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Elevated Level of Plasma Interleukin-18 (IL-18) in Leprosy Patients with Reversal (T1R) and Enl (T2R) Reactions

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Abstract

The clinical management of leprosy type 1 and type 2 reactions poseschallenges mainly because they can cause severe nerve injury and disability. No laboratory test or marker is available for the diagnosis or prognosis of leprosy. This study screened IL-18 level in plasma in samples to identify circulating biomarker associated with type 1 and type 2 lepra reactions. In this study IL-18 level was compared in T1R (10) and T2R (9) with NRL (n=20) and NHC (n=43). IL-18 level was measured using commercial kit. IL-18 level was significantly higher in the T1R and T2R groups when compared to the NRL and NHC groups (p=0.0001). The level was significantly different in T1R group and T2R group (p=0.0343). The BTR group showed the highest IL-18 plasma levels among the other groups in the disease spectrum in reactional leprosy patients (mean±sD)(3483.38±1035.63). Bacteriological index (BI), was showed negative correlation with IL-18 plasma levels. The results specify that IL-18 plasma levels are elevated during T1R and T2R leprosy reactions and thus may play a role in the regulation of inflammatory responses associated with reactions in leprosy.

Keywords: *Reactions; Cytokines; Interleukin-18 (IL-18), Bacterial Index (BI), Mycobacterium leprae (M. leprae)*

Introduction

Leprosy reactions are major causes of hospitalization and disability of patients with leprosy, a granulomatous disease of the skin and peripheral nerves caused by Mycobacterium leprae [1]. Leprosy patients show a spectrum of clinical manifestation which is largely depends on the immunological response of host to antigen of Mycobacterium leprae. Patients may undergo immunological changes known as "reactional state" (reversal reaction and erythema nodosum leprosum) that result major clinical deterioration. "Type 1"reaction (T1R), occurs in 30- 40% of borderline leprosy patients with heightened cellular immune response to M. leprae [2]. T1R usually develops abruptly as exacerbations of pre- exesting skin and nerve lesions. Hence, research activities have been dedicated toward a better understanding of T1Rs and their relationship with overall leprosy pathogenesis. The main dermato-pathological finding in T1R are an increased infiltration of lymphocytes in the dermis, interagranuloma edema with, loss of normal granuloma structure [3-4].

On the other hand, "Type 2" reactions (T2R), occur only in lepromatous (LL) and borderline lepromatous (BL) patients with a high bacterial load [bacterial index (BI)] with little or no cellular immunity to M. leprae. The occurrence of ulcerated necrotic, pustular and bullous forms has also been reported. Some

nodules may persist, as a chromic painful panniculitis leading to fibrosis and scaring [5].

Various risk factors such as female gender, multibacillary disease, skin lesions with high BI are reported to be associated with development of reversal reaction [6]. However, older age group patients more than 40 years have been shown a significantly decreased risk of ENL [7]. It has been reported that the incidence of T1R was highest during 6 to 12 months after multidrug therapy (MDT) [6]. ENL reaction was also noted, reversal reaction (RR) is known to occur mostly during second and third year of starting MDT [7]. Late reversal reaction (RR) is known to occur mostly within the first 3 to 4 years after RFT [8- 9] and 9.7% patients develop recurrent episode of RR. Female gender and pregnancy or lactation has also been reported to be risk factors for reactions changes which include alternation in lepromin reaction and in the reactivity of T cells to M. leprae antigens.

Cytokines play a critical role in regulating all aspects of immune responses, including lymphoid development, homeostasis, differentiation, tolerance, inflammation and memory [10]. Interleukin-17F has been reported to increase in type1 reaction [11]. T1R have been characterized by heightened in situ. Th1 immunity with infiltration of IFN-γ and TNF-α, secreting CD4T cells in skin lesion and serum. Plasma levels of CXCL10 were noted significantly higher in association with T1R, but plasma levels of IFN-Ɣ were not elevated (12). T2R is manifested by a systemic inflammatory response characterized by neutrophil infiltration, activation of complement deposition of extra vascular immune complexes with high levels of TNF-α in tissue lesion and in the circulation [13-15].

Interleukin-18 (IL-18), also known as interferon-γ inducing factor a cytokine that belongs to the IL-1 superfamily and is produced by macrophages and other cells. IL-18 works by binding to the interleukin-18 receptor and together with IL-12 it induces cell mediated immunity following infection with microbial products like lipopolysaccharide (LPS). After stimulation with IL-18, natural killer (NK) cells and certain T cells release another important cytokine called interferon-γ that plays an important role in activating the macrophages or other cells. IL-18 acts together with IL-12 as an early signal in the development of Th1 responses [16- 17]. However, IL-18 alone does not induce IFN-γ production by T lymphocytes [17]. The presence of secondary stimulants, particularly IL-12 or microbial agents, is required for IL-18 induced IFN-γ production [19]. Despite evidences for rise in the levels of various immunological and molecular mediators leprosy reactions, no individual marker or combination of markers has been sufficiently identified with reaction to enable its use as a laboratory test for the diagnosis or management of T1R and T2R . The goal of this study was to screen plasma levels of IL-18 and its association with in type 1 and type 2 leprosy reactions in leprosy.

Material And Methods

Patients / subjects

Patients and healthy volunteers consented to take part in this study. The study population designed of active untreated leprosy cases in T1R (n=10) the time of their initial diagnosis . These patients had severely in-durated and erythematous lesions at the site of previous indolent macules, according to the medical history. T2R (n=9) were selected among patients who had the T2R at diagnosis or during follow up (n=6), NRL cases (n=20) control and NL cases (n=43) (control group) recruited for estimation of normal values and threshold data). The demographic and clinical characteristic of the cases and control are shown in (Table-1).

The following case definitions were employed: T1R was defined as an acute the following clinical manifestation, usually characterized by the exacerbation of pre exiting lesion, the appearance of new lesions with or without reunites. T2R was characterized by the sudden appearance of tender erythematous skin nodules mainly accompanied by fever and other systemic symptoms such as joint pain , bone tenderness, neuritis, edema , malaise, anorexia patients with some histopathological classification as the reaction patients attending the out patients

department of National JALMA Institute for Leprosy and Other Mycobacterial disease, Taj Ganj , (ICMR) Agra 282004. The human ethics committee of our institute approved the study protocol. After the participant signed informed consent they underwent thorough clinical assessment for dermatological and medical complications, skin smears were collected and acid-fast staining was performed to determine the bacterial index (BI).

Following clinical and histopathology examination, the cases were divided based on the Ridely and Jopling classification into TT (tuberculoid) , BT (borderline tuberculoid) , BL (borderline lepromatous), LL (lepromatous) and PN (pure neuritic) groups. All cases on steroid therapy and pregnant lactating female patients were exempted from the study. Non- leprosy cases included healthy volunteers and cases with medical condition other dermatology and inflammatory complications. 5 ml of peripheral venous blood was collected in vial from each subject by using sterile syringe and plasma aliquots were stored in -80º C until IL- 18 ELISA was performed.

ELISA for IL-18

Sandwich enzyme linked immunosobent Assay (ELISA) for IL- 18 was carried out using commercial kits form MBL (Medical &Biological Laboratories) co. ltd, Naka –ku- Nagoya, JAPAN (code no. 7620) as per manufacturing instructions. Precoated wells coated with anti-human IL-18 monoclonal antibody, were loaded with 150 µl of diluted (1:5 in diluent) samples wells and incubated for 60 minutes at room temperature (20- 25ºC).After incubation the contents of the wells were discarded and washed (X4)) with wash solution. Wells were poured with 100 µl each well of peroxide conjugate anti-human IL-18 monoclonal antibody, diluted 1:100 with conjugate diluent and incubated plate for 60 minute at room temperature (20-25ºC).

After aspiration of reaction mixture wells were washed (X4) with washing solution and complete removal of the liquid was ensured at each step by inverting the plate and blotting them on a filter paper towel. Wells were poured with 100µl of prepared substrate reagent TMB (Tetra Methyl Benzadine) and incubated for 30 minutes at room temperature (20 -25º C). The reaction was stopped by adding 100µl of stop solution (0.5 mol/l H2SO4) in each well. The optical density (O D) of each was then measured at a wave length of 450 nm using a microplate reader. The concentration of human IL 18 was calibrated based on the references standards.

Statistical Analysis

The concentration of human IL- 18 in plasma in pg/ml each individual was used for data analysis. Descriptive statistics were applied to the patient characteristics. Exploratory data analysis, including box-plot, medians and standard deviation were calculated for the concentration of human IL-18 and results were stratified by groups non-reactional and reactional (T1R and T2R) and compared to plasma concentrations obtained in their respective control groups. Statistically significant was assessed by kruskall- wall is one way analysis of variance for comparisons of multiple groups and Mann- whitney comparison between two groups. P values < 0.05 were considered statistically significant result.

Results

Patients Characteristics

The main base line clinical characteristics of 39 leprosy patients tested during T1R (n=10), T2R n=9), NRL (non-reactional leprosy) (n=20) and NHC (normal healthy controls) (n=43) are shown in Table -1. Adult males around 40 years of age predominated in all groups. Patients with T1R were classified by clinical and bacteriological criteria as with BT and none had acid-fast bacilli in skin smears (BI=0). Patients with T2R had BL and LL disease; all had acid-fast bacilli in skin smears. For patients diagnosed during a reactional episode T1R (n=10) and T2R (n=9), a wide variation in the symptoms prior to diagnosis was reported. No clinical differences were identified between patients who had T2R on diagnosis compared to those who developed the reaction after starting MDT. NHC (n=43) non-leprosy were included in the study.

Table 1. *Demographic and clinical characteristics of all the patients enrolled in the study.*

Characteristic	Details	Type 1 Reaction	Type 2 Reaction	Non-reactional Leprosy patient	Non-Leprosy cases (NHC)
Nos. of subjects	19	10	9	20	43
Gender	Male Female	6 4	6 3	17 3	22 21
Age (yrs)	Mean±s D Range	37.92+10.54 21-52	39.77+14.09 26-70	36.3157+14.3024 14-60	27.1908+12.3969 2-59
RJ classification	BT BB BL LL PN	6 2 2 0 0	0 0 3 6 0	3 6 4 3 4	
Bacteriological Index (BI)	Negative (1+ to 2+) (3+to5+)	4 2 4	1 1 7	11 7 2	
No. of Nerve Involvement	1-2 3-4 5-6	4 5 1	1 3 5	5 7 8	
Nos. of Lesions	0-10 11-20 21-30	7 1 2	8 1 0	17 3	

TT:tuberculoid, BT:borderline tuberculoid, BL:borderline lepromatous, LL: lepromatous, PN: pure neuritis.

IL-18 Levels in Leprosy Reactions

The mean level of IL-18 in plasma sample was significantly higher in the T1R group ($p<0.0001$) when compared NRL and NHC groups. There was a significant ($p=0.0343$) in IL-18 plasma levels high in the T2R group when compared to the NRL group (Table-2).

A significant difference was also noted in IL-18 plasma levels in the T1R group compared to T2R group ($p=0.0381$) (Fig.: 2). IL-18 plasma levels when compared between NRL and NHC groups was noted significantly ($p=0.0038$) high in NRL group (Table- 2). There was a significant difference in IL-18 levels in plasma on the reactional leprosy and non-reactional leprosy patients (Fig.: 1).

Table 2. *Levels of IL-18 within the study groups T1R,T2R, NRL, and NHC.*

Subjects	Number of cases	IL-18 levels in pg/ml Mean±sD	P values*
• T1R	10	3449.32±1064.57	1 vs.3 and 1 vs.4 P=3.8566E-06
• T2R	9	2371.943±852.27	1 vs.2 and 2vs.3 P=0.0381, p=0.0343
• NRL	20	1610.13±7.26.16	3 vs. 4 P=0.0038
• NHC	43	259.45±44.5	2 vs. 4 P=0.0057

*$p<0.05$ is considered as statistically significant.

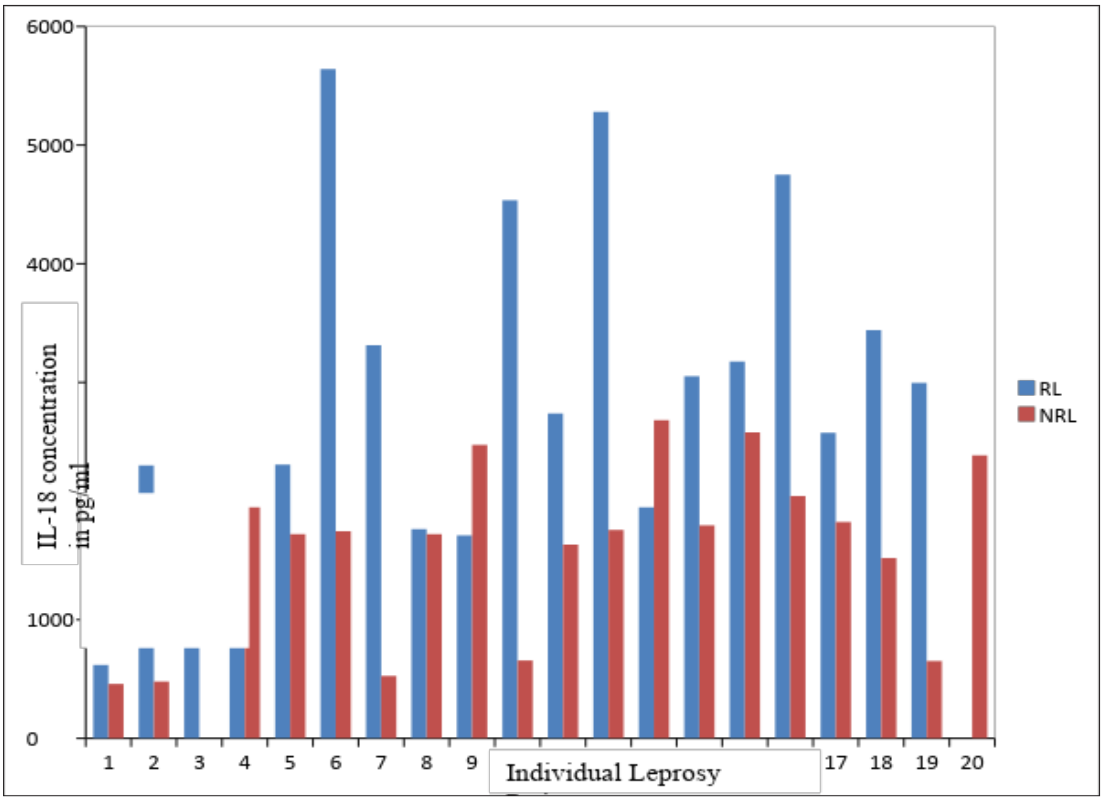

Fig 1. *IL-18 plasma levels in reactional Leprosy patients and non-reactional patients*

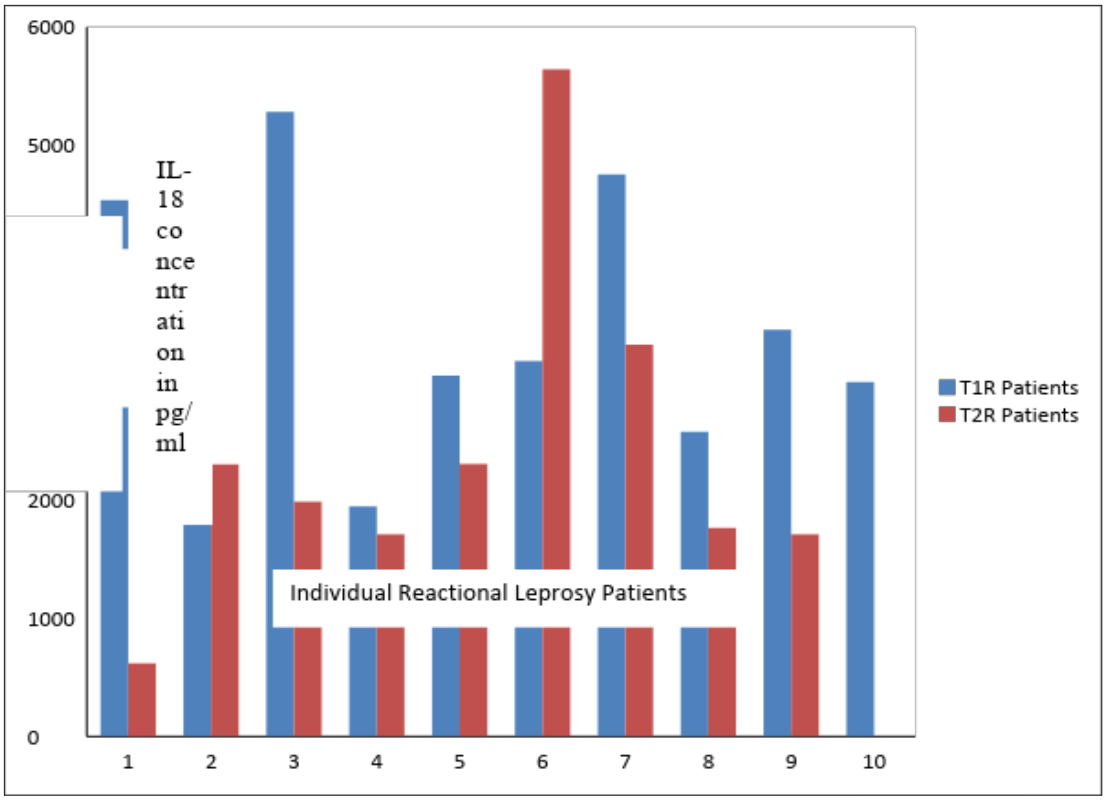

Fig 2. *IL-18 plasma levels in reversal reaction(T1R) and ENL reactions(T2R)*

IL-18 levels in different classes of Ridley DS and Jopling WH classification

IL-18 levels was found to be higher in LL group (1843.57±1183.8) (mean±sD) in pg/ when compared to BT, BB, BL and PN groups (Table-3).

In leprosy reactions IL-18 plasma levels in pg/ml (3483.38±1035.63) (mean+sD) showed higher levels in BT group when compared with BB, Bl and LL. However, there was no statistically significant difference was noted in IL-18 levels between BT, BB, BL and LL (Table-3).

Table 3. *Levels of IL-18 within the Ridley DS and Jopling WH classification*

Variables	Number of cases	IL-18 levels in pg/ml Mean±sD
Reactional Leprosy Cases:		
BT	8	3483.38±1035.63
BB	2	2867.22±183.82
BL	1	2681.69
LL	8	2574.50±1522.87
Non-Reactional Leprosy Cases:		
BT	3	1768.79±25.50
BB	6	1602.68±556.77
BL	4	1619.63±714.33
LL	3	1843.57±1183.08
N	4	1356.14±1022.44

IL-18 levels on the basis of Bacterial Index (BI)

Samples were classified based on the bacteriological index into three groups i.e BI – Negative, BI- 1-2 and BI -3-5. When compared in each group, the group BI negative has significantly higher levels of IL-18 p=0.0145 followed by BI (1-2) (p=0.121) and BI 3-5 (p=0.204) in non reactional leprosy patients.

In reactional leprosy patients stastically significant difference could not be found in IL-18 levels when compared among BI-negative, BI 1-2 and BI 3-5 groups (Table-4).

Correlation of IL-18 Levels with BI

Correlation of IL-18 levels with BI negative, BI 1-2 and BI 3-5 groups revealed that levels are reciprocal proportional to the BI and there was significant correlation (p=0.0145 and p=0.021) in the non-reactional patients.

No correlation was observed in IL-18 levels with BI negative, BI 1-2, and BI 3-5 groups in reactional leprosy patients (Table-4).

IL-18 Levels based on Nerve Involvement

Samples were also classified based on number of nerve involvement into three groups, 1-2, 3-4, and 5-6. Pair wise comparisons between three groups. None group was observed statistically significant for IL-18 plasma levels in non-reactional leprosy patients. In reactional leprosy patients IL-18 plasma levels was observed significantly (p=0.036) high when compared to 5-6 group with the 3-4 group. There was no significant difference observed between 1-2 and 3-4 groups (p=0.276 and p=0.1014 respectively) for IL-18 plasma levels in reactional leprosy patients (Table-4).

IL-18 Levels based on Lesions

Further samples were classified based on number of lesions into two groups, 0-10 and 11-20 for IL-18 plasma levels in non-reactional leprosy patients. When one group (0-10) compared with another group (11-20), IL-18 plasma levels was elevated significantly (p=0.05) high.

Similarly, samples of reactional leprosy patients also divided into three groups based on the numbers of lesions i. e. 0-10, 11-20, and 21-30. Para wise comparisons between three groups revealed that none group was observed statistically significant for IL-18 plasma levels in reactional-leprosy patients (Table-4).

Table 4. *Levels of IL-18 within the study groups Bacterial Index, No. of nerve involvement and No. of lesions in plasma of reactional and non-reactional leprosy patients*

	Number of cases	IL-18 levels in pg/ml Mean±sD	P values*
Bacterial Index			
1.BI negative (NRL)	7	1420.32±570.07	1 vs.2, 2 vs3 and1 vs. 3
			P=0.01457, p=0.0211 and p=0.2041
2.BI 1-2 (NRL)	5	2214.78±1064.57	
3.BI3-5(NRL)	8	999.61±736.409	
No. of Nerve involvement (NRL)			
• 1-2	4	1535.64±595.37	1 vs.2, 2 vs3 and1 vs. 3
• 3-4	7	1899.05±775.255	
• 5-6	9	1504.17±734.099	p=0.2207, p=0.1735 and p=0.4718
No. of lesions (NRL)			
• 0-10	17	1527.97±756.63	1 vs.2
• 11-20	3	2269.6±196.79	P=0.0505
Bacterial Index			
1.BI negative (RL)	5	2908.01±461.75	1 vs.2, 2 vs3 and1 vs. 3
2.BI 1-2 (RL)	3	2234.62±666.92	
3.BI3-5(RL)	11	3148.89±1670.83	P=0.0689, p=0.1913 and p=0.3799
No. of Nerve involvement (RL)			
1.1-2	5	3634.43±1451.96	1 vs.2, 2 vs3 and1 vs. 3
2. 3-4	8	3127.44±1456.37	
3.5-6	6	2188.12±599.0	P=0.2767, p=0.1014 and p=0.036
No. of lesions (RL)			
1.0-10	12	2896.14±1005.17	1 vs.2, 2 vs3 and1 vs. 3
2.11-20	5	3430.87±2092.32	
3.21-30	2	1966.33±28.40	P=0.2391, p=0.1962 and p=0.114

*p<0.05 is considered as statistically significant.

Discussion

This is the first description of the IL-18 plasma levels among T1R,T2R, and leprosy patients without reactions compared to well-matched normal healthy controls. The results indicate that IL-18 plasma levels are higher in reactional leprosy patients when compared with non-reactional leprosy cases and NHC.

Leprosy presents a spectrum of immunological groups between the two poles the tuberculoid pole (TT) with increased CMI (cell mediated immunity) which gradients towards the lepromatous pole (LL) though a series of borderline forms BT, BB, and BL with simultaneous increase in humoral immune response and increase in the bacillary load [19].

It has been demonstrated that the synergistic action of IL-18 play an important role in IFN-Ɣ production [20].The synergistic action can be reflected by the induction of the IL-18 receptors by IL-12; this in turn leads to the induction of IL-12 receptors in Th 1 cells. IL-18 mediated transcriptional regulation of IFN-Ɣ production plays an important role in the development of leprosy [21].

T1R are delayed type hypersensitivity reactions, which occur predominantly in the borderline forms of leprosy. M.leprae antigens have been demonstrated in the nerves and skin of patients experiencing T 1 R [22-23].This study also indicate that IL 18 plasma levels was noted significantly higher in number of nerve involvement when compared with pair wise in reactional patients aas well as also observed in without reactional leprosy patients (Table 4). A study of Brazilian patients with slit skin smear negative-single lesion paucibacillary leprosy that showed skin were more likely to experience a T 1R than those in whom M.leprae DNA was undetectable [24].Schwann cells also express toll like receptor TLR-2 [25]. M.leprae infection may lead to the expression of MHC II molecules on the surface of the cells and this may participate in antigen presentation which may trigger CD4 lymphocyte mediated killing of the cell in the presence of cytokines such as IL-1β and TNF-α [26]. Role of IL-18 in the generation of Type 1 responses to mycobacterial infection has been suggested by many researchers [27].Our results also indicate that the mean cytokine levels in all the groups of leprosy are significantly high when compared to the non-leprosy group. This may suggest that M.leprae stimulate the production of IL-18 across the spectrum of the disease.

Patients having BI 3-5 showed higher levels of IL-18 when compared to patients with negative BI indicating that the presence of optimum levels of M. leprae may activate Th 1 cells to produce IL-18. In the present situation increased nerve involvement in patients showed high levels of IL-18 production, whether the increase in lesions fall. Lowering levels of IL-18 production leads to a shift in Th1to Th2 responses, which is beyond the scope of this study and has to be explored. Our studies are concurrent with these finding owing to the significant upsurge in IL-18 levels in case of reactional-leprosy patients when compared to non-reactional leprosy and non leprosy cases.

Further functional analysis needs to be pursued in the context of understanding the IL-18 mediated immunomodulatory mechanisms involved in regulation of inflammation and such a study may provide deeper insight into the functional role of IL-18 in the control of leprosy reactions.

Conclusion

We investigated the role of IL-18 in the immune response to M. leprae in the hope of developing a model to explain the profound cytokine difference observed in the polar manifestation of leprosy. The local production of the type 1 cytokine pattern in T- Lep patients would promote CMI responses to M.leprae allowing them to control the growth of the bacteria and to respond to the infection. BTR patients showed high levels of IL-18 which indicates pathogenic role in developing Type 1 reaction in leprosy. Circulating profiles of the cytokines may act as potential plasma markers [28] to identified the disease early and predict the occurrence of reactions.

Various research groups [29-30] in this context have studied key pro-inflammatory and inflammatory cytokines but one has to explore the novel T cell subsets and their cytokines. In this study, we prorpose a possible association one of the cytokine IL-18 which is reported to have a potential role in the in the immunomodulation of inflammatory responses [31]. Our results are concurrent with our hypothesis that IL-18 levels increase in reactional state when compared with leprosy cases. Additional studies on IL-18 may help understand the immunopathogenic mechanisms of leprosy reactions and indicate their usefulness for the diagnosis and for the clinical management of these events.

Ethics Statement

This study was carried out in accordance with the recommendations of "Indian Council of Medical Research guidelines, National JALMA Institute for Leprosy & OMD Human Ethics Committee" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the "National JALMA Institute for Leprosy & OMD Human Ethics Committee".

Authors Contribution

US conceived and designed the study; ARY, KKM, MN, and USG performed the experiments and analyzed and interpreted the data; ARY drafted the manuscript; KKM, MN, , UG, and US critically reviewed the manuscript.

Declaration of Interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Abberviations

pg/ml	Picogram per milliliter
µl	Microliter
0 C	Degree Celsius
AFB	Acid fast bacilli
BB	Borderline-borderline
BI	Bacterial Index
BL	Borderline lepromatous
BT	Borderline tuberculoid
BTR	Borderline tuberculoid in reaction
CMI	Cell mediated immunity
ELISA	Enzyme-linked immunosorbent assay
ENL	Erythema nodosum leprosum
IFN	Interferon
IL	Interleukin
LL	Lepromatous leprosy
NHC	Normal healthy control
OD	Optical density
PBS	Phosphate buffered saline
RR	Reversal reaction
S. D.	Standared deviation
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TT	Tuberculoid
W.H.O	World Health Organization

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