Serum Th17 cytokines in leprosy: correlation with circulating CD4+ $CD25^{high}FoxP3^{+}$ T-regs cells, as well as down regulatory cytokines

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ORIGINAL PAPER

Serum Th17 cytokines in leprosy: correlation with circulating CD4$^+$ CD25^{high}FoxP3$^+$ T-regs cells, as well as down regulatory cytokines

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Abstract Leprosy is not only a bacteriological disease but also an immunological disease, in which T helper17 and CD4$^+$ CD25^{high}FoxP3$^+$ regulatory T cells (T-regs), among others, may play a role. We aimed to evaluate serum levels of interleukin (IL)-17, IL-22 (Th17 cytokines), IL-10 and transforming growth factor (TGF)-β (down regulatory cytokines) in 43 untreated leprosy patients and 40 controls by enzyme-linked immunosorbent assay, and to assess circulating CD4$^+$ CD25^{high}FoxP3$^+$T-regs in patients using flow cytometry. Patients were grouped into tuberculoid, pure neural, borderline, lepromatous, type 1 reactional leprosy, and erythema nodosum leprosum. IL-10 and TGF-β were significantly higher in patients as compared to controls ($p < 0.001$), while IL-17, but not IL-22, was significantly lower ($p < 0.001$), with no significant difference comparing patients' subgroups. Significantly higher CD4$^+$ CD25^{high}FoxP3$^+$T-regs levels was detected in tuberculoid, type 1 reaction and pure neural leprosy, while the lowest levels in erythema nodosum leprosum ($p < 0.001$). TregsFoxP3 expression% was significantly lower in pure neural leprosy than other patients' subgroups ($p < 0.05$). T-regs/T-effs was lowest in erythema nodosum leprosum ($p < 0.05$). TGF-β correlated negatively with TregsFoxP3 expression% and T-effs% ($p = 0.009$ and 0.018 respectively). Leprosy is associated with defective IL-17 and overproduction of IL-10 and TGF-β. Tuberculoid, type 1 reaction and pure neural leprosy express significantly higher circulating T-regs, consistent with effector immune mechanisms activation, but with lower TregsFoxP3 expression (in pure neural leprosy). Erythema nodosum leprosum is characterized by deficient T-regs and increased TregsFoxP3 expression%. The present study pinpointed a potential role of Th17, CD4$^+$ CD25^{high}FoxP3$^+$T-regs, and probably CD4$^+$ CD25$^+$IL-10$^+$ T regulatory cells 1 (Tr1), and Th3 in leprosy.

Keywords Interleukin-10 · Interleukin-17 · Interleukin-22 · Leprosy · T-regs · Transforming growth factor-β

Abbreviations

BB	Borderline borderline leprosy
BCG	*Bacillus Calmitte Guerin*
BI	Bacterial index
BL	Borderline lepromatous leprosy
BT	Borderline tuberculoid leprosy
CMI	Cell-mediated immunity
EDTA	Ethylene diamine tetra acetate
ELISA	Enzyme-linked immunosorbent assay
ENL	Erythema nodosum leprosum
FoxP3	Forkhead box P3
HBV	Hepatitis B virus
HCV	Hepatitis C virus
IFN	Interferon
IL	Interleukin
iTregs	In vitro induced T-regs
IQR	Inter-quartile range
LL	Lepromatous leprosy
MB	Multibacillary
M. leprae	*Mycobacterium leprae*
p	Probability factor

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PB	Paucibacillary
PBMN	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PNL	Pure neural leprosy
pT-regs	In vivo peripheral derived T-regs
r	Correlation factor
T-regs	Regulatory T cells
RL	Reactional leprosy
SPSS	Statistical program for social science
SSS	Slit skin smear examination
T-effs	Effector-memory T cells
TGF	Transforming growth factor
Th	T helper cells
Tr1	T regulatory1
tT-regs	Thymic derived T-regs
TT	Tuberculoid leprosy

Introduction

Despite the presence of extensive studies concerning the immunopathogenesis of leprosy, certain T cell subsets, apart from T helper (Th)1 and Th2 effector cells, were hardly investigated. Regulatory T cells (T-regs), a third subset of $CD4^+$ T cells, that regulate the activation and expansion of the aforementioned lineages, are among these. T-regs are believed to play a central role in inducing and maintaining immunologic tolerance and in the termination of immune responses [35]. Thus, deficiency or dysfunction of these cells may lead to autoimmunity or aggravated pathogen-induced inflammation [24]. Two main subsets of professional T-regs have been proposed that differ in terms of development, specificity, and effector mechanisms: naturally occurring and adaptive T-regs. The latter develop from mature T cells in peripheral tissues under certain conditions of antigen-specific stimulation. This subset includes the T regulatory1 (Tr1) and Th3 types of regulatory cells, which exert their suppressive function via the secretion of interleukin (IL)-10 and transforming growth factor (TGF-β), respectively [6]. However, macrophages also constitute a major source of IL-10 [33], and TGF-β1 is known to be a product of activated monocytes, among other inflammatory cells [3].

Naturally arising T-regs, which are induced in the thymus, display a constitutive high expression of CD25; the alpha chain of the IL-2 receptor, and are generally referred to as $CD4^+$ $CD25^{high}$T-regs. These T-regs suppress other T cells by cell–cell contact in a cytokine-independent fashion [5]. Forkhead box P3 (FoxP3) is a transcription factor shown to have a direct role in inducing immunosuppression and has been identified as a good marker for T-regs. Thus, they are known as $CD4^+$ $CD25^{high}FoxP3^+$T-regs [10]. T-regs may respond to an ample variety of auto-antigens, although there is evidence that they may also respond to antigens expressed by microbes [18]. In a previous publication, we demonstrated a possible role of $CD4^+$ $CD25^{high}FoxP3^+$T-regs in leprosy [4]. However, neither Tr1 nor Th3 or their effector cytokines were investigated in that report.

Recently, the identification of a novel lineage of helper T cells, Th17, has broken the long-held paradigm regarding the roles of the other three lineages (Th1, Th2, and T-regs). Th17 is among T cell subsets shown to produce cytokines that could not be classified according to the Th1–Th2 scheme. They are a branch of the $CD4^+$ T cell compartment, involved in host protection against bacterial and fungal infections, as well as in orchestration of chronic inflammation and autoimmunity [32]. Th17 cells produce IL-17 (also called IL-17A) and variable amounts of IL-17F, IL-21, IL-22 and IL-26 [19]. Few studies investigated Th17 cells and their effector cytokines in leprosy and results were somehow contradictory. While there were some reports of defective serum IL-17 [1, 7, 37], as well as low in situ expression [7], others reported increased lesional expression [26, 34, 37].

The aim of this study was to evaluate serum levels of IL-17 and IL-22 (Th17 cytokines), IL-10 and TGF-β (down regulatory cytokines), as well as circulating $CD4^+$ $CD25^{high}FoxP3^+$T-regs, in untreated patients with different types of leprosy.

Materials and methods

This study was conducted in Ain Shams University Hospital on 43 untreated leprotic patients attending at El Qal'ah (Citadel) Dermatology and Leprosy Hospital, Cairo, Egypt, and 40 healthy volunteers as a control group, after signing an informed consent, over a period of 14 months. The study was conducted according to the Declaration of Helsinki Principles, and was approved by the medical ethical committee of Ain Shams University. Patients were evaluated according to clinical examination, slit skin smear examination (SSS), and histopathological examination, and were divided into six groups: Group I: tuberculoid leprosy (TT); Group II: pure neural leprosy (PNL); Group III: borderline leprosy (borderline tuberculoid; BT, mid borderline; BB and borderline lepromatous; BL); Group IV: lepromatous leprosy (LL), Group V: reactional leprosy type 1; RL1; and Group VI: erythema nodosum leprosum; ENL. Patients were also grouped according to presence or absence of reactions, as well as bacillary load (paucibacillary; PB with negative SSS versus multibacillary; MB with positive SSS). In addition, 40 healthy age- and gender-matched subjects with almost with negative hepatitis C

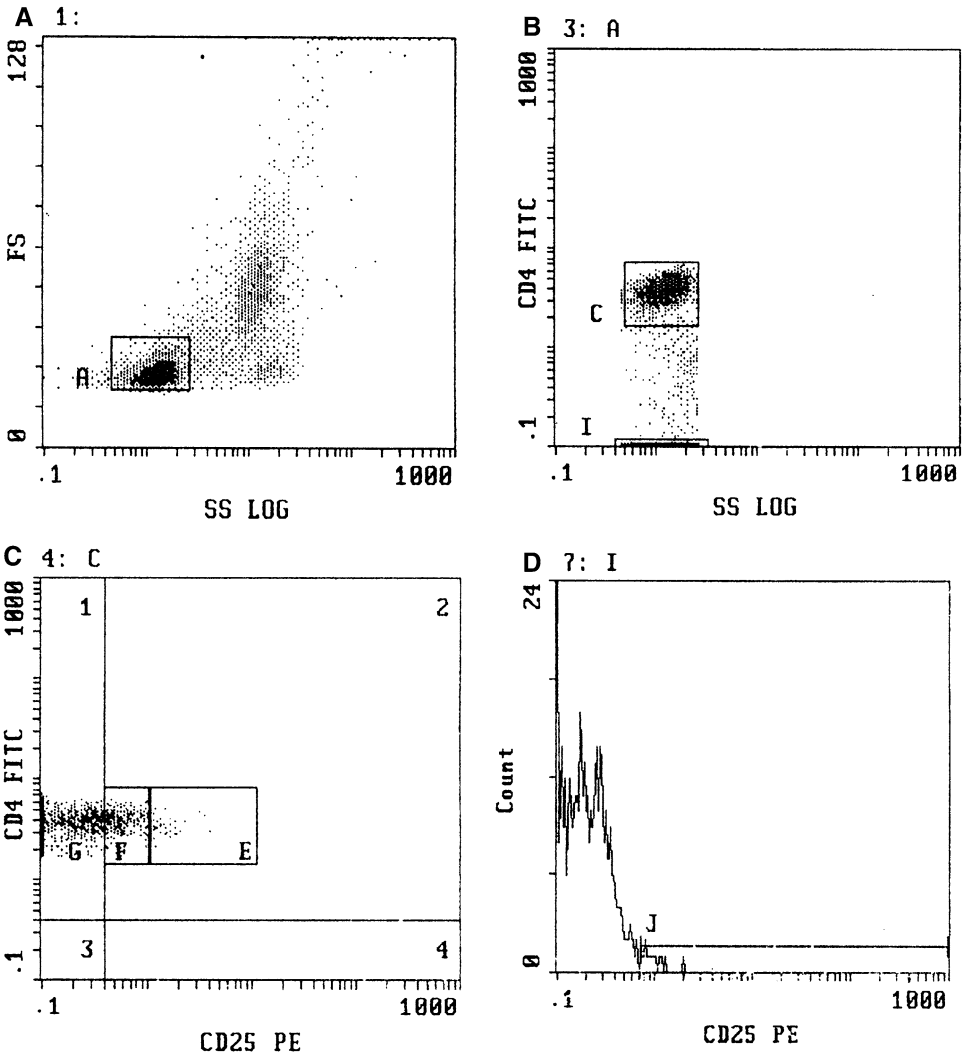

Fig. 1 The gating strategy; **a** Lymphocyte gating using foreword scatter versus side scatter. **b** $CD4^+$ cells gating: $CD4^+$ cells were acquired after gating lymphocyte population by forward- and side-scatter properties. **c** Tregs (Gate E region 2) and Teffs (Gate F region 2). **d** Gating approach: CD25 expression on non-$CD4^+$ T cells to discriminate between $CD4^+/CD25^+$ high and low cells: for discrimination of $CD25^{high}$ (Tregs) and $CD25^{low}$ (Teffs) cells were set using CD25 expression levels on non-$CD4^+$ T cells (low expression only), as described in "Materials and methods" section

virus (HCV) and hepatitis B virus (HBV) serology and negative tuberculin test, were also included in the study, comprising the control group.

Blood samples were collected from both patients and controls, provided that all subjects were free of any other systemic disease. Patients who started antileprotic treatment or were on any kind of immunomodulatory or immunosuppressive therapy likely to alter the results of the study, such as systemic corticosteroids, were excluded. For enzyme-linked immunosorbent assay (ELISA), blood was collected in sterile test tubes and centrifuged for 15 min at 50 g. Serum was separated and kept at −70 °C until used for estimation of IL-17 and IL-22, IL-10, and TGF-β, by the RayBio® human ELISA kits (Norcross City, Georgia, USA) according to manufacturer guidelines.

For flow cytometry, venous blood samples were collected using vacutainer containing anticoagulant potassium ethylene diamine tetra acetate (EDTA) in a final concentration of 1.5 mg/ml. Fifty micro liters of whole anti coagulated blood was lysed using 1 ml IQ test lysing reagent (Beckman Coulter, Miami, USA) followed by washing with phosphate buffer saline (PBS) (Oxoid, Hampshire, UK). After that, the cells were stained with combinations of the following antibodies (5 µl each): anti-CD25- PE, anti-CD4-FITC and isotype controls (FITC, PE and PE-Cy5) (Beckman Coulter). The test tubes were then incubated in dark for 20 min followed by washing with PBS. Intracellular staining FoxP3-PE-Cy5 (eBioscience, California, USA) was as follows: anticoagulated whole blood was fixed and permeabilized using FoxP3 Staining buffer Set (eBioscience) according to the manufacturer's instructions with certain modifications. Data acquisition and analysis were performed on EP-ICS XL flow cytometry using SYSTEM II version 3 software with a standard three-color filter configuration. A total of at least 10,000 $CD4^+$ cells were acquired after gating the lymphocyte population by forward- and side-scattered properties. Discrimination of $CD25^{high}$T-regs, $CD25^{low}$ activated effector-

memory T cells (T-effs) were acquired after gating the $CD4^+$ lymphocyte population as previously described by Zhang et al. [39]. The $CD25^{high}$ population was determined relative to the low intensity of CD25 staining found on non-$CD4^+$ T cells (low intensity only). Cells expressing CD25 at levels above those of the isotype control but at higher expression levels than the $CD25^{low}$ cells were considered as T-regs (Fig. 1).

Analysis of data was done by IBM computer using Statistical Program for Social Science version 15 (SPSS Inc., Chicago, IL, USA). Quantitative variables were described in the form of median and inter-quartile range (IQR). Qualitative variables were described as number and percent. Test of normality was done using Kolmogorov–Smirnov test, which revealed non-parametric distribution of the data. Comparisons between groups as regards categorical parameters were done using Chi square test (Fisher's exact test was used for comparing parameters with small sample size less than 5). Comparisons between groups as regards numerical parameters were done using Mann–Whitney and Kruskal–Wallis test. Spearman's correlation test was used to measure the correlation between the quantitative variables. A "p" value of ≤ 0.05 was considered significant, while ≤ 0.001 was highly significant.

Results

The present study included 43 leprotic patients; 16 females (37.2 %) and 27 males (62.8 %). Their ages ranged from 15 to 65 years (median of 33 years). Forty clinically free individuals served as gender- and age-matched controls; 14 females (35 %) and 26 males (65 %). Their age ranged from 15 to 60 years (median of 38.5 years). The patients were sub-grouped as follows: Group I: 6 patients (14 %) with TT, Group II: 5 patients (14 %) with PNL, Group III: 9 patients (20.9 %) suffering from borderline leprosy (five with BT, three with BB, and one with BL), Group IV: 11 patients (25.6 %) with LL, Group V: 6 patients (14 %) suffering from RL type1, and Group VI: 6 patients with ENL (14 %). SSS was negative in 16 patients (37.2 %), grouped as PB, while it was positive in 27 patients (62.8 %); grouped as MB. Clinical, demographic and histopathological features of the study subjects are summarized in Table 1. The presence of statistically highly significant higher mean FoxP3 expression% in T-regs versus T-effs, confirms proper gating process ($p < 0.001$).

Cytokines

Serum IL-10, TGF-β, were significantly higher in patients as compared to controls ($p < 0.001$), while IL-17, but not IL-22, was significantly lower ($p < 0.001$) (Table 2).

Table 1 Clinical, demographic and histopathological features of the study subjects

Parameter	Patients (43)	Controls (40)
Age		
Median (years)	33	38.5
Gender		
Males	27	26
Females	16	14
Family history of leprosy		
Negative	36	40
Positive	7	0
Clinico-histopathological diagnosis		
TT	6	–
PNL	5	–
Borderline	9	–
LL	11	–
RL1	6	–
ENL	6	–
Slit skin smear		
Negative	16	–
Positive	27	–
Paucibacillary/multibacillary		
Paucibacillary	16	–
Multibacillary	27	–

As regard the patients' subgroups, no statistically significant difference was noted among all subgroups regarding the studied cytokines (Table 3).

Comparing patients' subgroups with controls, all subgroups showed statistically higher levels compared with controls regarding IL-10 ($p < 0.001$, <0.001, =0.05, <0.001, =0.003 and <0.001 respectively) (Table 3). In addition, serum TGF-β was statistically higher in patients' subgroups compared to controls ($p < 0.001$ for all comparisons). However, statistically significant lower levels of serum IL-17 were found in patients' subgroups compared to controls ($p = 0.007$, 0.005, <0.001, 0.005, <0.001 and 0.016 respectively). On the other hand, comparing serum IL-22 level in different types of leprosy with controls, there

Table 2 Comparison between patients and controls as regards the studied cytokines

Cytokine (pg/ml)	Patients median (IQR)	Controls median (IQR)	Z	p
IL-10	10 (7–15)	2.5 (1.625–3.725)	−6.721	<0.001*
TGF-β	12 (10–18)	1.6 (0.635–2)	−7.233	<0.001*
IL-17	19 (14–23)	37.5 (34–42.563)	−5.4	<0.001*
IL-22	75 (54–104)	72.5 (28–100)	−0.694	0.488

* $p < 0.001$: highly significant difference

was statistically significant higher level only in ENL compared to controls ($p = 0.022$).

No statistically significant difference was found on comparing patients, based on the presence or absence of reaction, bacillary load, or gender, as regards the studied cytokines (data not shown).

T-regs and T-effs

Comparison between different clinical forms of leprosy revealed statistically significant differences in T-regs% ($p < 0.001$), absolute T-regs count ($p < 0.001$), FoxP3 expression% on T-regs ($p = 0.037$), and T-regs/T-effs ratio ($p = 0.002$), but not T-effs% ($p = 0.057$) or absolute T-effs count ($p = 0.163$) (Table 3). Group I (TT) and Group V (RL1) showed the highest T-regs% and absolute T-regs count (median of 5.8 %, 90.5/μl and 4.9 %, 118.25/μl respectively), followed by Group II (PNL) (median of 3.3 %, 54.9/μl), while the lowest detected levels were in Group VI (ENL) (median of 1.15 %, 29.3/μl). T-regs % was significantly higher in Group I (TT) than Groups II, III, IV and VI ($p = 0.006$, 0.001, 0.001 and 0.004 respectively), and in Group V (RL1) as compared to Groups III, IV, and VI ($p = 0.025$, 0.049 and 0.006 respectively). In addition, it was significantly higher in Group II (PNL) than Groups III and VI ($p = 0.037$ and 0.016 respectively). Similarly, T-regs absolute count was significantly higher in Group I (TT) than Groups II, III, IV and VI ($p = 0.006$, 0.001, 0.016 and 0.004 respectively), and in Group V (RL1) as compared to Groups III, IV, and VI ($p = 0.007$, 0.02 and 0.004 respectively). It was also significantly higher in Group II (PNL) than Groups III and VI ($p = 0.05$ and 0.006 respectively). The lowest FoxP3 expression% on T-regs was detected in Group II (PNL) (median of 50 %). FoxP3 expression% was significantly lower in Group II (PNL) than Groups I, III, IV and VI ($p = 0.027$, 0.009, 0.005 and 0.027 respectively). T-regs/T-effs was highest in Group VI (RL1) and lowest in Group V (ENL) (median of 0.075), with statistically significant less value in ENL compared to the other patients' subgroups ($p = 0.005$, 0.006, 0.011, 0.002 and 0.01 respectively). Moreover, it was statistically significant lower in Group III compared to Groups I and II ($p = 0.039$ and 0.031 respectively).

No statistically significant difference was found on comparing patients, based on bacillary load (PB versus MB), the presence or absence of reaction, or gender, as regards the studied T-regs and T-effs data (data not shown).

Correlations

Serum TGF-β correlated negatively to T-regsFoxP3 expression% and T-effs% ($r = -0.339$, $p = 0.026$, and $r = -0.360$, $p = 0.018$ respectively) (Table 4).

Table 3 Comparison between patients' subgroups as regards the medians and inter-quartile range (IQR) of the studied parameters as well as between patients' subgroups and controls regarding the medians of the studied cytokines

Parameter	Group I: TT (6)	Group II: PNL (5)	Group III: Borderline (9)	Group IV: LL (11)	Group V: RL1 (6)	Group VI: ENL (6)	X^2	p	Controls	X^2	p
IL-10 (pg/ml)	10.25 (6.58–24.25)	3.9 (3.8–10.25)	17 (9.25–17)	10 (7–10)	10.75 (6.95–18.75)	9 (2.25–15.5)	7.743	0.171	2.5	49.36	<0.001**
TGF-β (pg/ml)	13.9 (9.13–22.75)	14 (8.55–20)	11.5 (9.25–25)	12 (10–17)	14 (7.28–20.5)	12 (9–16.25)	0.415	0.995	1.6	52.79	<0.001**
IL-17 (pg/ml)	21 (18.5–29.5)	14 (12.5–22)	19 (16–41.5)	12.5 (12–38)	19.5 (16.63–28.25)	20 (19–21.38)	6.973	0.223	37.5	32.08	<0.001**
IL-22 (pg/ml)	61 (48–91)	40 (24–89.5)	75 (61–96)	62 (54–120)	67 (36–148.5)	102 (89.25–170)	7.522	0.185	72.5	7.24	0.299
T-regs%	5.8 (5.225–7)	3.3 (2.8–3.3)	2.3 (1.9–2.7)	2.8 (2.3–4.3)	4.9 (3.75–10.725)	1.15 (0.85–1.8)	26.308	<0.001**			
T-regs absolute count/μl	90.5 (81–163.75)	54.9 (54.9–59)	47.9 (26.7–54.5)	27.7 (26.9–67.7)	118.25 (67.925–175.125)	29.3 (9–41.7)	22.259	<0.001**			
T-regs FoxP3 expression%	72 (61.975–79.475)	50 (44–50)	62.3 (59.7–68.35)	68.7 (62.3–78.2)	59.45 (31.2–68.075)	71.9 (50.65–89.4)	11.862	0.037*			
T-effs%	11.25 (7.2–20.275)	9.2 (6.8–9.2)	12.7 (6.45–13.5)	12.2 (9.2–15.6)	7.25 (5.7–12.525)	16.4 (13.6–21.9)	10.748	0.057			
T-effs absolute count/μl	238.5 (137.075–346)	153.2 (143.2–153.2)	321 (105.5–358.5)	154.4 (38.7–227.1)	189.5 (139.875–319.525)	373 (213.95–488.625)	7.875	0.163			
T-regs/Teffs ratio	0.515 (0.26–0.975)	0.36 (0.36–0.41)	0.21 (0.145–0.325)	0.3 (0.18–0.4)	0.88 (0.2–1.475)	0.075 (0.035–0.125)	18.589	0.002*			

* $p < 0.05$: significant difference

** $p < 0.001$: highly significant difference

Table 4 Results of correlation between all the studied parameters

Parameter	Spearman's correlation; significance (p)			
	IL-10 (pg/ml)	TGF-β (pg/ml)	IL-17 (pg/ml)	IL-22 (pg/ml)
T-regs%	0.181 (0.247)	−0.054 (0.729)	−0.006 (0.971)	−0.206 (0.184)
T-regs absolute count/μl	0.191 (0.219)	0.002 (0.992)	0.082 (0.599)	−0.172 (0.27)
T-regs FoxP3 expression%	0.01 (0.948)	−0.339 (0.026)*	−0.076 (0.63)	0.269 (0.081)
T-effs%	0.048 (0.758)	−0.360 (0.018)*	−0.071 (0.649)	0.021 (0.896)
T-effs absolute count/μl	0.209 (0.179)	−0.273 (0.077)	−0.041 (0.792)	0.106 (0.5)
T-regs/Teffs ratio	0.057 (0.715)	0.218 (0.161)	0.062 (0.694)	−0.113 (0.472)
IL-22 (pg/ml)	0.07 (0.657)	−0.254 (0.1)	−0.056 (0.722)	
IL-17 (pg/ml)	0.216 (0.165)	0.253 (0.101)		
TGF-β (pg/ml)	0.118 (0.453)			

* $p < 0.05$: significant difference

Discussion

In a previous work, we studied the frequency and FoxP3 expression of circulating $CD4^+$ $CD25^{high}FoxP3^+$T-regs in leprosy patients [4]. In agreement with our previous findings [4], we found increased frequencies of T-regs in TT patients, and in patients with RL1 and PNL. These disease forms are known of relatively high cell-mediated immunity (CMI) against *Mycobacterium leprae* (*M. leprae*) [28]. Therefore, expansion of T-regs could be interpreted as a protective counter-mechanism trying to regulate effective anti-pathogen immune response and to attenuate the *M. leprae*-induced chronic immune activation. In accordance, ENL patients showed T-regs depletion, consistent with disease progression and humoral immune hyperactivation [13]. This was also supported by our current finding of significantly increased suppressive marker; TregsFoxP3 expression% in this category of patients, in accordance with our previous work [4]. In contrast, PNL patients showed the lowest FoxP3 expression%, reflecting a state of hypersensitivity, consistent with greater nerve damage in this category [23]. T-regs/T-effs ratio was lowest among ENL patients, as a reflection of depressed patients' T-regs count, consistent with our previous results [4].

Our results revealed that serum IL-10 was significantly elevated in patients versus controls, in agreement with Moubasher et al. [28] and Trombone et al. [37] findings. However, we did not report significant difference among patients' subgroups, in contrast to Moubasher et al. [28] and Trombone et al. [37] findings who revealed higher serum IL-10 in LL and ENL compared to TT and RL1 and in MB compared to PB cases respectively, with significant positive correlations with the bacterial index (BI). On the other hand, Misra et al. [27] showed that in vitro infected monocytes contribute to the development of T cell anergy in LL by releasing IL-10, while Mutis et al. [29], Sieling et al. [36] and Machado et al. [22] revealed that clones from different disease forms produced comparable amounts of IL-10. Nevertheless, the aforementioned in vitro studies adopted different methodologies with regard to activation or no activation of PBMCs. Further in vivo and in vitro studies on larger populations, thus, are warranted.

IL-10 is known to regulate various processes involved in generation of Th17 responses, a Th subset with possible synergistic value to Th1 in leprosy [1, 31]. In our study, high serum IL-10 in leprosy patients was associated with low serum IL-17, compared to controls. Therefore, we speculate that high IL-10 and deficient IL-17 production can contribute to the development of leprosy, or even lead to disease progression towards the MB immunocompromised pole. In accordance, da Motta-Passos et al. [7] concluded that low expression of IL-17A in leprosy patients may be a constitutive genetic feature of patients or a circumstantial event induced by the local presence of the pathogen, as an escape mechanism. Likewise, Lim et al. [20] revealed that susceptibility to pulmonary disease due to *Mycobacterium avium-intracellulare* complex might reflect low IL-17 and high IL-10 responses rather than Th1 deficiency. In accordance, inhibition of IL-10 signaling during *Bacillus Calmitte Guerin* (BCG) vaccination enhanced host-generated antigen-specific interferon-γ (IFN-γ) and IL-17A responses [31]. Thus, antagonists of IL-10 may be of great benefit as adjuvants in preventive vaccination against leprosy, as well as in controlling progressive down grading disease.

In contrast, IL-17A was consistently expressed in skin biopsies before and after thalidomide treatment for ENL [26]. Moreover, IL-17 isoforms showed significantly higher expression and release in supernatants of antigen stimulated PBMC cultures and dermal lesions of healthy contacts and TT as compared to LL leprosy [34]. In addition, Trombone et al. [37] demonstrated that IL-17 and TGF-β expression prevail in diseased sites versus controls, but no significant differences were found between the clinical forms. Further in situ and in vitro studies are recommended for better understanding of these cytokines' expression in leprosy.

Regarding serum IL-22, we did not find a statistically significant difference between levels in patients compared to controls. Although antimicrobial peptides, associated with host defense, are cooperatively induced by IL-22, IL-17A and IL-17F, IL-22 alone is not as strong as IL-17A or IL-17F in inducing such peptides [19]. Moreover, previous studies revealed that during experimental tuberculosis, IL-22 was not essential for the generation of CMI [15]. In contrast, Trombone et al. [37] showed that serum IL-10 and IL-22 were significantly higher in leprosy patients than in controls. Though secreted also by Th17, it is now becoming apparent that the IL-22 expression profile differs from that of IL-17A [40].

Interestingly, we found that serum IL-22 was significantly higher in patients with ENL compared to controls. In a previous study on chronic hepatitis, IL-22 was found to help to limit damage and allow survival of damaged hepatocytes [14]. Therefore, we speculate that our finding can be interpreted as counter mechanisms to limit damage during ENL; the more systemic reaction of leprosy reactions.

TGF-β1 plays roles in the suppression of T cell responses, inhibiting both IFN-γ and IL-2 expression, and has the ability to inhibit the lytic activity of macrophages [8]. Our leprosy patients showed higher serum TGF-β than controls, with no difference comparing patients' subgroups, indicating active anti-inflammatory response throughout leprosy spectrum. In accordance, serum TGF-β correlated negatively to T-effs%. Previous reports showed higher TGF-β1 concentrations and/or expression in LL, BL and ENL forms than BB, BT and TT cases and/or control, with positive correlation to BI [11, 12, 30, 38]. However, Kiszewski et al. [16] reported that the three TGF-β isoforms and their receptors were expressed in both polar forms of leprosy, in agreement with our results. Besides, Andersson et al. [2] found that circulating cytokine profiles were similar in patients with and without RL1, with or without treatment, denoting compartmentalization of pathology in some forms of leprosy that necessitates studying the immune response in the skin.

TGFβ is known to be needed for maintenance of T-regs, because in its absence the peripheral numbers goes down in spite of normal thymic output [25]. However, TGF-β did not significantly correlate to T-regs number in our study. This discrepancy can be explained by the fact that T-regs constitute various subsets. Subsets of T-regs are now including tT-regs = thymic derived, pT-regs = in vivo peripheral derived, and iT-regs = in vitro iT-regs [9]. Thus, the detected circulating and lesional T-regs in various forms of leprosy need further identification studies.

In our study, TGF-β correlated negatively to T-regs FoxP3 expression%. Apparent contradiction may be encountered while reviewing other related reports. However, Kumar et al. [17] revealed that low ubiquitination of FoxP3 in T-regs is a major driving force in conferring stability to FoxP3 which in turn is linked to suppressive potential of T-regs. In addition, while we reported elevated serum TGF-β in leprosy, we did not know its exact source (from Th3 or other cells), and whether it is a cause or an effect within the disease process. Thus, more in-depth research is needed to clarify such apparent contradiction within the related reports.

The average levels of the studied cytokines were low when compared to previous reports in leprosy. In addition to different methodology, commercial kits and exposure levels of the study samples, differential cytokine expression in different ethnic and racial groups should be considered. In 2013, a bibliographic search was performed on Medline and ISI databases and included studies on cytokine expression in response to *M. tuberculosis*, published between January 1980 and October 2011. Consequently, different susceptibility, cytokine expression and immunogenetics were reported in different populations [21].

One limitation of the study is the lack of antigen specific responses of in vitro cultures. Further studies where T-regs are isolated and expanded with *M. leprae* specific antigens and supernatants are assessed for cytokines are, thus, recommended. Another limitation is the small sample size so that stratification of data rendered statistical significance limited. Thus, further studies on larger population are warranted.

In conclusion, active leprosy is associated with defective secretion of IL-17 but not IL-22, and overproduction of IL-10 and TGF-β, denoting defective host-protective cytokines and overproduction of host-impairing cytokines respectively. TT, RL1 and PNL express significantly higher frequency of $CD4^+$ $CD25^{high}FoxP3^+$T-regs, consistent with effector immune mechanisms activation, but with lower FoxP3 expression (in PNL), which was negatively correlated to TGF-β, for further studies. On the other hand, LL and ENL forms are characterized by disease progression and immune hyperactivation, marked by deficient T-regs, and increased TregsFoxP3 expression% with low T-regs/T-effs ratio (in ENL). The present study pinpointed a potential role of Th17, $CD4^+$ $CD25^{high}FoxP3^+$T-regs, and probably $CD4^+$ $CD25^+IL\text{-}10^+$ T regulatory cells 1(Tr1), and Th3 in leprosy. These findings may open a door to understand part of the immunological course of this disease, for better control where more research is mandatory.

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Conflict of interest All authors have no conflict of interest.

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