

## Expansion of T regulatory Cells in Lepromatous Leprosy is Mediated by Phenolic Glycolipid-1

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### Abstract

In leprosy, lepromatous form of the disease is more severe and results from suppression of T cell response. T regulatory cells which suppress T cell response has been found in higher frequency in blood and at the site of infection in leprosy. Therefore, present study was carried out to evaluate the role of *Mycobacterium leprae* antigens whole cell sonicate (WCS) and especially phenolic glycolipid-1 (PGL-1), which is known for its suppressive nature, in the induction of T regulatory cells expansion in peripheral blood of leprosy patients. For this purpose peripheral blood mononuclear cells (PBMCs) of different category of leprosy patients and healthy controls were stimulated with *M. leprae* antigens *in vitro* and percentage of T regulatory cells was determined by flow cytometry. We found higher frequency of T regulatory cells in PBMCs of untreated borderline lepromatous/lepromatous leprosy (BL/LL) patients. Further, PBMCs of untreated BL/LL patients also showed higher percentage of T regs after stimulation with PGL-1. Antigen mediated expansion of T regulatory cells was also supported by results of Carboxy fluorescein succinimidyl ester (CFSE) proliferation assay. None of the antigen induced T regs expansion in healthy controls, untreated tuberculoid/borderline tuberculoid (TT/BT) leprosy patients and treated leprosy patients. Therefore it is suggested that increased frequency of T regs in BL/LL patients may be due to the induction of T regs expansion mediated by PGL-1 of *M. leprae* and this high percentage of T regs resulted in T cell suppression in lepromatous disease.

**Keywords:** T regulatory cells; PGL-1; Lepromatous leprosy; T cell suppression

**Abbreviations:** BL: Borderline Lepromatous Leprosy; BT: Borderline Tuberculoid Leprosy; CFSE: Carboxy Fluorescein Succinimidyl Ester; LL: Lepromatous Leprosy; *M. leprae*: *Mycobacterium leprae*; PBMCs: Peripheral Blood Mononuclear Cells; PGL-1: Phenolic Glycolipid-1, T regs: T Regulatory Cells; TT: Tuberculoid Leprosy; WCS: Whole Cell Sonicate

### Introduction

Leprosy is a chronic infectious disease caused by an intracellular obligate organism *Mycobacterium leprae* (*M. leprae*) that affects mainly nerves, skin and mucous membrane. It is well known that immune response of the host determines the outcome of clinical manifestation of the disease. At tuberculoid (TT) pole patients develop strong delayed type of immune response and limit the growth of the bacteria while lepromatous (LL) pole cellular immune response of the patients selectively fails to respond to the antigens of *M. leprae* which results in uncontrolled bacterial growth. Between these polar forms borderline tuberculoid (BT), borderline borderline (BB) and borderline lepromatous (BL) forms also occur [1-2].

T lymphocytes play crucial role in directing cell mediated immune response to intracellular pathogens. A recently discovered subpopulation of T cells which is CD4+ CD25+ (IL-2 receptor  $\beta$  chain) regulatory T cell expressing Fork head box protein (FoxP3) transcription factor play role in the suppression and regulation of immune response to self and nonself antigens. FoxP3 is a transcription factor which is not only a phenotypic marker of T regulatory cells but also essential for their development and function [3]. CD4+ CD25+ T regulatory cells suppress activation and proliferation of other CD4+ and CD8+ T cells [4-5]. Their suppressive function needs direct cell-cell contact or release of anti-inflammatory cytokines interleukine-10 (IL-10) and transforming growth factor- $\beta$  (TGF- $\beta$ ) or both [6-

9]. Generally T regulatory cells need to be activated to exert their suppressive function [10].

T regulatory cells have been shown to be essential for establishing and maintaining persistent infection by *Leishmania major* [11]. In human tuberculosis various studies have also shown the increase number of T regulatory cells in blood and at site of infection during the active disease [12-14]. However, there are three reports on increased number of T regulatory cells in leprosy patients [15-17], two of them reported increased T regulatory cells in lepromatous leprosy patients and one reported increased T regulatory cells in tuberculoid leprosy patients. PGL-1 is *M. leprae* specific lipid which has been reported to be immunosuppressive [18] and highest rate of leprosy cases was detected in PGL-1 seropositive BCG unvaccinated contacts [19], therefore, we designed the present study to evaluate the percentage of T regulatory cells in different categories of leprosy patients and healthy controls to delineate their role in T cell suppression in leprosy. Further, frequency of T regulatory cells was detected in PBMCs after stimulation with *M. leprae* antigens to evaluate role of these antigens, especially PGL-1 in the induction of T regulatory cells.

### Materials and Methods

#### Study subjects

Ten freshly diagnosed untreated TT/BT (Sex male 7 & female 3,

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Age 20-50yrs), BL/LL (Sex male 8 and female 2, Age 19-45yrs) patients and ten completely treated TT/BT Sex male 8 and female 2, Age 18-50yrs), BL/LL (Sex male 8 and female 2, Age 20-55 yrs) patients attending OPD of National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra, India were enrolled in the study. Patients were characterized on the basis of clinical and skin smear examination. Treated patients had received the treatment till the reduction of clinical activity and skin smear negativity. None of fully treated patients received immunosuppressive therapy. Ten healthy (Sex male 6 and female 4, Age 20-44yrs) individuals working at the Institute were included as exposed healthy control. None of healthy individuals had history of TB or leprosy and also did not suffer from any infectious disease at the time of blood collection. Blood samples were collected after taking informed written consent and study was approved by Institutional ethics committee.

### Antigens

*M. leprae* antigens whole cell sonicate (WCS) and Phenolic Glycolipid-1 (PGL-1) was procured from the laboratory of Dr. PJ Brennan under WHO contract no. NIH-N01-A1-25469.

### Peripheral blood mononuclear cells (PBMCs) isolation and stimulation with antigens

PBMCs were isolated from heparinized blood by Ficoll-hypaque (Sigma, USA) density gradient centrifugation method [19] and suspended in RPMI (Sigma, USA) supplemented with 5% fetal bovine serum (Sigma, USA), 100 U/ml penicillin, 100 ug/ml streptomycin and 2 nM L-glutamine (Sigma, USA). For Carboxy fluorescein succinimidyl ester (CFSE) cell proliferation assay  $1 \times 10^6$  cells were incubated with 5  $\mu$ M CFSE (Sigma, USA) in 37°C for 15 min in dark. Cells were then washed twice with 10% FBS containing RPMI. CFSE stained and unstained cells were then stimulated with whole cell sonicate (10 $\mu$ g/ml) and phenolic glycolipid-1 (PGL-1) (10  $\mu$ g/ml) of *M. leprae* and incubated at 37°C with 5% CO<sub>2</sub> for 24 hrs. Four hrs before the termination of incubation 2  $\mu$ M monensin was added to CFSE unstained cells.

### Flow cytometry

After incubation, CFSE stained and unstained cells were stained for surface marker with the combination of following antibodies anti CD3 APC (BD-Biosciences, USA), anti CD4 PerCP (BD-Biosciences, USA), anti CD25 PECy7 (BD-Biosciences, USA), CD127 PE (BD-Biosciences, USA) for 30 min in dark and then cells were fixed with 4% formaldehyde. CFSE unstained cells were permeabilized using FoxP3 buffer set (BD-Biosciences, USA) and stained with anti FoxP3 Alexa flour-488 (BD-Biosciences, USA). Stained cells were acquired in FACS Aria and data was analyzed by FACS Diva Software.

### Statistical analysis

Difference between the groups was calculated by non-parametric Mann Whitney test using GraphPad Prism 3.0 software.

## Results

### Increased frequency of T regulatory cells in untreated lepromatous leprosy patients

To study the difference in T regulatory cells frequency among different category of untreated leprosy patients, completely treated leprosy patients and healthy controls, we studied the frequency of CD3+CD4+CD25+FoxP3+ T regulatory cells by flowcytometry in the

blood of these study subjects and percentage was determined using FACS Diva software (Figure 1). Significantly higher frequency of T regulatory cells was noted in untreated BL/LL patients as compared to untreated BT/TT patients and healthy subjects with p value 0.0144 and 0.0262 respectively (Figure 2). Frequency of T regulatory cells in treated TT/BT and BL/LL patients did not differ significantly than healthy controls (Figure 2).

### Expansion of T regulatory cells by *M. leprae* antigens

To determine whether the *M. leprae* antigens induce the expansion T regulatory cells we first isolated PBMCs from the blood of untreated TT/BT and BL/LL patients, treated TT/BT and BL/LL patients and healthy controls and stimulated with WCS and PGL-1 for 24 hrs and the frequency of CD3+CD4+CD25+FoxP3+ T regulatory cells was detected by flow cytometry. Percentage of T regulatory cells stimulated with antigens in different study subjects was calculated by subtraction of percentage CD3+CD4+CD25+FoxP3+ T regulatory cells without stimulation from T regulatory cells after stimulation and compared. WCS induced significantly higher percentage of T regulatory cells in untreated TT/BT patients than treated TT/BT patients (p=0.0376) and untreated BL/LL patients than in treated BL/LL patients (p= 0.0144) (Figure 3a). Significantly higher percentages of T regulatory cells were noted after the stimulation of PBMCs with PGL-1 in untreated BL/LL patients compared with untreated TT-BT patients (p= 0.0376) and healthy controls (p=0.0005) (Figure 3b). Percentage of PGL-1 induced T regulatory cells was also high in untreated TT/BT patients than treated TT/BT patients (p=0.0093) and untreated BL/LL patients compared to treated BL/LL patients (p=0.0034) (Figure 3b).

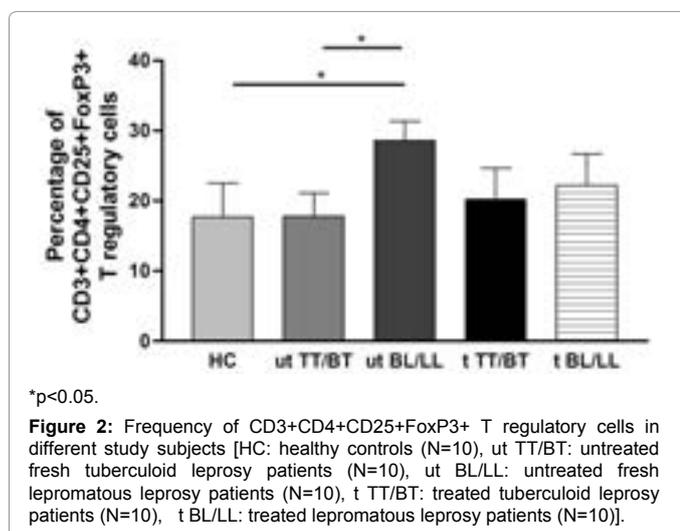
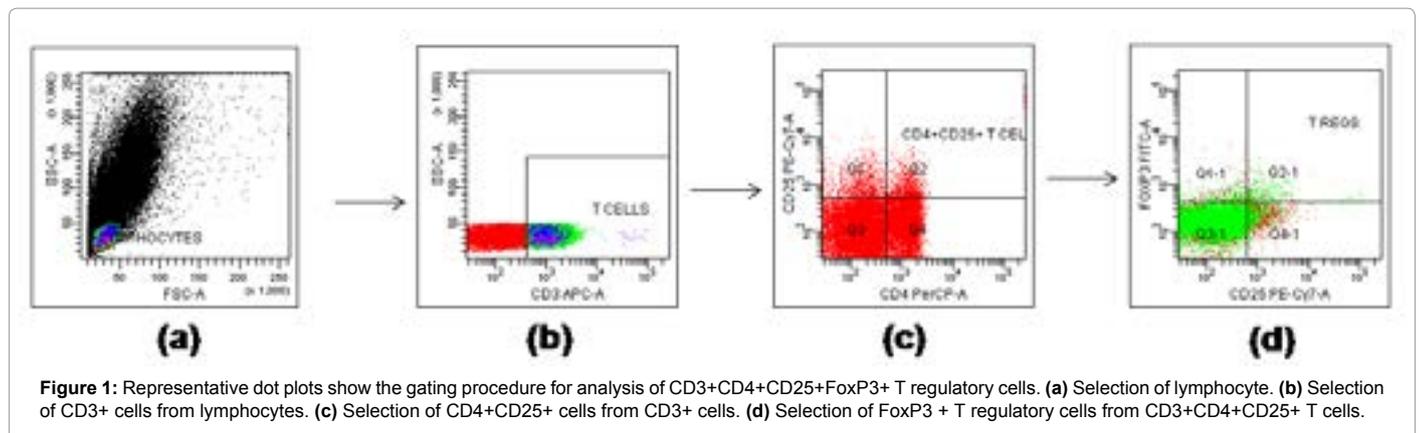
To differentiate T regulatory cells from effector or memory T cells, regulatory T cells were stained for CD127. When FoxP3+ T regulatory cells were gated for CD127, more than 80% cells were CD127low in each experiment (data not shown).

### CFSE cell proliferation assay

To prove antigen mediated expansion of T regulatory cells, CFSE cell proliferation assay was performed and percentage of CFSE+CD3+CD4+CD25+CD127 low cells was determined by FACS Diva software (Figure 4). No proliferation was noted with WCS in CD3+CD4+CD25+CD127 low cells in healthy controls and untreated TT/BT and BL/LL patients (Figure 5a) whereas PGL-1 was found to proliferate CD3+CD4+CD25+CD127 low cells in BL/LL patients than TT/BT patients (p=0.0376) and healthy controls (p=0.0034) (Figure 5b). No proliferation was noted after the stimulation with antigens in treated TT/BT and BL/LL patients (data not shown).

## Discussion

When naïve CD4+ T cell recognize peptide on MHC molecules, they proliferate and differentiate in Th1/Th2 effector cells. In addition to Th1 and Th2, naïve CD4+ T cells can also differentiate in regulatory T cells by antigen recognition. T regulatory cells play role in preventing autoimmunity, but in addition to suppressing autoimmunity T regulatory cells also suppress immune response to infections thereby play role in the pathogenesis of various diseases. There are at least three subsets of CD4+ T regulatory cells involved in negative regulation of immune response, which include natural T regulatory cells (CD4+ CD25high Foxp3+)- generated in thymus, Tr1-IL-10 producing regulatory T cells generated in periphery and Th3 regs-TGF- $\beta$  producing regulatory T cells generated in periphery [20,21]. Regulatory T cells suppress the activation and proliferation of other CD4+ and CD8+ T cells [4,5]. Increased frequency of T regulatory



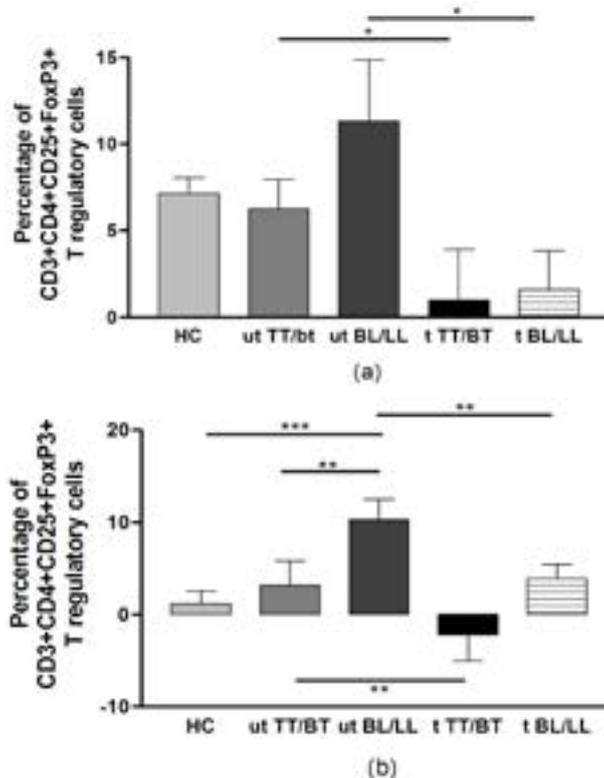
cells has been reported in blood and site of infection of leprosy patients [15-17]. To confirm that the increased frequency of T regulatory cells in leprosy patients is due to the exposure of *M. leprae* or *M. leprae* antigens mediated expansion of T regulatory cells; for the first time we studied the frequency of CD3+CD4+CD25+FoxP3+ T regulatory cells in untreated and treated leprosy patients to correlate these cells with immunosuppression seen in leprosy. Further, we also evaluated the role of WCS and PGL-1 of *M. leprae* in induction of these cells.

On comparing the frequency of CD3+CD4+CD25+FoxP3+ T regulatory cells, significantly higher frequency was found in blood of BL/LL cases as compared to TT/BT cases and healthy controls in the present study. High frequency of T regulatory cells in lepromatous leprosy patients has also been reported by Maria et al. in 2012 [16]. A recent study has also reported increased percentage of FoxP3+ T regulatory cells in blood and skin biopsies of lepromatous leprosy patients [17]. In contrast to these two studies and present study higher frequency of T regulatory cells in blood of tuberculoid leprosy patients as compared to lepromatous leprosy patients and healthy controls has been reported by Attia et al. [15]. Our result suggests that lepromatous leprosy patients have high frequency of T regulatory cells in their blood and this higher frequency may be one of the factors responsible for suppression of T cell responses in lepromatous leprosy. To check whether this increase in the T regulatory cells frequency is due to exposure of *M. leprae*, effect of two *M. leprae* antigens WCS and PGL-1 on expansion of these cells was detected in the present study. Significantly high percentage

of CD3+CD4+CD25+FoxP3+ T regulatory cells was noted after the stimulation of PBMCs with PGL-1 in untreated BL/LL cases compared to untreated TT/BT cases and healthy controls. Increase in T regulatory cells number after exposure to *M. leprae* cell wall antigen (MLCwA) in lepromatous leprosy patients than in tuberculoid leprosy patients and healthy controls has been reported [16]. None of the *M. leprae* antigens induced the percentage of T regulatory cells in treated leprosy patients in our study. Furthermore, percentage of WCS and PGL-1 induced T regulatory cells was higher in untreated TT/BT patients than treated TT/BT patients and untreated BL/LL patients than in treated BL/LL patients. WCS induced percentage of T regulatory cells was higher in untreated patients than treated patients but it was not significantly higher than the percentage in healthy controls. Therefore, higher percentage of T regulatory cells in lepromatous leprosy could be due to the exposure of T cells to *M. leprae* PGL-1 as it induced the percentage of T regulatory cells in lepromatous leprosy patient's PBMCs than TT/BT patients and healthy controls. Suppressive nature of PGL-1 for lymphocytes in leprosy has also been reported by Mehra et al. [18] and PGL-1 has also been reported in a study to play role in immune invasion of host by activating a complement system pathway which further leads to differentiation of IL-10 positive T regs [22]. Earlier regulatory T cell expansion by *M. tuberculosis* Mannose-capped lipoarabinomannan (ManLAM) has been reported in healthy tuberculin reactors [23]. It was shown that ManLAM induces prostaglandin E2 production which leads to T regulatory cells expansion. The mechanism of regulatory T cells induction by PGL-1 in leprosy patients has not been evaluated in our study. It is also noted in the present study that frequency of T regulatory cells goes down after completion of the multi-drug therapy as frequency of T regulatory cells in treated BL/LL patients was not significantly higher than healthy controls. It is also evident that this increased population of T regulatory cells is inducible regulatory T cells as the frequency increases after the exposure with *M. leprae* antigens.

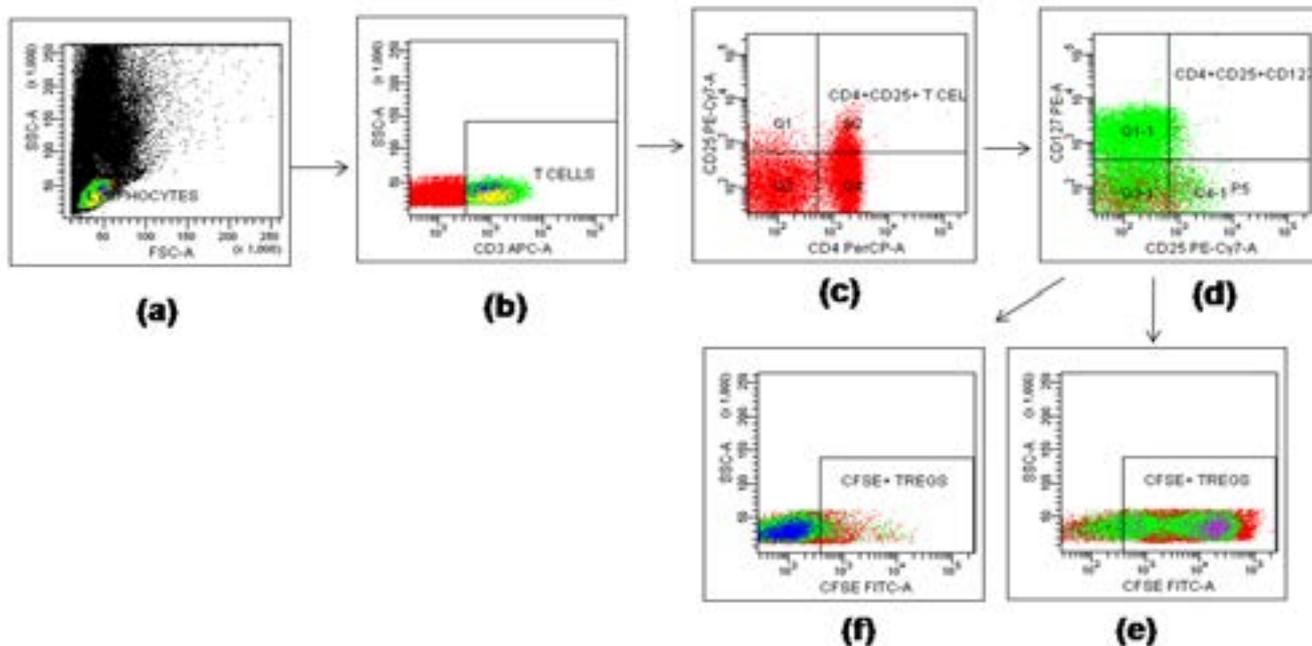
To prove that the increased percentage of T regulatory cells after stimulation is due to antigen mediated proliferation, we also performed CFSE experiment to detect cell proliferation in response to *M. leprae* antigens. CD3+CD4+CD25+CD127low T cells were found to proliferate in response to PGL-1 in untreated BL/LL patients than in TT/BT patients and healthy controls. Proliferation of these cells was not detected in cells from treated TT/BT and BL/LL patients. The results of CFSE proliferation experiments support the result of antigen mediated expansion of T regulatory cells.

Untreated lepromatous leprosy patients have high frequency of T regulatory cells, and *M. leprae* antigen PGL-1 induces the percentage

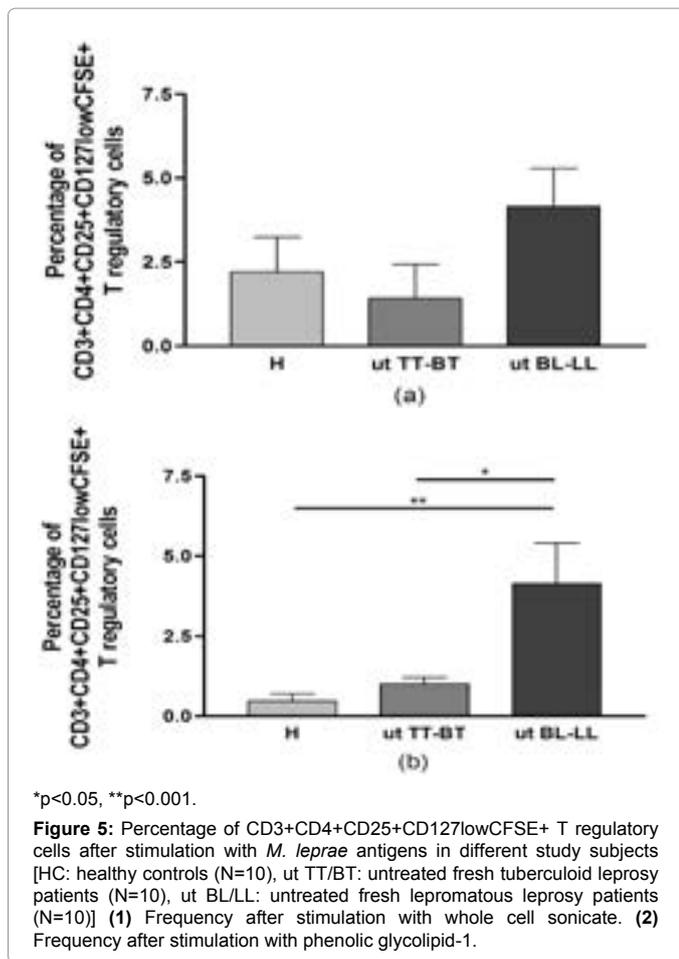


\*p<0.05, \*\*p<0.001 & \*\*\*p<0.0001

**Figure 3:** Percentage of CD3+CD4+CD25+FoxP3+ T regulatory cells after stimulation with *M. leprae* antigens in different study subjects [HC: healthy controls (N=10), ut TT/BT: untreated fresh tuberculoid leprosy patients (N=10), ut BL/LL: untreated fresh lepromatous leprosy patients (N=10), t TT/BT: treated tuberculoid leprosy patients (N=10), t BL/LL: treated lepromatous leprosy patients (N=10)]. (a) Frequency after stimulation with whole cell sonicate. (b) Frequency after stimulation with phenolic glycolipid-1.



**Figure 4:** Representative dot plots show the gating procedure for analysis of T CD3+CD4+CD25+CD127lowCFSE+ T regulatory cells. (a) Selection of lymphocyte. (b) Selection of CD3+ cells from lymphocytes. (c) Selection of CD4+CD25+ cells from CD3+ cells. (d) Selection of CD127low cells from CD3+CD4+CD25+ cells. (e) Selection of CFSE+ cells from CD3+CD4+CD25+CD127low unstimulated cells. (f) Selection of CFSE+ cells from CD3+CD4+CD25+CD127low stimulated cells.



of T regulatory cells in untreated patients, therefore PGL-1 may be one of the factors responsible for the increased frequency of T regulatory cells and thereby leading to immunosuppression in lepromatous leprosy patients. Multi drug therapy brings down the frequency of T regulatory cell in treated leprosy patients. These findings give lead to understanding the mechanism of PGL-1 mediated induction of T regulatory cells in future studies.

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