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# Increased IL-35 producing Tregs and CD19$^+$IL-35$^+$ cells are associated with disease progression in leprosy patients

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ABSTRACT

*Background:* The clinical forms of leprosy consist of a spectrum that reflects the host's immune response to the *M. leprae*; it provides an ideal model to study the host pathogen interaction and immunological dysregulation in humans. IL-10 and TGF-β producing Tregs are high in leprosy patients and responsible for immune suppression and *M. leprae* specific T cells anergy. In leprosy, involvement of IL-35 producing Tregs and Bregs remain unstudied.
*Objective:* To study the role of IL-35 producing Tregs and Bregs in the human leprosy.
*Methods:* Peripheral blood mononuclear cells from leprosy patients were isolated and stimulated with *M. leprae* antigen (MLCwA) for 48 h. Intracellular cytokine IL-35 was evaluated in CD4$^+$CD25$^+$ Tregs, CD19$^+$ cells by FACS. Expression of PD-1 on CD4$^+$CD25$^+$ Tregs, CD19$^+$ cells and its ligand (PD-L1) on B cells, CD11c cells were evaluated by flow cytometry (FACS). Serum IL-35 level was estimated by ELISA.
*Results:* The frequency of IL-35 producing Tregs and Bregs cells were found to be high in leprosy patients ($p < 0.0001$) as compared to healthy controls. These cells produced suppressive cytokine IL-35 which showed positive correlation with bacteriological index (BI) and TGF-β producing Tregs, indicating its suppressive nature. We found higher expression of PD-1 on Tregs, B cell and its ligand (PD-L1) on antigen presenting cells in leprosy patients.
*Conclusion:* This study point out a shift in our understanding of the immunological features that mediate and regulate the immune suppression and the disease progression in leprosy patients with a new paradigm (IL-35 producing Tregs and Bregs) that is beyond TGF-β and IL-10 producing Treg cells.


## 1. Introduction

Leprosy is a bacterial disease caused by *Mycobacterium leprae*, the immunological spectrum firmly correlating with the pathological and clinical manifestation of the disease and bacterial load (BI). Leprosy is classified into tuberculoid (BT/TT) and lepromatous leprosy (BL/LL) forms, in between these two spectrum lies borderline (BB) leprosy which is unsteady with patients moving towards tuberculoid or lepromatous pole. Tuberculoid leprosy (BT/TT) shows restricted bacterial growth, few bacilli in the lesions, early nerve damage and good recall T cell mediated immune response ($Th_1$ type, IFN-γ, IL-2) and poor antibody responses to the *M. leprae* antigens. Whereas, lepromatous leprosy (BL/LL) patients show multibacillary skin lesions having abundant bacilli along with involvement of other organs and $Th_2$ (IL-4, IL-5) immune response [1,2]. Moreover, lepromatous leprosy patient exhibit specific T cell unresponsiveness to the *M. leprae* and the mechanisms underlying the antigen specific T cell anergy are being intensely investigated. However, genesis of $Th_1/Th_2$ like effector cells only cannot fully explain the polarized state of immunity and T cell unresponsiveness. Besides $Th_1$, $Th_2$ other subsets of T cells (Th3, Th17 and Tregs) have been reported which play important role in determining host immunity during progression of the disease [3–5].

Initially, Ottenhoff et al., described the concept of suppressor T cells and its possible function in immune suppression in leprosy patients [3] and these suppressor cells are now known as Treg cells. In the mid-1990s Sakaguchi and colleagues, who specifically identified these population as CD4$^+$CD25$^+$ T cells that had suppressive function and maintains homeostasis. Tregs represent 2–6% of the total CD4$^+$ T cell population in humans [6,7] and later identified to also express intracellular transcription factor forkhead box P3 (FoxP3) [8]. Nevertheless, mice constitutively express CD25, while

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in humans only Treg cells that highly express CD25$^{hi}$ have suppressive activity [9]. T regulatory cells mediate suppression primarily by secreting immunosuppressive cytokine TGF-β and IL-10. Recently, Dario Vignali et al., reported that iTr35 regulatory cells mediate immune suppression by secreting regulatory cytokine IL-35 [10]. Interleukin (IL)-35, is a member of the IL-12 cytokine family, a dimeric protein composed of IL-12α and IL-27β chains, which are encoded by two separate genes *IL12A* and *EBI3*, respectively. IL-35 is a potent immunosuppressive cytokine secreted by Treg cells, functions as an important immunosuppressive factor in immune-mediated diseases, and suppresses T cell proliferation and effector T cell functions [11]. B lymphocytes have critical roles as positive and negative regulators of immunity. The inhibitory function of B cell has so far been associated primarily with IL-10 and IL-35 a newly reported regulatory B (Breg) cells, because Breg derived IL-10 and IL-35 can defend against autoimmune disease thereby increase susceptibility towards pathogens [12]. Here we identify IL-35 producing Tregs and B cells which are associated with progression of the disease and show positive correlation with bacteriological index.

It has been reported that several mechanisms may devote to the dysfunction of *M. leprae* specific T cells, such as high bacterial load and, immune suppressive cytokines IL-10 and TGF-β produced by regulatory T cells, result in a gradual loss of T-cell function and cause *M. leprae* specific T cells to become exhausted. In this environment, T cells have also shown to upregulate inhibitory molecules, like cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1), which resembles T-cell exhaustion in leprosy patients [4,13]. Tregs induced by the Programmed Death-1 (PD-1) pathway that play important role in maintaining immune homeostasis and prevent autoimmune [14] also lead to *M. leprae* specific T cell anergy in lepromatous leprosy [13]. PD-1 is an inhibitory costimulatory molecule which exerts its effect on T cells by down regulating the cytokine production (IFN-γ, TNF-α and IL-2) and cell proliferation [14]. PD-1 expression may exert its impact on cell differentiation and survival through induction of apoptosis. The PD-1-PD-L1 pathway also plays an important role in chronic infections and in the suppressive tumor microenvironment [15,16] by T cell exhaustion and loss of immune response [17]. PD-1-PD-L1 pathway is important in dampening the T cell immune responses during infections.

Here, we made an attempt to identify the IL-35 producing Tregs, Bregs in leprosy patients and we found an increase in these cells from tuberculoid to lepromatous pole of leprosy. Furthermore, PD-1 expression on Tregs and B cells, expression of its ligand (PD-L1) on antigen presenting cells in stimulated PBMC cultures, increase in leprosy patients. Taken together, our results suggested that IL-35 may play an important role in the disease progression.

## 2. Materials and methods

### 2.1. Patients and controls

In this study we have recruited 40 newly diagnosed leprosy patients, 20 borderline tuberculoid (BT) and 20 Lepromatous leprosy (BL/LL) from Department of Dermatovenerology, AIIMS, New Delhi, India. Patients below 18 years of age, tuberculosis, pregnant women, HIV and MDT treatment were not included in this study. Leprosy patients were determined by clinical and histological criteria on the basis of Ridley-Jopling classification. In addition, age match 15 healthy volunteers were recruited after receiving the written consent (Table 1).

### 2.2. Ethics

Ethical approval of this study was obtained from the Institute Ethics Committee, All India Institute of Medical Sciences (AIIMS), New Delhi, India (IESC/T-417/01.11.2013). Written consent was obtained from the patients after counseling and prior to blood sampling.

### 2.3. Isolation the peripheral blood mononuclear cells (PBMCs) and in vitro culture

Blood samples layered on ficoll-hypaque (Sigma Aldrich, USA) and mononuclear cells were isolated by centrifugation at 2000 rpm for 20 min. Cells were washed thrice in sterile PBS by centrifugation at 1600 rpm for 10 min. Washed cells were re-suspended in RPMI 1640 along with 10% fetal calf serum (Gibco, CA, USA) and cell viability was done by 0.2% trypan blue using haemocytometer. $2 \times 10^6$ cells/ml were stimulated with MLCwA (15 μg/ml) (*M. leprae* cell wall antigen). All the cultures were stimulated with rIL-2, anti-CD3/CD28. After stimulation, cultures were incubated in 5% $CO_2$ incubator at 37 °C for 48 h. After harvesting the culture cells were processed for FACS staining.

### 2.4. Flowcytometer staining

After 48 h, cultured cells were harvested and stained with surface antibodies anti-Human CD4 FITC (Clone: OKT4), anti-Human CD25 PerCP-Cyanine5.5 (Clone: BC96), anti-Human PD-1(CD279) PE-Cyanine7 (Clone: eBioJ105), anti-Human CD11c PE (Clone: S-HCl-3), anti-Human CD274 (PD-L1, B7-H1) PE-Cyanine7 (Clone: MIH1) and anti-Human CD19 FITC (Clone: HIB19) for 30 min at 4 °C in the dark. Intracellular staining of anti-Human IL-35-APC (Clone: 27537) and anti-Human TGF-β PE-Cyanine7 (Clone: FNLAP) were performed according to the specifications of the manufacturer. The cells were fixed with 2% paraformaldehyde and stored at 4 °C. For intracellular staining cultures were incubated with Protein Transport Inhibitor containing Monensin (BD GolgiStop) for 4 h prior to harvest to block secretion of cytokine. The data were collected using FACS Canto flow cytometer (BD Biosciences) and analyzed with FACS DIVA software (BD Bioscience, USA).

### 2.5. ELISA

TGF-β and IL-35 cytokines were estimated by ELISA (Ready Set Go, e-Bioscience, San Diego, CA, USA) as per manufacturer's instructions. Serum was tested in duplicate in 96-well plates (Nunc, Rochester, USA) pre-coated with biotin conjugated anti

**Table 1**
Clinical details of 40 newly diagnosed untreated leprosy patients and 15 healthy control subjects.

| Clinical types | Number of patients | Sex | | Age | BI | Duration of disease |
|---|---|---|---|---|---|---|
| | | M | F | (years) | | |
| Tuberculoid leprosy (BT) | 20 | 12 | 08 | 18–58 | 0–0.4 | 0.4–2.5 Yrs. |
| Lepromatous leprosy (BL/LL) | 20 | 13 | 07 | 19–55 | 4.8–6 | 0.6–1.8 Yrs. |
| Healthy controls (HC) | 15 | 09 | 06 | 20–50 | – | – |

Patients were typed on the basis of Ridley Jopling classification [1], BI; Bacillary Index (mean of six lesional sites) and skin lesions. M; male, F; female. BT: Borderline Tuberculoid, BL: Borderline Lepromatous, LL: Lepromatous Leprosy, HC: Healthy controls.