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IL-12 and IL-23 modulate plasticity of FoxP3$^+$ regulatory T cells in human Leprosy

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ABSTRACT

Leprosy is a bacterial disease caused by *M. leprae*. Its clinical spectrum reflects the host's immune response to the *M. leprae* and provide an ideal model to investigate the host pathogen interaction and immunological dysregulation. Tregs are high in leprosy patients and responsible for immune suppression of the host by producing IL-10 and TGF-β cytokines. In leprosy, plasticity of Tregs remain unstudied. This is the first study describing the conversion of Tregs into Th1-like and Th17-like cells using *in vitro* cytokine therapy in leprosy patients. Peripheral blood mononuclear cells from leprosy patients were isolated and stimulated with *M. leprae* antigen (MLCwA), rIL-12 and rIL-23 for 48 h. Expression of FoxP3 in CD4$^+$CD25$^+$ Tregs, intracellular cytokines IFN-γ, TGF-β, IL-10 and IL-17 in Tregs cells were evaluated by flow cytometry (FACS) after stimulation. rIL-12 treatment increases the levels of pStat4 in Tregs and IFN-γ production. In the presence of rIL-23, pStat3$^+$ and IL-17A$^+$ cells increase. rIL-12 and r—IL-23 treatment downregulated the FoxP3 expression, IL-10 and TGF-β production by Tregs and enhances the expression of co-stimulatory molecules (CD80, CD86). In conclusion rIL-12 converts Tregs into IFN-γ producing cells through STAT-4 signaling while rIL-23 converts Tregs into IL-17 producing cells through STAT-3 signaling in leprosy patients. This study may helpful to provide a new avenue to overcome the immunosuprression in leprosy patients using *in vitro* cytokine.

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1. Introduction

Leprosy is a bacterial disease caused by *Mycobacterium leprae*, which affects the skin, peripheral nerves and cooler body parts (WHO, 2013). 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 because each clinical manifestation is associated with different levels of immune responses to *M. leprae*. Based on histopathological criteria, Ridley and Jopling classified leprosy into five forms: tuberculoid (TT) and lepromatous (LL), in between these two clinical forms lies borderline tuberculoid (BT), borderline-borderline (BB), borderline lepromatous (BL) (Ridley and Jopling, 1966). The tuberculoid type (BT/TT) leprosy patients show good recall of cell-mediated immune (CMI) with prevalence of CD4$^+$ T cells, Th1 cytokines in the lesions and restricted growth of *M. leprae*, whereas lepromatous (BL/LL) patients have foamy macrophages in lesions, high bacterial load with numerous lesions consisting of CD8$^+$ T cells, Th2 cytokines (IL-4, IL-10) and defective cell mediated immunity (Modlin, 1994, 2010; Scollard et al., 2006).

T regulatory cells (CD4$^+$CD25$^+$FoxP3$^+$) represent approximately 2–10% of the total CD4$^+$cell population in humans that maintains immune homoeostasis (Sakaguchi et al., 2008). FoxP3$^+$Tregs have been characterized as one of the most forceful hierarchic suppressing effector T cell function with ultimate control of immune response elicited by the host during infections of intracellular pathogens such as leishmaniasis (Mendez et al., 2004) and tuberculosis (Sharma et al., 2009). Immunosuppressive cytokines TGF-β and IL-10 secreted by Tregs attribute suppressive activities of Treg cells (Annacker et al., 2003). It was reported that TGF-β secreting Tregs associated with antigen specific T cells anergy in lepromatous leprosy by down regulating T cell response (Saini et al., 2014). Our laboratory demonstrated for the first time that leprosy progression finally leads to the expansion of Th3 immune responses (Kumar et al., 2011a). In continuation, we also reported that high levels of

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TGF-β induce the expression of FoxP3 in memory and naive T cells during progression of disease (Kumar et al., 2013a), (Kumar et al., 2013b). Recently, A new lineage of effector T cells, Th17 play important role in immune responses to autoimmune diseases (Korn et al., 2009) and against intracellular pathogens (Lin et al., 2009) has been reported. Th17 cells secrete IL-17, has a protective role in the onset of infection arbitrate their pro-inflammatory function by activating macrophages, recruiting neutrophils and enhancing Th1 effector cells, leading to control of bacillary load (Bettelli et al., 2008; de Almeida-Neto et al., 2014). Saini et al. identified $CD4^+$ Th17 cells in tuberculoid patients and highlighted their importance in leprosy and they also reported higher expression and release of IL-17A in *M. leprae* antigen stimulated PBMC cultures and in skin lesions of tuberculoid leprosy (Saini et al., 2013).

IL-12, a heterodimer cytokine composed of two subunits p35 and p40, chain sharing is a key characteristic of the IL-12 family (IL-12, IL-23, IL-27 and IL-35 etc.) of cytokines and receptors (Vignali and Kuchroo, 2012). IL-12 promotes antitumor immunity by activating natural killer (NK) cells and Th1 cells to produce of IFN-γ, an important cytokine for *M. leprae* clearance (Bobosha et al., 2014) and antitumor immunity (Kortylewski et al., 2009) .It also promote the activity and expansion of cytotoxic T lymphocytes (CTLs)(Colombo and Trinchieri, 2002). IL-23, like IL-12, is a pro-inflammatory cytokine; more recently discovered IL-12 family member cytokine, composed of two subunits, a unique p19 and p40 subunit common with IL-12 receptor (Vignali and Kuchroo, 2012). IL-23 has also been shown to promote the expansion of Th17 cells that are characterized by production of a number of specific cytokines including IL-17A, IL-17F, IL-21, and IL-22(Langrish et al., 2005).

Treg cells inhibit the function of different effector T cells such as Th1 and Th17 cells (Ogino et al., 2010). Presence of Th17 cytokines considerably decrease the number of $FoxP3^+$Treg cells as determined by reduced number of $CD4^+CD25^+$ cells with concurrent increase of $IL\text{-}17^+$ CD4T cells(Sadhu et al., 2016). Recent findings suggested that $Foxp3^+$ Treg cells treated with IL-12, show phenotypic and functional plasticity and are able to secret pro-inflammatory cytokines(Feng et al., 2011).Th1 (IFN-γ) and Th17 (IL-17) cells, therefore two lymphocyte subsets play protective role during *Mycobacterium leprae* infection while $FoxP3^+$ Tregs increase during the disease progression and suppress the host immune system. Here, we made an attempt to convert the $FoxP3^+$Treg cells to $FoxP3^+IFN\text{-}\gamma^+$Treg (Th1 like) phenotype in leprosy patients in the presence of IL-12 and, $FoxP3^+$Treg cells acquire $FoxP3^+IL\text{-}17A^+$Treg (Th17 like) character in the presence of IL-23 that is beneficial for host. IL-12 and IL-23 have impact on $FoxP3^+$ Tregs cell numbers and function and whether this can be modulated for benefit of the host.

2. Materials and methods

2.1. Patients and controls

We have considered newly diagnosed 20 paucibacillary, borderline tuberculoid (BT) and 20 multibacillary, Lepromatous leprosy (BL/LL) without history of MDT treatment recruited from Department of Dermatology, AIIMS New Delhi. Leprosy patients were determined by clinical and histological criteria on the basis of Ridley-Jopling classification(Ridley and Jopling, 1966). In the addition age match 10 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).

2.3. Reagents

We obtained Ficoll-Hypaque, RPMI-1640 (Sigma Aldrich, USA), anti-Human CD4-APC (RPA-T4), Anti-human CD25-FITC (CD25-4E3), Anti-human Foxp3-PE (236A/E7), Anti-Human IFN-γ PE-Cyanine7 (4S.B3), Anti-Human IL-17A PerCP-Cyanine5.5 (eBio64DEC17) were procured from eBioscience, USA. Anti-Human CD80 –FITC (Clone L307.4), Anti-Human CD86-PE (Clone 2331), Anti-Human $CD11_C$-APC (Clone B-ly6), Purified Mouse Anti-Human Stat4 (pY693) (Clone 38/p-Stat4) and Mouse Anti-human- PE Stat3 (pY705) (Clone 4/P-STAT3), Monensin (BD GolgiStop) were purchased from BD Biosciences, USA. rIL-2, anti-CD3/CD28 was purchased from Life Technology, USA. rIL-12 and rIL-23 were purchased from Prospec protein specialist, USA. *M. leprae* derived total cell wall antigen (MLCwA) was a kind gift from Dr. Patrick Brennan, Colorado State University, Colorado, USA, and NIH, NAIAID "Tuberculosis Research Materials and Vaccine Testing Organization".

2.4. PBMCs isolation and in-vitro culture

Blood samples layered on ficoll-hypaque (Sigma Aldrich, USA) and mononuclear cells were isolated and washed thrice in RPMI media. After the last wash cells were re-suspended in RPMI 1640 along with 10% fetal calf serum (Gibco, CA, USA) and cell viability and enumeration were estimated by 0.2% trypan blue exclusion using haemocytometer. Tregs were isolated by using Stem cells Technologies (USA) kit. 2×10^6 cells/well were stimulated with recombinant protein of IL-12(5 ng/ml), IL-23(3 ng/ml) (Prospec, USA) to reduce the FoxP3 expression, MLCwA (15 μg/ml), and various combinations of MLCwA (*M. leprae* cell wall antigen) with rIL-12 and rIL-23. 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. Monensin (BD GolgiStop) added 8 h prior to harvest to block secretion of cytokine. After harvesting the culture cells were taken for FACS staining and western blot analysis.

2.5. Flowcytometer staining

Harvested cells were labeled with surface antibodies (CD4, CD25, CD80, CD11c and CD86) for 30 min at 4 °C in the dark. Intracellular labeling of FoxP3, pSTAT-3, IL-17A, IFN-γ, TGF-β and IL-10 were performed according to the specifications of the manufacturer. The cells were fixed in 2% paraformaldehyde and stored at

Table 1
Clinical details of 40 newly diagnosed untreated leprosy patients and 10 healthy control subjects.

Clinical types	Number of Patients	Sex		Age	BI
		M	F	(years)	
Borderline Tuberculoid (BT)	20	12	08	19–56	0–0.3
Lepromatous Leprosy (BL/LL)	20	11	09	22–53	4.3–6
Healthy controls (HCs)	10	07	03	21–54	–

Patients were typed on the basis of Ridley Jopling classification, 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.

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