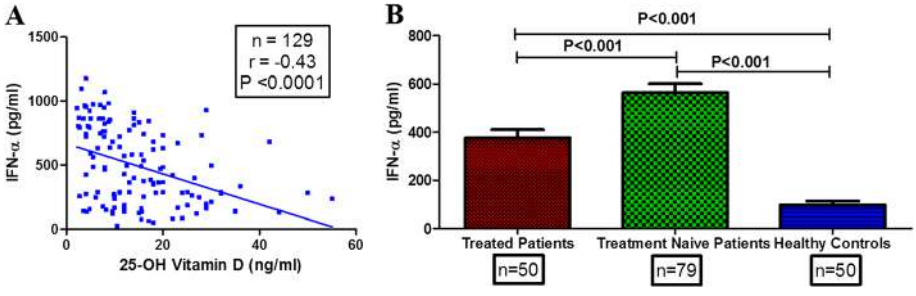

Figure 3 Correlation of plasma IFN-α with 25-OH vitamin D_3 and its levels in SLE patients and controls. (A) Plasma 25-OH vitamin D_3 levels correlated negatively with IFN-α levels. Dots represent individual samples. Correlation analysis was performed by Spearman's correlation coefficient. **(B)** Treatment-naïve patients displayed significantly higher levels of IFN-α compared to treated cases and healthy controls. Mean plasma levels of IFN-α in different clinical categories were compared by ANOVA followed by Tukey's multiple comparisons test. A *P* value less than 0.05 was considered as significant. IFN-α, interferon alpha; SLE, systemic lupus erythematosus.

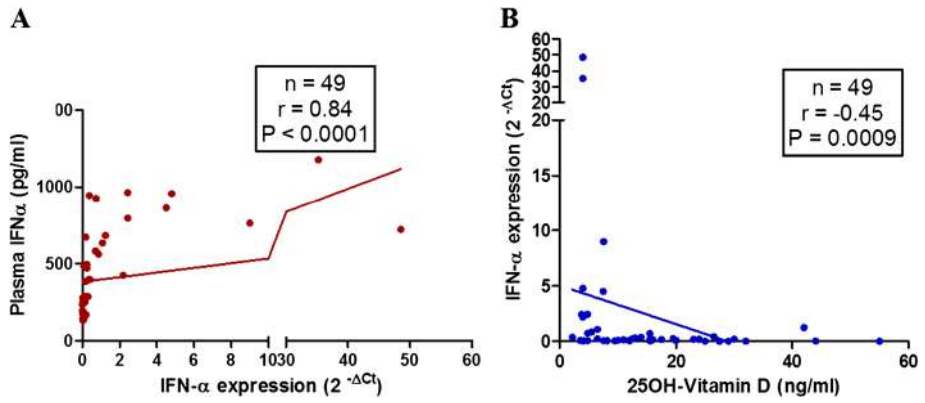

Figure 4 Correlation of IFN-α gene expression with plasma IFN-α levels and 25-OH vitamin D_3. Plasma levels of IFN-α and gene expression levels were quantified by ELISA and RT-PCR respectively. **(A)** Plasma levels of IFN-α positively correlated with gene expression ($2^{-\Delta Ct}$) of IFN-α. **(B)** IFN-α gene expression ($2^{-\Delta Ct}$) correlated negatively with plasma levels of 25-OH vitamin D_3. Dots represent individual samples. Correlation analysis was performed by Spearman's correlation coefficient. A *P* value less than 0.05 was considered as significant. IFN-α, interferon alpha.

reported to be widely prevalent in the Indian subcontinent irrespective of the social class [23]. Two groups of healthy controls were analyzed, which included medical students (HCA), who led a lifestyle marked by poor exposure to sunlight and irregular dietary habits, and a group of healthy subjects from the same locality (HCB). Interestingly, 63% of healthy medical students were deficient and 37% were insufficient of vitamin D_3. Furthermore, 94% of the other groups of healthy controls were either deficient or insufficient of vitamin D_3. This was an important observation considering India being a tropical country with lots of sunshine. However, the facts were contrary and several hypotheses have been discussed to explain the discrepancy. Higher melanin concentration in the skin [35], current lifestyle changes, avoidance of sunlight and poor food habits are some of the causes attributed to the widespread prevalence of low vitamin D_3 among Indians. Low vitamin D_3 may not be cause for development of SLE but persons with low serum levels are likely to suffer from severe disease. The current cross-sectional study does not address the issue of cause and effect relationship between vitamin D_3 and SLE.

There are several interesting observations in the current study that points to an important role for vitamin D_3 in disease modulation. One of them being a significant inverse correlation between vitamin D_3 and SLEDAI scores and the other association is between vitamin D_3 and anti-dsDNA. Association between plasma vitamin D_3 and SLEDAI scores has not been uniform across observations: several studies have reported a negative correlation [15-17], while others have found none [36-39].

One of the important functions of vitamin D_3 is maintenance of homeostasis of B cells [40]. Low levels of vitamin D_3 contribute to hyperactivity of B cells and enhanced production of autoantibodies [41]. Furthermore,

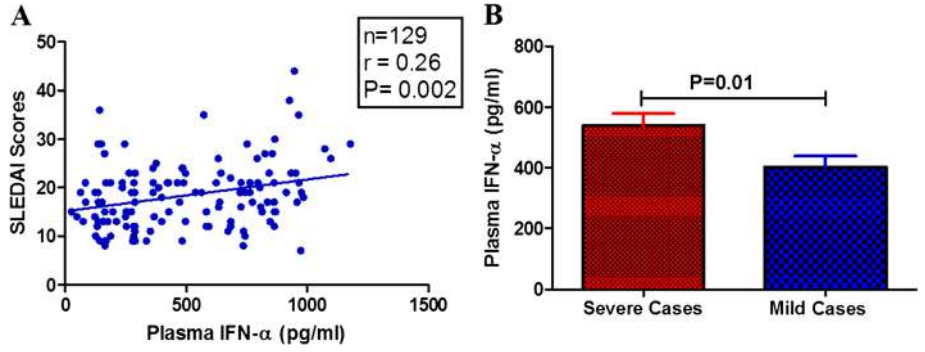

Figure 5 Correlation of plasma IFN-α with SLEDAI scores and association with organ involvement. (A) Plasma levels of IFN-α positively correlated with SLEDAI scores. Dots represent individual samples. Correlation analysis was performed by Spearman's correlation coefficient. A *P* value less than 0.05 was considered as significant. **(B)** Based on clinical phenotype SLE patients were categorized into two broad groups and plasma levels of IFN-α were compared. Patients with major disease manifestation displayed significantly higher plasma levels of IFN-α than those with minor disease manifestation. Mean plasma levels of IFN-α were compared by unpaired *t* test and a *P* value less than 0.05 was taken as significant. IFN-α, interferon alpha; SLEDAI, systemic lupus erythematosus disease activity index; SLE, systemic lupus erythematosus.

vitamin D_3 is known to modulate various immunological pathways [33] and thus could have a defining role in the development, progression and pathogenesis of SLE. Vitamin D_3 also inhibits differentiation of dendritic cells (DCs) and T-helper cells (CD4+) [42], enhances T regulatory cell proliferation and suppresses release of inflammatory mediators [43], which collectively help in control of autoimmune disorders.

In recent years, the role of interferon in the pathogenesis of lupus has been widely investigated. Higher levels of IFN-α were observed in our SLE patients compared to healthy controls, corroborating earlier observations [32,44-46]. The interferon levels were significantly low in patients under treatment compared to treatment-naïve cases, supporting its possible role in disease modulation. Furthermore, IFN-α could be a marker of disease activity and low levels in treated patients could indicate response to therapy.

Interestingly, our study revealed a strong negative correlation of vitamin D_3 with IFN-α (P <0.0001, r = –0.52). The robustness of the assay was validated by assessment of IFN-α gene expression, which corroborated with the earlier observations on the association between plasma IFN-α and vitamin D_3. There are no reports in the literature assessing the association between IFN-α and vitamin D_3.

In active SLE overexpression of interferon-inducible genes (IFN signature) has been reported [47]. The major source of IFN-α in SLE patients are activated DCs. Maturation/activation of DCs and production of IFN-α has been observed to be inhibited by vitamin D in *in vitro* studies [48,49]. A direct role for vitamin D_3 in modulating lupus activity has been demonstrated in animal models [11-13]. Our observations, although cross-sectional, and studies on experimental models, provide evidence for a disease-modulating role for vitamin D_3, which could be a promising therapeutic adjunct in the treatment of SLE. In view of the limited number of drugs available for the treatment of lupus and the low cost of vitamin D_3 therapy, there is a strong case for its use routinely.

Conclusions

To conclude, vitamin D deficiency is prevalent among healthy Indians as well as among SLE patients. The significant inverse correlation of vitamin D_3 with SLEDAI scores, anti-dsDNA and IFN-α highlights its immune-modulatory role contributing to disease outcome. Although the present study indicates a necessity for vitamin D_3 supplementation in the management of SLE patients, larger randomized controlled trials would be necessary to define the daily requirement and optimum blood levels of vitamin D_3 that are effective in influencing disease outcome.

Abbreviations

ACR: American College of Rheumatology; C3: complement component 3; C4: complement component 4; DCs: dendritic cells; dsDNA: double-stranded DNA; ELISA: enzyme-linked immunosorbent assay; HC: healthy controls; IBD: inflammatory bowel disease; IFN-α: interferon alpha; MS: multiple sclerosis; NPSLE: neuropsychiatric systemic lupus erythematosus; RT-PCR: real-time polymerase chain reaction; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SLEDAI: systemic lupus erythematosus disease activity index.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MM, SD and SSP were involved in samples collection, data management and clinical categorization of samples. AdKP carried out quantification of IFN-α by ELISA, real-time PCR, data interpretation and wrote the first draft of the manuscript. AnKP and SC quantified IFN-α gene expression and interpreted the results. RT performed all routine tests including measurement of vitamin D. RT, BR and BKD made a contribution in the design, data interpretation, work supervision and critically revising the manuscript. All authors read and approved the manuscript.

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Author details

[1]Department of Medicine, SCB Medical College, Medical College Road, Cuttack, OD 753007, India. [2]Department of Biochemistry, SCB Medical College, Medical College Road, Cuttack, OD 753007, India. [3]Infectious Disease Biology Group, Institute of Life Sciences, Nalco Nagar Road, Bhubaneswar, OD 751023, India. [4]Department of Gene Function and Regulation, Institute of Life Sciences, Nalco Nagar Road, Bhubaneswar, OD 751023, India.

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PAPER

Diminished IL-17A levels may protect filarial-infected individuals from development of rheumatoid arthritis and systemic lupus erythematosus

AK Panda[1] and BK Das[2]
[1]Centre for Life Sciences, Central University of Jharkhand, Brambe, Ranchi, India; and [2]Department of Medicine, SCB Medical College, Cuttack, Odisha, India

Nematode infections have been observed to inversely correlate with autoimmune disorders. Recently, we have shown the absence of filarial infection in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) who live in filarial-endemic areas. The mechanism(s) by which filarial-infected individuals are protected against the development of RA or SLE are unknown. In mice CIA, an experimental model for RA, ES-62, an execratory product of rodent filarial nematode, has been shown to improve arthritis through suppression of the IL-17 pathway. A total of 160 individuals, 40 each of endemic normal, filarial-infected cases, SLE and RA patients, from filarial-endemic areas, were enrolled in the study. Plasma levels of IL17-A, IFN-α and TNF-α were quantified by enzyme-linked immunosorbent assay (ELISA). RA and SLE patients displayed significantly higher plasma IL-17A, IFN-α and TNF-α levels compared to endemic normal and infected individuals. Furthermore, IL-17A levels were significantly low in participants with filarial infection compared to endemic controls ($p < 0.05$). Interestingly, plasma IL-17A levels correlated inversely with circulating filarial antigen (CFA) ($p = 0.004$, Spearman $r = -0.51$). Filarial infection was associated with low plasma IL-17A levels, a mechanism by which it possibly protects individuals in filarial-endemic areas from the development of autoimmune disorders like RA and SLE. *Lupus* (2016) **0,** 1–7.

Key words: Filariasis; rheumatoid arthritis; systemic lupus erythematosus

Introduction

The prevalence of autoimmune disorders is worldwide and is now considered to be a silent epidemic. The load of autoimmune diseases in developed countries is higher than in tropical countries.[1] This variation in the prevalence of autoimmune diseases in different geographic regions is believed to be associated with nematode load in respective populations,[1] which is the basis of the concept of the Hygiene Hypothesis. Epidemiological studies are limited but demonstrate a trend and an inverse association between nematode infections and autoimmune disorders. Recently, we observed the absence of filarial infection in patients with systemic lupus erythematosus (SLE)[2] and rheumatoid arthritis (RA)[3] living in filarial-endemic areas where the infection rate among the general population, demonstrated by the presence of circulating filarial antigen (CFA), was 40%–42%. Another independent study from South India has demonstrated an inverse relationship between the prevalence of filariasis and type-1 diabetes.[4] The precise mechanism(s) involved in protection against development of autoimmune disease by filarial infection in humans has not been clearly defined. It is believed that nematode-derived products protect the host against autoimmunity through modulation of the various components of the immune system. A seminal work on a mouse model of RA showed that ES-62, an excretory-secretory (ES) product of the rodent filarial nematode *Acanthocheilonema viteae,* effectively reduces the severity of inflammation in murine collagen-induced arthritis (CIA).[5] The administration of ES-62 in the CIA model was found to significantly reduce interleukin-17

Correspondence to: Bidyut K Das, Department of Medicine, SCB Medical College, Cuttack, Odisha, 753007 India.
Email: bidyutdas@hotmail.com
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(IL-17)-producing cells and suppression of IL-17 production.[6]

IL-17 is a proinflammatory cytokine associated with the pathogenesis of autoimmune diseases which includes RA, SLE, systemic sclerosis, inflammatory bowel disease (IBD) and multiple sclerosis (MS). IL-17A is produced by a wide variety of cells, namely CD4+ T cells (Th17 cells), CD8+ T cells, natural killer cells, γδ-T cells, mast cells and CD4-CD8-CD3+ (double-negative, DN) T cells. IL-17 participates in tissue inflammation and destruction by enhancing the expression of other pro-inflammatory cytokines and matrix metalloproteases. Increased IL-17 cells were reported in peripheral blood of RA patients compared to healthy controls.[7,8] Furthermore, high levels of IL-17 have been reported in synovial fluid of RA patients.[9] In SLE, high plasma levels of IL-17A and a positive correlation with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) have been reported.[10] In addition, an increased number of IL-17-producing cells have been previously observed in the peripheral blood[11]and infected organs of SLE patients.[11,12]

There are no epidemiological studies demonstrating the possible association of IL-17 vis a vis autoimmune disorders in filarial-endemic areas. This hospital- and rural-based case-control study has made an effort to demonstrate the possible mechanism(s) associated with filarial infection-mediated modulation of immune response in RA and SLE, in the background of the role demonstrated by IL-17 in an experimental model of CIA.

Materials and methods

Participants

We enrolled only female individuals with RA and SLE admitted to or attending the specialty unit of Clinical Immunology and Rheumatology, Department of Medicine, SCB Medical College, Cuttack, Odisha, India, to maintain the homogeneity of the cohort. The diagnoses of RA and SLE were based on the American College of Rheumatology (ACR) classification criteria of 1987 and 1997, respectively. These patients lived in the coastal districts of Odisha endemic to Bancroftian filariasis.[13] Healthy individuals residing in the same villages, without a clinical history suggestive of filariasis and with no evidence of infection (confirmed by absence of circulating microfilariae or CFAs) were included as endemic controls (ECs). Individuals positive for circulating microfilariae and/or CFAs were considered filarial-infected cases (ICs). Parasitological examination of controls was performed by means of microscopic examination of Giemsa-stained finger prick blood smear samples (20 µl) obtained by night blood survey. The study was approved by the Institutional Human Ethics Committee of the SCB Medical College, Cuttack, Odisha.

Determination of circulating filarial antigenemia

The level of CFAs was measured with the Trop Bio enzyme-linked immunosorbent assay (ELISA) test kit (Trop Bio Pvt Ltd) in accordance with the manufacturer's protocol.

Quantification of plasma IL-17A, interferon-alpha (IFN-α) and tumour necrosis factor-alpha (TNF-α) levels

Plasma levels of IL-17A, IFN-α and TNF-α were quantified by ELISA according to the manufacturers' instructions (IL-17A: R&D Systems; IFN-α: eBiosciences; TNF-α: eBiosciences).

Statistical analysis

Statistical analyses were performed by using GraphPad Prism (version 5.01). Distribution of plasma IL-17A, IFN-α or TNF-α in different clinical categories were assessed by the D'Agostino & Pearson omnibus normality test. Based on results of the normality test, the association of plasma IL-17A, IFN-α or TNF-α levels with various clinical categories were analysed by analysis of variance (ANOVA) or Kruskal-Wallis test followed by an appropriate post-test. The correlation between plasma cytokines and circulating filarial antigenemia units was analysed by Spearman's correlation test.

Results

Baseline characteristics of enrolled individuals

A total of 160 individuals (40 from each category) were enrolled in the present study. Baseline data are shown in Table 1. Mean duration of disease of SLE patients and RA patients was 2.3 and 6.7 years, respectively. Similar to our earlier observations,[2,3] SLE and RA patients were negative for the Og4C3 test. In filarial-infected cases CFA varied from 230 to 32,000 units.

Table 1 Baseline characteristics of studied individuals

	EN (n = 40)	*Filariasis-infected cases (n = 40)*	*SLE (n = 40)*	*RA (n = 40)*
Sex (M/F)	0/40	0/40	0/40	0/40
Mean age in years (range)	23.6 (11–69)	30.3 (13–67)	26.4 (14–45)	43.2 (20–66)
Duration of disease in years (mean ± SEM)	–	–	2.30 ± 0.31	6.78 ± 1.10
Og4C3 positivity (CFA unit range)	0	40 (100%) (230–32000)	0	0

EN: endemic normal; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; SEM: standard error of mean; CFA: circulating filarial antigen.

Filarial-infected cases display lower plasma IL-17A than EN, RA and SLE patients

Mean plasma IL-17A levels were compared in ECs, ICs, SLE and RA patients, and the results are shown in Figure 1 (a). ICs displayed significantly lower levels of plasma IL-17A compared to ECs ($p < 0.05$), SLE ($p < 0.001$) and RA patients ($p < 0.001$). Furthermore, patients with RA and SLE showed elevated levels of plasma IL-17A compared with ECs. However, mean plasma IL-17A levels were comparable in RA and SLE patients.

Plasma TNF-α and IFN-α are elevated in RA and SLE patients

Plasma levels of TNF-α and IFN-α were quantified by ELISA in ECs, ICs, SLE and RA patients. As shown in Figure 1 ((b) and (c)), SLE and RA patients displayed significantly higher plasma levels of TNF-α and IFN-α compared to ECs and ICs. No significant difference in mean plasma TNF-α and IFN-α levels was observed either between ECs and ICs or between RA and SLE patients.

Correlation of plasma cytokines with disease severity

Furthermore, we analysed the correlation of plasma parameters with disease severity scores of RA and SLE patients. Similar to earlier observations,[14–18] plasma TNF-α and IFN-α correlated positively with DAS28 and SLEDAI scores (Figure 2). However, plasma levels of IL-17A did not show any significant correlation with disease severity scores of RA and SLE patients (Figure 2).

Correlation of CFA units with plasma IL-17A levels

Out of the 40 ICs, plasma IL-17A could be measured in 29 cases while in the remaining 11 cases it was below the detectable range of the ELISA kit used for estimation. Correlation between CFA units and plasma IL-17A levels were analysed by Spearman rank correlation test (Figure 3(a)). There was a significant inverse correlation between plasma IL-17A and CFA units ($p = 0.004$, $r = -0.51$). However, there was no significant correlation between CFA units and plasma levels of TNF-α and IFN-α (Figure 3 (b) and (c)).

Discussion

This study was undertaken on the premise that parasitic infections have a role in delaying, preventing or modulating autoimmune diseases.[19] This has been supported by epidemiological observations and in experimental models. We have previously demonstrated the absence of filarial infection in RA and SLE patients in filarial-endemic areas of Odisha, India.[2,3] The mechanism(s) involved in the protection of humans with nematode infection from development or modulation of autoimmune disorders is not clearly known. However, elegant studies on mouse models have demonstrated that the derivatives of parasitic worms can suppress inflammation in a significant manner. The CIA model, which is similar to human RA, provided direct evidence of a rodent filarial nematode ES-62 antigen that suppressed arthritis by modulating IL-17 levels. In our study, besides looking at the association of human filarial ES product (CFA) with IL-17, we also investigated its possible relationship with key cytokines associated with RA and SLE, namely TNF-α and IFN-α, respectively. There was a clear association between human filarial infection (CFA) and IL-17A in patients with RA and SLE: higher levels of CFA correlating significantly and inversely with IL-17A levels.

The role of IL-17 has been widely investigated both in human and experimental autoimmune disorders. Plasma levels of IL-17A have been found to

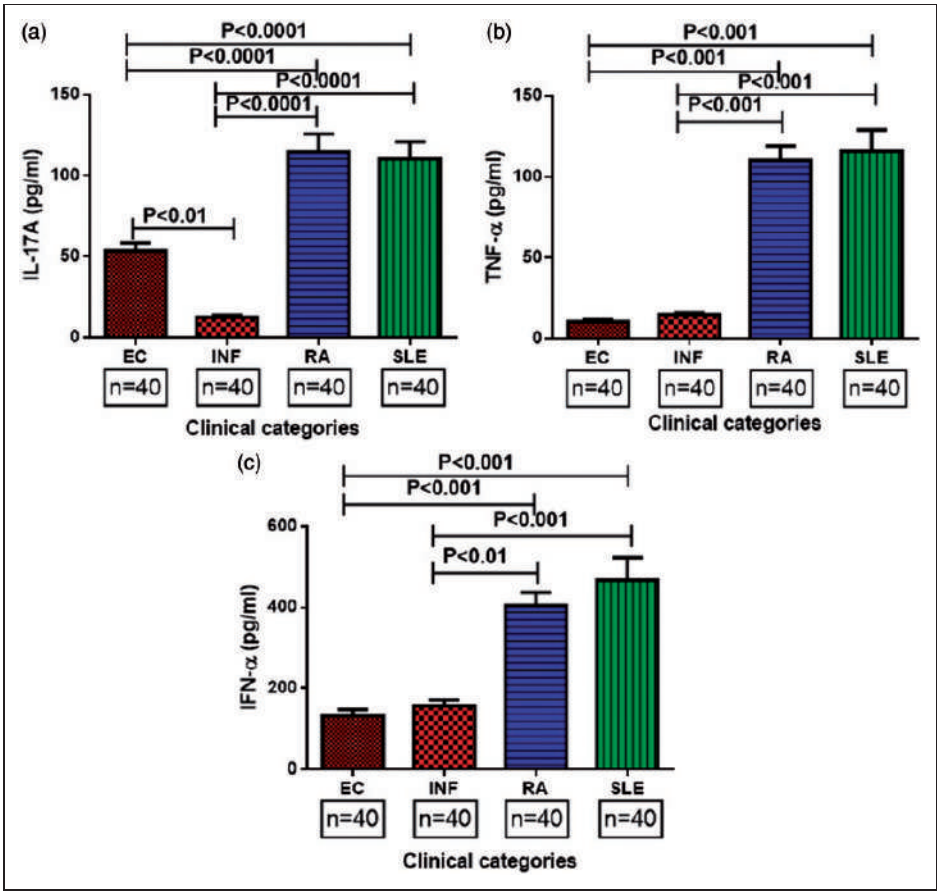

Figure 1 Plasma IL-17A, TNF-α and IFN-α levels in different clinical categories. Plasma levels of IL-17A (a), TNF-α (b) and IFN-α (c) were quantified by ELISA in healthy controls, filarial-infected cases, rheumatoid arthritis and systemic lupus erythematosus patients (40 each case). Mean plasma levels of cytokines among different clinical categories were compared by ANOVA followed by Tukey's multiple comparison post-test. A p value less than 0.05 was considered significant. IL-17A: interleukin 17A; TNF-α: tumour necrosis factor alpha; IFN-α: interferon alpha; ELISA: enzyme-linked immunosorbent assay; ANOVA: analysis of variance.

be elevated in RA and SLE patients compared to normal controls.[20] This cytokine has been demonstrated to have an important contributory role in disease pathogenesis. Our observations corroborated the earlier reports of high IL-17 in RA and SLE. In the context of a filarial-endemic area, IL-17A levels were significantly low in individuals infected with the filarial parasite compared to ECs. Furthermore, ICs displayed the lowest level of IL-17A compared to the other three clinical categories, suggesting a possible suppressive role of filarial worm products in the production of IL-17A cytokine in the host. This observation is supported by an earlier study in the CIA mouse model in which treatment with parasite antigen ES-62 significantly reduced plasma levels of IL-17 in arthritic animals compared to control mice.[6] Importantly, we observed a significant inverse correlation between plasma levels of IL-17A and CFA. In 11 ICs, the IL-17A levels were below detectable range and nine of them had very high CFA unit levels. These observations are significant, indicating that levels of CFA and/or parasite load could contribute to the control of IL-17A production in the host.

Although studies on animal models have indicated that nematode derivatives work on several immune pathways both of the innate and adaptive immune system,[19] precise demonstration of the effect of filarial ES product on the experimental mouse model of CIA supports our observation that products of the filarial worm act specifically on the Th17 pathway and reduce IL-17 production. It may have other immunomodulatory effects as well through other mechanisms but clearly administration of ES-62 to a CIA model significantly reduces plasma IL-17 levels, and diminishes its action on synoviocytes and production of pro-inflammatory molecules that damage joints

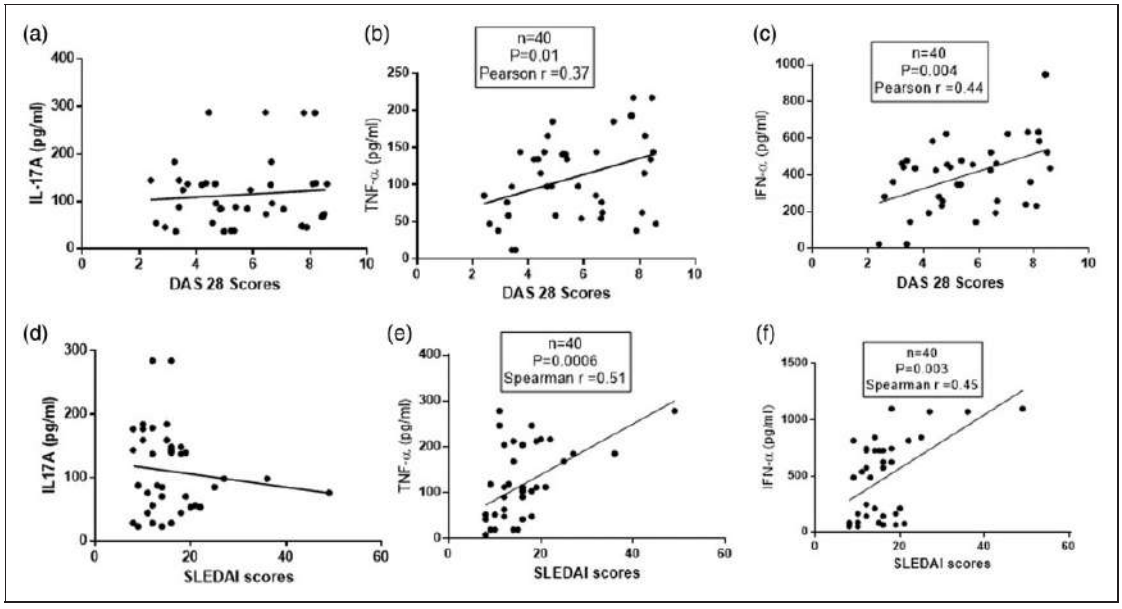

Figure 2 Correlation of plasma cytokines with disease severity. Plasma levels of IL-17A, TNF-α and IFN-α were quantified and correlated with DAS28 ((a), (b) and (c)) and SLEDAI scores ((d), (d) and (f)) based on sample distribution Pearson/Spearman rank correlation and a *p* value less than 0.05 taken as significant. IL-17A: interleukin 17A; TNF-α: tumour necrosis factor alpha; IFN-α: interferon alpha; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

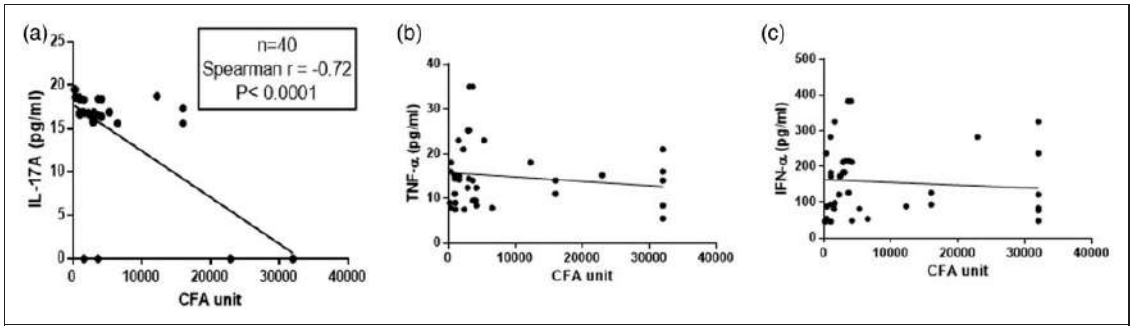

Figure 3 Correlation of plasma parameters with circulating filarial antigen levels (CFA). CFA levels were significantly correlated with plasma levels of IL17A (a) but not with TNF-α (b) and IFN-α (c). Dots represent individual sample. Correlation analysis was performed by Spearman correlation coefficient. A *p* value less than 0.05 was considered significant. IL-17A: interleukin 17A; TNF-α: tumour necrosis factor alpha; IFN-α: interferon alpha.

through Toll-like receptor (TLR)-dependent pathways in RA.[6] Furthermore, ES-62 reduces IL-17 production by CD4+ γδT cells and prevents conversion of naïve CD4+ cells to IL-17 phenotypes.[6] In vitro, ES-62 also suppresses IL-1/IL-23-stimulated production of IL-17 by γδT cells.[6]

The role of TNF-α and IFN-α have also been well documented in autoimmune disorders like RA and SLE, respectively. Plasma levels of TNF-α and IFN-α have been found to be elevated in SLE and have been associated with severity of disease.[21] Similarly, RA patients display higher plasma TNF-α levels compared to healthy controls.[22] Our observations corroborate earlier reports of increased levels of TNF-α and IFN-α in RA and SLE, but in the context of filariasis they were low both in ECs and ICs. However, in acute attacks of filariasis, we had demonstrated high plasma TNF-α compared to ECs, asymptomatic carriers and ICs[23] presumed to be triggered by filarial antigens or secondary bacterial infection. Importantly, there was no significant association between CFA units, TNF-α and IFN-α levels.

Humans and helminths have co-evolved over millions of years in a symbiotic relationship perhaps for mutual benefit; the gain to the

human host – a reduced incidence of allergic and autoimmune disorders.[1] This has been apparent in epidemiological studies demonstrating an inverse relationship between nematode infection and autoimmune diseases – a phenomenon apparent in Western countries where the level of hygiene and sanitation has practically eradicated parasite infections.[24] The filarial parasite is fairly robust and can survive in the human host for many years without causing disease, a situation described as asymptomatic carriers and cryptic infective state. This was demonstrated by our group by following asymptomatic microfilaremic individuals for 13 years.[25] Recently, its role in modulating the host immune system for its survival is being increasingly analysed and the regulation of the Th17 pathway and upregulation of regulatory T (Treg) cells have come into focus.[19] Unregulated IL-17 production is perhaps an important driver in the genesis of autoimmune diseases. The practical application of these observations can have far-reaching consequences in the management of autoimmune diseases if validated by long-term studies on a larger number of cases from different geographical areas.

This study is the first to demonstrate a possible mechanism of action, although association, of a human filarial infection that possibly affects the susceptibility to RA and SLE. Helminth product(s) could be an option in the treatment of autoimmune disorders in the future if interventional studies could be designed in a rational and scientific manner to prove its efficacy.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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TNF-α promoter polymorphisms (G-238A and G-308A) are associated with susceptibility to Systemic Lupus Erythematosus (SLE) and *P. falciparum* malaria: a study in malaria endemic area

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Harishankar Mahto[1,2], Rina Tripathy[3], Biswa Ranjan Meher[4], Birendra K. Prusty[5], Meenakshi Sharma[2], Divya Deogharia[2], Anjana Kumari Saha[2], Aditya K. Panda[1,2] & Bidyut K. Das[6]

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine associated with autoimmune and infectious diseases. Importance of TNF-α in *P. falciparum* malaria and systemic lupus erythematosus (SLE) have been demonstrated. However, association of functional promoter variants with SLE and malaria is lacking in malaria endemic population. A total of 204 female SLE patients and 224 age and sex matched healthy controls were enrolled in the study. Three hundred fourteen *P. falciparum* infected patients with different clinical phenotypes were included. TNF-α polymorphisms (G-238A & G-308A) were genotyped by PCR-RFLP. Plasma levels of TNF-α was quantified by ELISA. Heterozygous mutants and minor alleles of TNF-α (G-238A and G-308A) polymorphisms were significantly higher in SLE patients compared to healthy controls and associated with development of lupus nephritis. In addition, both promoter variants were associated with severe *P. falciparum* malaria. SLE patients demonstrated higher levels of plasma TNF-α compared to healthy controls. TNF-α (G-238A and G-308A) variants were associated with higher plasma TNF-α. In conclusion, TNF-α (G-238A & G-308A) variants are associated with higher plasma TNF-α levels in SLE patients residing in malaria endemic areas and could be a contributing factor in the development of SLE and susceptibility to severe *P. falciparum* malaria.

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine produced by wide range of cells such as macrophages, B cells, T cells and mast cells[1]. TNF-α is primarily produced as a trans-membrane protein that gets released from the membrane by a metalloprotease- TNF alpha converting enzyme (TACE), to form soluble 17 kDa protein[2]. TNF-α is a pleotropic cytokine with wide range of biological functions: it can initiate host defense against infectious diseases and along with it involved in toxicity and inflammatory processes[1]. TNF-α exerts its biological effect through specialized types of receptors viz. TNF receptor 1 (TNFR-1) and TNFR-2[3]. Expression of TNF receptors is tissue specific. TNFR1 is normally observed in most tissues but TNFR2 is restricted to cells of the immune system[3]. TNF-α has both a beneficial and deleterious role and it has been linked with infectious diseases and autoimmune disorders[4–7]. The TNF-α gene is located in short arm of chromosome 6 at position 21.3 and spans about 12 kilobase (kb) length[8]. Till date, 43 single nucleotide polymorphisms (SNPs) at promoter

[1]Department of Bioscience and Bioinformatics, Khallikote University, GMax Building, Konisi, Berhampur, 761008, Odisha, India. [2]Centre for Life Sciences, Central University of Jharkhand, Brambe, Ranchi, 835205, Jharkhand, India. [3]Department of Biochemistry, S.C.B. Medical College, Cuttack, 753007, Odisha, India. [4]Computational Biology and Bioinformatics Laboratory, Department of Botany, Berhampur University, Berhampur, Odisha, 760007, India. [5]Infectious Disease Biology Group, Institute of Life Sciences, Bhubaneswar, Odisha, India. [6]Department of Medicine, S.C.B. Medical College, Cuttack, 753007, Odisha, India. Correspondence and requests for materials should be addressed to A.K.P. (email: adityarmrc@gmail.com) or B.K.D. (email: bidyutdas@hotmail.com)

region of TNF-α gene with minor allele frequency data have been reported (https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=7124). Although there are contradictory reports, some SNPs at promoter region of *TNF-α* have been shown to regulate TNF-α expression and/or soluble TNF-α levels viz. TNF-α G-238A (rs361525), TNF-α G-308A (rs1800629), TNF-α T-857C (rs1799724), and TNF-α T-1031C (rs1799964)[9]. However, large number of genetic association studies have focused on two common promoter polymorphisms of TNF-α gene (G-238A and G-308A) and these have shown a significant association with SLE as well as *P. falciparum* infection in different populations[10,11].

Malaria infection is believed to be an important selection pressure during human evolution and subjects with possible survival advantage genotypes against lethal malaria are more prevalent in malaria endemic areas[12]. This was true across the continents where malaria was endemic. *Plasmodium falciparum* infection is a life-threatening disease with diverse clinical manifestations[13–15]. TNF-α is an important molecule that works like a double-edged sword in malaria infection[16]. TNF-α can protect an individual against severe infection[17] but when production is unregulated it could be damaging to the host. Low levels of TNF-α has been associated with susceptibility to *P. falciparum* infection. While various reports have demonstrated elevated plasma levels of TNF-α in severe malaria compared to uncomplicated infection[18,19]. Mortality in *P. falciparum* infection is also associated with very high plasma levels of TNF-α[18,20]. These observations collectively indicate the importance of TNF-α in *P. falciparum* malaria: optimum levels are essential for protection against infection. Recently, numerous studies have been carried out in different populations to established possible link between TNF-α gene polymorphisms and susceptibility/resistance to *P. falciparum* infection and/or clinical severity[21–24]. Most of the reports[22–24] have included TNF-α promoter polymorphisms, that are believed to affect mRNA expression and alter plasma levels of protein molecule. TNF-α (G-308A) mutants have been associated with susceptibility to *P. falciparum* infection[23], higher levels of parasitaemia[22] and severe malaria[25]. However, an independent study on South-West Nigerian infected patients failed to demonstrate such association[24]. Another common TNF-α promoter (G-238A) variant is also linked to elevated parasitaemia[22] and severe *P. falciparum* malaria[24].

SLE is characterized by production of autoantibodies against self-antigens, formation of immune complexes, deposition of these complexes in tissues leading to organ damage and its failure[26]. Lupus nephritis remains one of the severe clinical manifestations and contributes to significant morbidity and mortality[27,28]. About 50–60% of SLE patients present with kidney dysfunction and the rate of renal affection is higher in Asian population[29]. Although type I interferons have been shown to play an important role in the pathogenesis of lupus nephritis[30], there are cumulative evidence to suggest that TNF-α may also have a crucial role in renal dysfunction[6,31]. This has been demonstrated in the mouse model of SLE (MRL/lpr): elevated levels have been reported in serum and kidney tissue such as glomeruli, vascular smooth muscle, perivascular infiltrating cells and tubular epithelial cells[32,33]. Furthermore, the severity of proteinuria has been found to correlate with the degree of TNF-α expression in the kidney[32]. There are several reports of elevated TNF-α in SLE patients[34–37]. In a study involving African American, European American and Hispanic American SLE patients, high levels of plasma TNF-α was observed and a positive correlation with IFN-α was demonstrated[37]. A significant positive correlation between plasma TNF-α, with clinical severity and anti-ds DNA has also been reported in several studies[34–36]. Interestingly, TNF-α expression was found to be high in the renal tissue of patients with lupus nephritis[38,39]. Defective clearance of apoptotic bodies has been suggested to be an important factor in the pathogenesis in SLE[40,41]. TNF-α has been shown to induce apoptosis[42–44]. Elevated TNF-α in SLE patients could be one of the reasons for increased apoptosis, elevated production of nuclear debris followed by defective clearance of dead or dying cells. However, anti TNF-α therapy has provided no therapeutic advantage in the treatment of SLE[45,46].

Importance of TNF-α in *P. falciparum* malaria has been widely investigated[16,47,48] and it has been demonstrated that TNF-α is an important molecule for parasite clearance[49]. However, uncontrolled production of this cytokine during *P. falciparum* infection can lead to clinical complications[50,51]. In malaria endemic areas, subjects with moderate TNF-α producing genotypes have survival advantage[12]. The association of TNF-α in mouse models of lupus[6,52] and in the clinical manifestations in humans has been documented[34–37]. For instance, higher expression of TNF-α has been associated with lupus nephritis[38,39]. Since TNF-α appears to be important to some aspects of the pathogenesis in both SLE and *P. falciparum* malaria, we hypothesized a possible relationship between TNF-α promoter variants with predisposition to SLE, notably lupus nephritis, in patients residing in a malaria endemic area.

There are limited studies in the Indian population[10,53,54], especially, in the malaria endemic belts, for possible association between TNF-α polymorphisms (G-238A and G-308A) and SLE. A recent study has shown a significant association between heterozygotes and minor allele to SLE[10]. A study from south of India demonstrated an association between TNF-α promoter haplotype and protection against SLE[53]. In our study, we have enrolled SLE patients and controls from malaria endemic areas of Eastern India and investigated the association of TNF-α promoter variants with SLE. Furthermore, we have quantified plasma levels of TNF-α to assess the genotype-phenotype relationship. The novelty of our study relies on the enrolment of SLE patients and *P. falciparum* infected cases from malarial endemic areas. The question we have addressed is whether individuals from malarial endemic areas are vulnerable to the development of SLE if genetically susceptible.

Results

Baseline characteristics. In the present study, we enrolled 428 female subjects (224 healthy controls and 204 SLE patients) and 314 *P. falciparum* infected patients including 103 uncomplicated malaria (UM), 68 cerebral malaria (CM), eighty four multi organ dysfunctions (MODs) and 59 non-cerebral severe malaria (NCSM) (Table 1).

Prevalence of TNF-α promoter (G-238A & G-308A) polymorphisms. Prevalence of heterozygous (GA) and homozygous mutants (AA) for G-238A polymorphism was 12% and 1% respectively (Table 2).

Clinical profiles	SLE (n = 204)	Healthy control (n = 224)	*P. falciparum* infected patients (n = 314)			
			UM (n = 103)	CM (n = 68)	MOD (n = 84)	NCSM (n = 59)
Sex (male/female)	0/204	0/224	84/19	52/16	69/15	47/12
Age in years (mean ± SD)	27.84 ± 8.83	29.56 ± 5.48	33.18 ± 13.60	32.91 ± 14.89	34.54 ± 14.24	33.36 ± 13.28
Duration of disease in years (mean ± SD)	2.07 ± 1.13	—	—	—	—	—
ACR criteria						
Photosensitivity rash	63 (31)	—	—	—	—	—
Malar rash	84(41)	—	—	—	—	—
Discoid rash	28 (14)	—	—	—	—	—
Oral ulcer	97 (48)	—	—	—	—	—
Arthritis	103 (50)	—	—	—	—	—
NPSLE	12 (6)	—	—	—	—	—
AIHA	6 (3)	—	—	—	—	—
Serositis	9 (4)	—	—	—	—	—
Nephritis	83 (41)	—	—	—	—	—
Pneumonitis	9 (4)	—	—	—	—	—

Table 1. Clinical baseline characteristics of SLE patients, *P. falciparum* infected cases and healthy controls. Note. Data are no. (%) of participants unless otherwise specified. NPSLE, Neuropsychiatric systemic lupus erythematosus; AIHA, autoimmune hemolytic anemia.

SNPs	Genotype or allele	HC (n = 224)	SLE (n = 204)	P value	OR (95% CI)
G-238A	GG	195 (87)	159 (78)	1	Ref.
	GA	26 (12)	43 (21)	0.008	2.02 (1.19–3.44)
	AA	3 (1)	2 (1)	1.000	0.81 (0.13–4.95)
	G	416 (93)	361 (88)	1	Ref.
	A	32 (7)	47 (12)	0.032	1.69 (1.05–2.71)
G-308A	GG	194(87)	153 (75)	1	Ref.
	GA	25 (11)	43 (21)	0.005	2.18 (1.27–3.73)
	AA	5 (2)	8 (4)	0.261	2.02 (0.65–6.32)
	G	413 (92)	349 (86)	1	Ref
	A	35 (8)	59 (14)	0.002	1.99 (1.28–3.10)

Table 2. Distribution of TNF-α (−308G/A and −238G/A) polymorphisms in SLE patients and healthy controls. Note: Data are no. (%) of participants unless otherwise specified. HC = healthy control; SLE = systemic lupus erythematosus; OR = odds ratio; 95% CI = 95% confidence interval.

Similarly, GA and AA genotype frequency for G-308A polymorphism was 11% and 2% respectively. Distributions of TNF-α(G-308A) polymorphism in healthy female controls deviated from Hardy-Weinberg equilibrium (HWE) (G-308A: $\chi^2 = 11.35$, P value = 0.0007; G-238A: $\chi^2 = 3.5$, P value = 0.061).

TNF-α (G-238A and G-308A) polymorphism are associated with SLE. As shown in Table 2, the prevalence of GA and minor allele 'A' for TNF-α (G-308A) polymorphism were significantly high in SLE patients compared to healthy controls (GA: P = 0.005, OR = 2.18; A: P = 0.002, OR = 1.99). Similarly, frequency of GA for TNF-α (G-238A) polymorphism were more frequent in SLE patients than healthy female controls (P = 0.008, OR = 2.02). Although minor allele for TNF-α (G-238A) polymorphism was more frequent in SLE patients compared to controls, the difference was not significant after Bonferroni correction (P = 0.032, OR = 1.69).

Furthermore, haplotype analysis (G-308A/G-238A) showed significantly higher prevalence of A-G and A-A in SLE patients compared to healthy controls (A-G: P = 0.049, OR = 1.63; A-A: P = 0.029, OR = 2.57) (Supplementary Table 1).

Distribution of TNF-α (G-238A and G-308A) polymorphism in patients with nephritis. Since the study revealed a significant association between TNF-α promoter (G-238A and G-308A) polymorphisms and SLE, we analyzed the association of these polymorphisms with organ involvement. Lupus nephritis was the most important clinical phenotype observed and often associated with increased mortality in SLE. In our study, 41% of SLE patients had lupus nephritis. We categorized the patients into two broad groups: (1) Patients with lupus nephritis (LN+), and (2) patients without nephritis (LN−). As depicted in Table 3, GA genotype and minor allele (A) of TNF-α (G-238A) polymorphism was more frequent in patients with lupus nephritis (LN^+) compared to those patients without renal involvement (LN^-) (GA: P = 0.002, OR = 2.89;A: P < 0.001, OR = 2.92). However, distribution of TNF-α (G-308A) polymorphism was comparable among both groups.

SNPs	Genotype or allele	LN^- (n = 121)	LN^+ (n = 83)	P value	OR (95% CI)
G-238A	GG	104 (86)	55 (66)	1	Ref.
	GA	17 (14)	26 (31)	0.002	2.89 (1.44–5.78)
	AA	0 (0)	2 (3)	0.123	9.41(0.44–199.7)
	G	225 (93)	136 (82)	1	Ref.
	A	17 (7)	30 (18)	<0.001	2.92 (1.55–5.49)
G-308A	GG	97 (80)	56 (66)	1	Ref.
	GA	19 (16)	24 (30)	0.034	2.18 (1.10–4.34)
	AA	5 (4)	3 (4)	1.000	1.03 (0.23–4.51)
	G	213 (88)	136 (82)	1	Ref
	A	29 (12)	30 (18)	0.11	1.62 (0.93–2.82)

Table 3. Prevalence of TNF-α (G-308A and G-238A) polymorphisms in lupus nephritis and non-nephritis SLE patients. Note: Data are no. (%) of participants unless otherwise specified. LN^+ = lupus nephritis; LN^- = non-lupus nephritis; OR = odds ratio; 95% CI = 95% confidence interval.

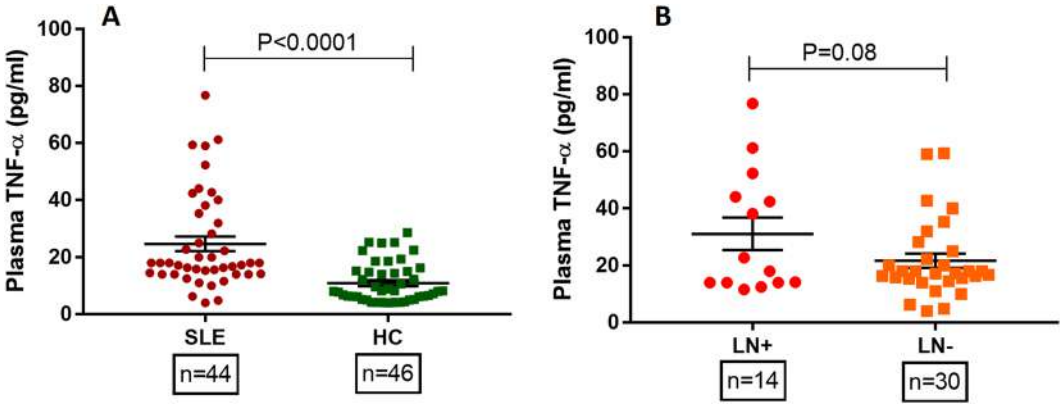

Figure 1. Plasma TNF-α levels in SLE patients and healthy controls. (**A**) Plasma TNF-α levels was quantified by ELISA in SLE patients (n = 44) and healthy controls (n = 46) and the mean TNF-α were compared by student's t test. SLE patients displayed significantly higher TNF-α levels compared to healthy controls ($P < 0.001$). (**B**) SLE patients were categorized in to two broad group, presence (n = 14) or absence of kidney involvement (n = 30) and mean TNF-α levels was compared. P value less than 0.05 was considered as significant. LN^+: lupus nephritis patients; LN^-: SLE patients without kidney involvement.

Plasma TNF-α level in SLE patient & healthy controls. We quantified plasma TNF-α in 90 samples (SLE: 44, HC: 46) by ELISA and mean TNF-α in each group was compared by unpaired 't' test. SLE patients had significantly higher levels of TNF-α compared to healthy controls ($P < 0.0001$). Plasma levels of TNF-α were compared between LN^+ and LN^- by student's t test and results are shown in Fig. 1. The difference in mean TNF-α levels among LN+ and LN^- SLE patients was not statistically significant ($P = 0.08$).

Genotype-phenotype association of TNF-α (G-238A and G-308A) polymorphisms. Several studies have demonstrated a functional relevance of TNF-α promoter polymorphisms (G-238A and G-308A) with expression of TNF -α. We compared plasma levels of TNF-α among different genotypes of TNF-α (G-238A and G-308A). As shown in Fig. 2A,B, for both promoter polymorphism (G-238A and G-308A) the major genotype GG expressed significantly lower levels of plasma TNF-α compared to heterozygous mutant (GA)($P < 0.0001$) and homozygous minor genotypes (AA) (G-238A: $P = 0.005$; G-308A: $P = 0.002$). Furthermore, we analyzed association of both promoter polymorphism with plasma levels of TNF-α in SLE patients and healthy controls independently (data not shown) and interestingly the observations remained consistent.

Association of TNF-α (G-238A and G-308A) polymorphisms with P. falciparum malaria. Association between TNF-α (G-238A and G-308A) polymorphisms and susceptibility to *P. falciparum* malaria have been well documented. In the present study, we enrolled 314 *P. falciparum* infected cases comprising of 103 uncomplicated cases and 211 severe malaria patients and genotyped for TNF-α (G-238A and G-308A) polymorphisms. As shown in Table 4, heterozygous genotype for TNF-α (G-238A) polymorphism and minor allele of G-308A polymorphism were more frequent in SM than UM (GA: $P = 0.02$, OR = 2.09; A: $P = 0.02$, OR = 2.05).

Severe malaria patients were further sub-categorized in to CM, MOD and NCSM and distributions of genotypes and allele were compared with UM cases. Results are shown in Table 4. Distributions of heterozygous genotype (GA), minor allele (A) were significantly higher in MOD compared to UM for both TNF-α promoter

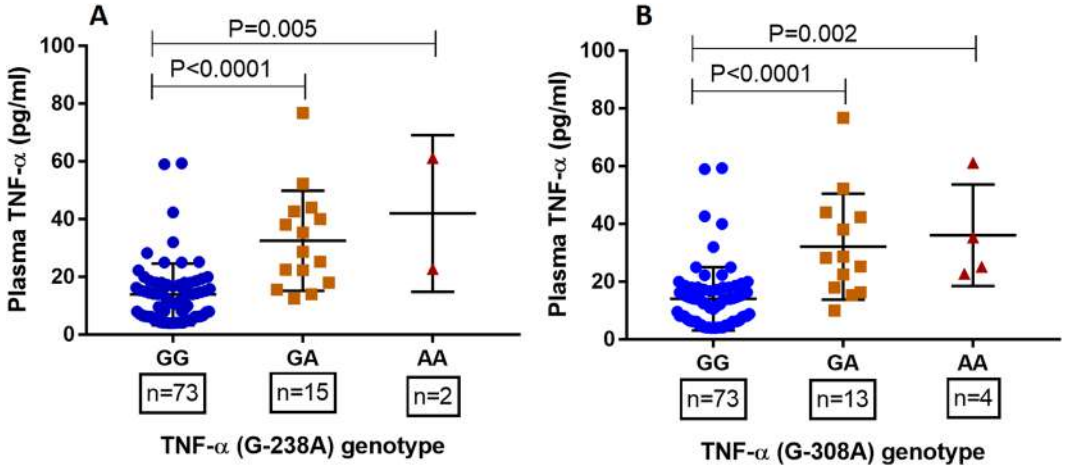

Figure 2. Association between TNF-α polymorphisms and levels of plasma TNF-α in SLE patients and control. Plasma TNF-α levels was measured by ELISA, based on availability of plasma samples (SLE: n = 44; HC: n = 46), and correlated with TNF-α genotypes (**A)** G-238A polymorphism and (**B**) G-308A polymorphism). Mean plasma TNF-α levels of various genotypes was compared by ANOVA followed by tukey's multiple comparison post-test. P value less than 0.05 was considered as significant.

polymorphisms (G-238A and G-308A). Prevalence of TNF-α (G-308A) heterozygous genotype (GA) was significantly higher in CM cases compared to UM (P = 0.02, OR = 2.65). Comparison of haplotype distribution revealed a significant association of A-A haplotype with predisposition to SM (P = 0.047, OR = 2.33) and MOD (P = 0.011, OR = 3.35) development (Data not shown).

Severe malaria patients displayed higher plasma TNF-α than uncomplicated cases. Severe malaria patients displayed significantly higher plasma TNF-α when compared to uncomplicated *P. falciparum* infected patients (P = 0.003) (Fig. 3A). Based on various organs involvement, severe malaria patients were further categorized into a) CM [n = 16], b) MOD [n = 21] c) NCSM [n = 18] and compared with uncomplicated cases. Patients with MOD displayed significantly higher levels of plasma TNF-α compared to NCSM (P = 0.004) and UM (P = 0.0002). In addition, a significant difference in mean plasma levels of TNF-α was also observed among CM and UM (P = 0.04) (Fig. 3B).

***In silico* analysis.** We observed a significant correlation between TNF-α polymorphisms (G-238A and G-308A) and plasma levels of TNF-α. To validate the above findings, we analyzed functional relevance of these variants *in silico*. SNPs (rs1800629 and rs361525) were submitted to the FuncPred program and results obtained are shown in Supplementary Table 2. Both the SNPs were found to affect transcription factor binding site (TFBS). However, none of them affect miRNA binding site. SNP with ID rs1800629 was found to have a regulatory potential (RegPot) of 0.0401, which was also an indication of regulatory effects on binding and expression of gene targets.

RegulomeDB database has divided both the SNPs into two distinct categories (Category 1d and Category 4 as shown in Supplementary Table 3). rs1800629 showed RegulomeDB score of 1d and rs361525 which has minimal binding evidence (Category 4). The top ranked SNP rs1800629 had annotation for eQTL + TF binding + any motif + DNase peak and thus very likely to have regulatory functions.

Resampling analysis. As the samples size investigated in the present study was smaller, we performed a resampling analysis and data are shown in Supplementary Table 4. TNF-α (G-308A and G-238A) variants and minor alleles were more frequent in SLE cases and lupus nephritis cases suggesting an important role of TNF-α variants with predisposition to SLE and clinical manifestations.

Discussion

TNF-α is an important cytokine in the pathogenesis as well as control of *P. falciparum* infection[16]. Therefore, higher levels observed in malaria infection is a protective phenomenon but very high levels can contribute to severity and mortality. The role of TNF-α in SLE is still conjectural but there are studies implicating it as a contributory factor in the pathogenesis based on experimental and associational studies[6,55]. It is important to understand the link between TNF-α and SLE in patients residing in malarial endemic areas. In the present study, we observed elevated plasma levels of TNF-α in SLE patients. Furthermore, TNF-α promoter polymorphisms (G-238A and G-308A) were significantly associated with higher plasma levels. These observations provide evidence of a possible role for TNF-α in the pathogenesis of SLE but the precise mechanism(s) is not yet known[6,55]. TNF-α is a pleotropic cytokine and acts at multiple levels[1]. In genetically susceptible SLE individuals, malaria might be a trigger for increased production of TNF-α, besides other cytokines, triggering a cascade of events contributing to the development of SLE[10,56–58].

TNF-α polymorphisms		Clinical categories, (%) of subject				UM Vs CM		UM Vs MOD		UM Vs NCSM		UM Vs SM	
TNF-α G-238A	UM (n = 103)	CM (n = 68)	MOD (n = 84)	NCSM (n = 59)	SM (n = 211)	OR (95% CI)	P Value	OR (95% CI)	P Value	OR (95% CI)	P value	OR (95% CI)	P value
GG	88 (85)	50 (73)	56 (67)	50 (84)	156 (74)	1	Ref	1	Ref	1	Ref	1	Ref
GA	14 (14)	17 (25)	27 (32)	8 (14)	52 (25)	2.13 (0.97 to 4.70)	0.06	**3.03 (1.46 to 6.27)**	**0.002**	1.00 (0.39 to 2.56)	1.00	**2.09 (1.09 to 3.99)**	**0.02**
AA	1 (1)	1 (2)	1 (1)	1 (2)	3 (1)	1.76 (0.10 to 28.77)	1.00	1.57 (0.09 to 25.65)	1.00	1.76 (0.10 to 28.77)	1.00	1.69 (0.17 to 16.52)	1.00
G	190 (92)	117 (86)	139 (83)	108 (91)	367 (87)	1	Ref	1	Ref	1	Ref	1	Ref
A	16 (8)	19 (14)	29 (17)	10 (9)	55 (13)	1.92 (0.95 to 3.89)	0.07	**2.47 (1.29 to 4.73)**	**0.006**	1.10 (0.48 to 2.50)	0.83	1.78 (0.99 to 3.19)	0.05
TNF-α G-308A													
GG	91 (88)	52 (76)	57 (68)	52 (88)	161 (76)	1	Ref	1	Ref	1	Ref	1	Ref
GA	10 (10)	15 (22)	24 (28)	7 (12)	46 (22)	**2.65 (1.11 to 6.33)**	**0.02**	**3.83 (1.70 to 8.60)**	**0.001**	1.23 (0.44 to 3.44)	0.79	0.91 (0.39 to 8.09)	0.83
AA	2 (2)	1 (2)	3 (4)	0 (0)	4 (2)	0.88 (0.07 to 9.99)	1.00	2.42 (0.39 to 14.94)	0.37	0.35 (0.01 to 7.48)	0.53	1.14 (0.20 to 6.36)	1.00
G	192 (93)	118 (87)	138 (82)	111 (94)	367 (87)	1	Ref	1	Ref	1	Ref	1	Ref
A	14 (7)	18 (13)	30 (18)	7 (6)	55 (13)	2.09 (1.00 to 4.36)	0.05	**2.98 (1.52 to 5.83)**	**0.001**	0.86 (0.33 to 2.20)	0.81	**2.05 (1.11 to 3.79)**	**0.02**

Table 4. Distribution of TNF-α (G-238A and G-308A) polymorphisms in *P. falciparum* malaria. Note: Data are no. (%) of participants unless otherwise specified. UM = uncomplicated malaria; CM = cerebral malaria; MOD = multi organ dysfunction; NCSM = non cerebral severe malaria; OR = odds ratio; 95% CI = 95% confidence interval.

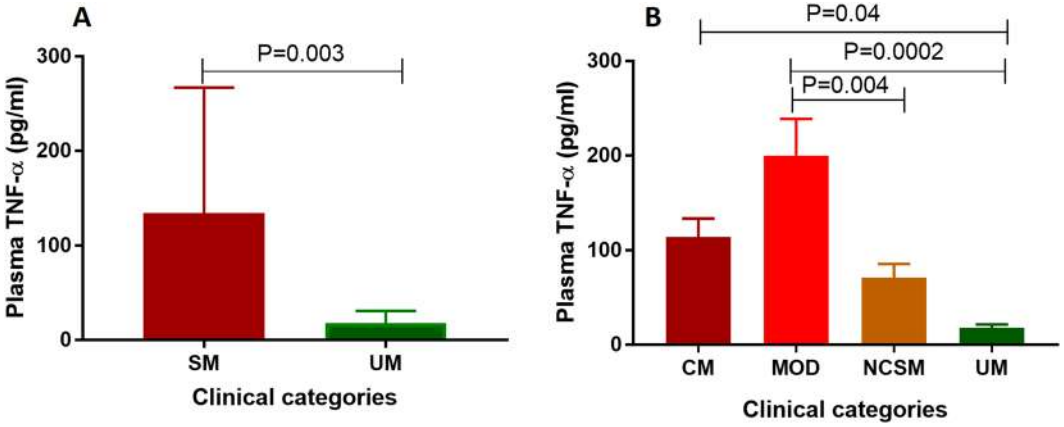

Figure 3. Plasma TNF-α in different clinical categories of *P. falciparum* malaria. (**A**) Plasma TNF-α levels was quantified by ELISA in uncomplicated malaria cases (UM) (n = 12) and severe malaria patients (SM) (n = 55) and the mean TNF-α values were compared by student's t test. Severe malarial cases displayed significantly higher TNF-α levels compared to uncomplicated malaria (P = 0.0003). (**B**) Severe malaria cases were further categorized clinically into four sub groups viz. cerebral malaria (CM, n = 16), multi organ dysfunction (MOD, n = 21), non-cerebral severe malaria (NCSM, n = 18) and mean TNF-α levels was compared among them. P value less than 0.05 was considered as significant.

P. falciparum malaria is predominantly endemic in the Eastern and Northeastern parts of India[59]. But it remains endemic in most parts of the country. Prevalence of TNF-α promoter polymorphisms (G-238A and G-308A) have been reported in Indian population. Distributions of TNF-α (G-238A and G-308A) genotypes were comparable with previous reports from different parts of the country[60],- South-West[61], North[62] and North-West[63] regions. In most of the earlier reports[10,60–63] distribution of TNF-α promoter variants were compatible with HWE in healthy controls. In the present study distribution of TNF-α (G-308A) genotypes deviated from HWE in healthy females (P = 0.0007). The geographical area of Odisha is highly endemic for *P. falciparum* malaria which contributes to high mortality due to malaria in the country[64]. Deviation of genotype distribution has been attributed to several factors, and selection pressure remains one of the important causes[65]. The studied population is endemic to various infectious diseases other than malaria and could be the reason for increased selection pressure on host genome[66]. Interestingly, two independent studies from South[67] and North India[68] have reported higher prevalence of heterozygotes (GA) compared to homozygous (GG or AA). They have also shown a deviation of TNF-α G-308A genotypes from HWE. These abnormalities in observations could be due to genotyping methods (ARMS PCR/sequence specific primer PCR) which could give spurious results.

Role of TNF-α promoter variants in SLE have been widely investigated. A recent meta-analysis including 41 published studies worldwide, showed association of minor allele (A) and AA genotype of TNF-α (G-308A) polymorphism with susceptibility to development of SLE[11]. In the present analysis, we observed higher prevalance of heterozygous (GA) and minor allele (A) in SLE patients compared to healthy females, suggesting a possible

role of TNF-α (G-308A) polymorphism in susceptible to SLE. Similar observations have been reported in SLE patients from different geographical areas such as Brazil, Colombo, Mexico, North America, Spain, Taiwan[11] and South India[10]. However, contradictory results have also been reported in Portugese, Thai, Chinese, Italian, African American, Japanese and Argentenian populations[11]. These discrepancies have been attributed to ethnicity of subjects enrolled for case-control studies and further supported by ethnicity related meta-analysis which revealed significant link between allele 'A' with predisposition to SLE in Europeans, Asians and South and North Americans but surprisingly not in African population[11]. In the present study, TNF-α (G-238A) heterozygous and minor allele (A) were also associated with susceptibility to SLE and it corroborated with other observations. Although the exact mechanism related to TNF-α polymorphism and SLE is yet to be understood, results of the present study and previous reports across the world indicates a strong association of TNF-α promoter variants, higher expression of TNF-α m-RNA and elevated levels of plasma TNF-α in SLE patients from malaria endemic regions.

We analysed the possible association of TNF-α polymorphisms with clinical manifestations of SLE, namely lupus nephritis which is one of the major clinical phenotypes linked to SLE mortality. We observed that heterozygous (GA) and minor allele 'A' of TNF-α (G-238A) polymorphism were significantly associated with lupus nephritis. These observations have been corroborated by a recent study on Chinese SLE patients[11]. However it contradicts an observation from South Indian population[10] and a recent meta-analysis[11]. Furthermore, patients with lupus nephritis had higher levels of plasma TNF-α than those without nephritis.

Functional relevance of TNF-α promoter polymorphisms (G-308A and G-238A) have not been widely investigated. Minor allele for TNF-α (G-308A) polymorphism has been observed to enhance the binding of transcription factors and is associated with increase in mRNA production compared to major allele (G)[69]. *In vitro* stimulation of peripheral blood mononuclear cells (PBMC) derived from heterozygous subjects (GA) with lipopolysaccharide, displayed higher TNF-α than those of wild type individuals (GG)[70]. Furthermore, elevated plasma TNF-α has been associated with mutants for TNF-α (G-308A) polymorphism[10]. In the present study, we observed higher plasma levels of TNF-α in GA and AA genotypes compared to GG, corroborating earlier observations. Interestingly, other TNF-α promoter polymorphism (G-238A) also revealed similar results: mutants (GA and AA) were associated with higher plasma TNF-α than wild type (GG), which corroborates with an earlier report[71]. Furthermore, we performed *in silico* analysis which revealed regulatory effect in binding of transcription factors and enhanced expression of TNF-α gene. Results of the present investigation and earlier reports demonstrate signifcant regulatory role of promoter polymorphisms.

Investigations on possible link between malaria and SLE are limited and contradictory. Epidemiological data have shown lower prevalence of autoimmune diseases in areas where malaria incidence is high[72]. However, in an earlier observation, we have demonstrated protection against severe malaria and malarial death in complement receptor 1 variants and concluded a possible reason for higher prevalence of CR1 mutants in malaria endemic areas[14]. We had also observed that CR-1 mutants are susceptible to development of SLE and lupus nephritis since they expressed lower surface CR1 which affects clearance of apoptotic debris[73]. Furthermore, similar association of FcγRIIb variant (codon 232) with susceptibility to SLE and protection against *P. falciparum* malaria has been reported[74]. Lower parasitaemia and minimal clinical severity has been reported in FcγRIIb deficient mice when infected with non-lethal murine *plasmodium* strain indicating protective nature of the truncated or deficient FcγRIIb against malaria[74]. This observation has been further supported by higher prevalence of FcγRIIb codon 232 mutant in African and Asian population when compared to other populations across the world where malaria is endemic. The results of the present study and earlier reports collectively demonstrate that certain genotypes are beneficial in protecting humans against *P. falciparum* malaria and are highly prevalent in malaria endemic areas. Unfortunately, subjects genetically susceptible to SLE and residing in malarial endemic areas have a greater chance to develop SLE compared to those residing in non-endemic areas.

In conclusion, elevated plasma TNF-α is observed in SLE patients and associated with clinical severity. Furthermore, promoter variants of TNF-α gene, associated with higher TNF-α expression, were more prevalent in SLE patients. TNF-α is essential for clearance of malarial parasites[49] and people residing in malarial endemic areas often produce optimal levels of TNF-α[19] which could be helpful in combating the infection. It could also be one of the contributory factors for inducing SLE in genetically susceptible individuals. Further studies from other malarial endemic areas in the world are important to validate our findings.

Materials and Methods

Subjects. Gender wise analysis has been recommended in numerous earlier reports of genetic association studies[73,75,76]. SLE is a chronic inflammatory autoimmune disorder and mostly prevalent in females[77]. In the present study, we enrolled 428 female subjects (224 healthy controls and 204 SLE patients) to investigate possible association of TNF-α polymorphism in SLE. Patients of SLE were diagnosed based on the revised American College of Rheumatology (ACR) classification criteria[78] and analyzed based on various clinical manifestations (Table 1). In addition, we enrolled 314 *P. falciparum* infected patients who reported to or were admitted to Department of Medicine, SCB Medical College, Cuttack, Odisha. Clinical categorization of *falciparum* infected patients were performed as described earlier[14,15,73,79]. Healthy females, age matched and residing in the same geographical areas, with no prior history of autoimmune disorders were enrolled as controls (HC). About 5 ml blood was collected from each participant. Plasma was separated and stored at −80 degrees centigrade for later use. The study was approved by the Institutional Human Ethics Committee of Central University of Jharkhand, India and S.C.B. Medical College Cuttack, Odisha, India. Informed written consent was obtained from each patient. The study was conducted in accordance with methods approved by IHECs.

DNA isolation and genotyping of TNF-α (G-238A and G-308A) polymorphisms. Whole genomic DNA was purified from blood samples using Gen Elute Blood Genomic DNA mini Kit (Sigma-Aldrich) according to manufacturer's instructions. TNF-α promoter polymorphisms (G-238A & G-308A) were genotyped by

polymerase chain reaction followed by restriction fragment length polymorphism method as described earlier (Galbraith *et al*. 1998).

TNF-α quantification. The plasma TNF-α levels in SLE patients, healthy controls and *P. falciparum* infected cases were quantified by enzyme linked immunosorbent assay (ELISA) kit (eBiosciences) according to manufacturer's instructions.

Non-coding SNP functional analysis. In order to recognize the effect of SNPs in non-coding regions, tools predicting probable functional effect of SNPs at transcription factor binding sites (TFBS), Intron/exon border consensus sequences (splice sites), Exonic splicing enhancers (ESEs), and miRNA binding were utilized. SNPinfo (FuncPred) and RegulomeDB offer a pool of functional information using series of tools. The SNPs functionality was defined by SNPinfo (FuncPred) (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.php) web server[80], which helps in selecting SNPs for genetic association studies. For the current study, two SNPs (rs1800629 and rs361525) were uploaded for batch analysis with the default settings. The output information was a list of SNPs with possible functional effect.

To supplement SNP ranking, SNPs were further analyzed by RegulomeDB (http://regulomedb.org/)[81]. RegulomeDB categorizes variants into six categories ranging from 1 to 6, where category 1 variants are 'likely to affect binding and linked to expression of a gene target', category 2 variants are 'likely to affect binding', Category 3 variants are 'less likely to affect binding', and Category 4, 5 and 6 variants have 'minimal binding evidence'. RegulomeDB also allocates a score of 7 for variants with no annotation data available. dbSNP rsIDs were utilized as input for the current study.

Statistical analysis. Genotype and allele distribution among different clinical categories was compared by Fisher's exact test. P value less than 0.02 was taken as significant (Bonferroni correction for two SNPs $0.05/2 = 0.02$). The mean plasma levels of TNF-α in SLE patients and healthy controls was compared by student's t test and analysis of variance (ANOVA) was employed for study difference in plasma TNF-α in different clinical categories of *P. falciparum* malaria. The association of TNF-α (G-238A and G-308A) genotypes with plasma TNF-α levels were analyzed by unpaired 't' test or ANOVA followed by an appropriate post-test. Graph Pad Prism 5.01 software was used for these statistical analyses. Haplotype analysis was performed by SNAP Stats online tool. Resampling analysis was performed by bootstrap method in Microsoft excel sheet attached as supplementary file-1.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

H.M., B.K.P., M.S., D.D. and A.K.S. performed genotyping and analysis of data. B.R.M. performed in silico analysis, H.M. wrote first draft of the manuscript. B.K.D. and R.T. enrolled patients, clinical categorization and maintain clinical data of patients. A.K.P., R.T. and B.K.D. designed, work supervised and interpret and finalized the paper.

Additional Information

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