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FOXP3 expression and frequency of regulatory T cells in healed individuals from *Leishmania major* infection and the asymptomatic cases

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ABSTRACT

Two groups of residents in an endemic area of *Leishmania major* infection in Iran with positive leishmanin skin tests who were either asymptomatic or had healed cutaneous leishmaniasis lesions were compared with respect to their T helper responses. The percentages of regulatory T cells (T_{reg}; $CD4^+CD25^{high}$ $FoxP3^+$) from the peripheral blood and $CD4^+$ T cells producing intracellular cytokines (IL-4, IL-10, IL-17 and IFN-γ) from the stimulated PBMCs were evaluated by flow cytometry and the expressions of *RORC* and *FOXP3* genes were quantified by real-time RT-PCR. T responder ($CD4^+CD25^-$) and T_{reg}-enriched ($CD4^+CD25^+$) cells were isolated magnetically and the suppressive capacity of the latter and the cytokines (IFN-γ, TGF-β and IL-10) secreted from them were evaluated by *in vitro* assays. The results showed that the frequency of T_{reg} in the studied groups were similar and T_{reg} from both groups exhibited high yet similar suppressive capacities while significantly higher levels of *FOXP3* expression was observed in the asymptomatic group. Taken together, similar frequency and suppressiveness of T_{reg} combined with high ratios of IFN-γ/IL-10 producing $CD4^+$ T cells were common in both groups; however the members of the asymptomatic group appeared to require higher expression of *FOXP3* to maintain their immunity to re-infection.

1. Introduction

Leishmaniases are a spectrum of parasitic infections caused by the genus *Leishmania* which have a digenetic life cycle, alternating from flagellated motile promastigotes inside their vectors (sandflies) to intracellular amastigotes within mononuclear phagosomes such as macrophages of their mammalian hosts [1,2]. The global incidence of these debilitating infections such as cutaneous leishmaniasis (CL) has been on the increase in recent years, mostly due to the rise in urbanization, international travel and military operations [3,4]. CL is a public health concern in Iran which is among the 7 countries where 90% of the global infections are occurred [5,6]. The infections are mostly caused by *Leishmania major* and *Leishmania tropica* although the former being the most frequent parasite in CL endemic areas of the country [7]. Since CL is dependent on complex interactions of multiple parameters, namely the parasite, the host, the vector and the environment, *L. major* infections may culminate in diverse clinical forms in the endemic areas. In one extreme, the infection could be manifested in form of cutaneous lesions that may appear in different parts of the body whereas in the other extreme, it is detected as subclinical infections that remain asymptomatic for many years. The lesion usually heals within a few months and leaves a defined scar at the inoculation site. One of the valuable tools for research in leishmaniasis, especially in terms of epidemiology and the evaluations of new vaccine candidates, is leishmanin skin test (LST) [8] which its response remains positive for many years or even decades after the infection [8–10]. The subclinical infections are detected by positive LST [11,12] and their etiology is poorly understood [13].

Natural regulatory T cells (T_{reg}), delineated as $CD4^+CD25^+FoxP3^+$ cells are the major population of T_{reg} in the immune system and are widely known as the maintainers of tolerance and the homeostatic preventers of excessive damages during the inflammatory responses [14–16]. The presence of T_{reg} in lesions of patients suffering from CL due to *Leishmania braziliensis* and *L. major* has been verified [17,18]. Although the precise suppressive mechanisms of T_{reg} has not yet been clarified, the current hypotheses account for contact-dependent interactions and secretion of inhibitory cytokines such as IL-10 and TGF-β, as two main routes to exert

Abbreviations: CL, cutaneous leishmaniasis; T_{reg}, regulatory T cells; T_{resp}, responder T cells; LST, leishmanin skin test.

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suppression in CL [19]. Efforts to elucidate the roles of T_{reg} in leishmaniases and particularly CL have been largely confined to the studies performed by means of animal models [20–23]. Preliminary human studies in the New World have reported the accumulation of functional T_{reg} at lesion sites in CL due to *L. braziliensis* [17]. Investigations on CL caused by *Leishmania guyanensis* have also pointed to the recruitment of functional T_{reg} from the peripheral blood to the skin lesions where such intralesional T_{reg} are considered to have a role in immunopathogenesis in the chronic cases [24] and a correlation between the unresponsiveness to the treatment and intralesional IL-10 and *FoxP3* mRNA expressions were detected in the acute cases [25].

In recent studies, the relevance of T_{reg} to human CL has been readdressed. For instance, impaired T_{reg} function in people suffering from chronic human dermal leishmaniasis due to *Leishmania* (*Vianna*) spp. has been accounted for the pathogenesis of the disease [26]. Moreover, increased gene expression and protein staining of T_{reg} markers in late (more than 6 months) compared to early (less than 4 months) lesion biopsies of *L. major*-infected CL patients have been demonstrated [18]. In the present study, instead of focusing on patients with active CL, we investigated the role of T_{reg} compared to other $CD4^+$ T helper subsets (i.e. Th1, Th2 and Th17) in two groups of *L. major*-infected volunteers who are immune to re-infection. The members of these groups were residents of a CL endemic area who had either recovered from their CL lesions or were asymptomatic.

2. Materials and methods

2.1. Human subjects

Three groups of volunteers, residing in an endemic rural area of zoonotic CL around the city of Damghan (approximately 340 km east of Tehran) were included in this study. First, 16 volunteers (10 females, 6 males, age range: 13–45, mean: 32.4 ± 10.1) recovered from *L. major* infection with positive LST response, called the healed group. Second, 18 healthy volunteers (11 females, 7 males, age range: 12–59, mean: 36.8 ± 14.3) with no sign or history of dermal lesions with LST positive response, called the asymptomatic group. Third, 15 healthy individuals (11 females, 4 males, age range: 19–47, mean: 33.8 ± 8.5) with LST equal to zero, called the control. All volunteers were clinically examined and those who met the inclusion but not the exclusion criteria (major illnesses such as immunosuppression, HIV infection, cancer, renal failure, tuberculosis along with recent major surgery and pregnancy) were enrolled in the study. Those who were older than 60 and younger than 12 years of age were excluded after the selection procedure. All participants provided written informed consent. The study protocol, questionnaire, consent forms and all procedures were approved by the Ethics Committee of Pasteur Institute of Iran.

2.2. Leishmanin skin test (LST)

The TDR/WHO reference leishmanin produced under guidelines of good manufacturing practices at Pasteur Institute of Iran [27], was used for skin testing according to the procedure described previously [28]. Briefly, 0.1 ml of leishmanin was injected intradermally into the inner surface of the left forearm. The diameter of indurations at injection site was measured by a millimeter graduated ruler 48 h later using the ball-point pen technique [29]. Indurations $\geqslant$5 mm were considered as positive LST.

2.3. Isolation of T cell subsets

Peripheral blood samples were collected in heparinized tubes on the field and were transferred to the laboratory for the same-day-processing. PBMC from each individual were separated from fresh whole blood using Lymphosep lymphocyte separation media (Biosera Ltd., East Sussex, UK) and density gradient centrifugation (270*g*). The PBMC (on average $\sim 25 \times 10^6$) were used to isolate $CD4^+CD25^-$ (T_{resp}) and $CD4^+CD25^+$ (T_{reg}) T cells by MACS using human $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany), according to the manufacturer's instructions.

2.4. Lymphocyte proliferation assays

The magnetically sorted $CD4^+CD25^-$ (T_{resp} cells; 25×10^3) alone or in addition to $CD4^+CD25^+$ (T_{reg}-enriched cells) at a ratio of 1:1 or 4:1 were stimulated in duplicates with 1 μg/ml of anti-CD3 mAb, anti-CD28 mAb and IL-2. The cells were harvested after 96 h of incubation (37 °C, 5% CO_2, 95% humidity) in flat-bottomed 96-well plates and an additional 16-h pulse with [methyl]-^{3}[H]-thymidine (0.5 μCi/well). Thymidine incorporation into DNA was measured by scintillation in count per minute (CPM). The percentages of proliferation for each group were calculated by dividing their co-cultures CPM values by their corresponding T_{resp} CPM values, multiplied by 100.

2.5. Quantitative detection of cytokines secreted from lymphocytes

Before the thymidine pulse (above section), 100 μl supernatant from each well containing the magnetically separated $CD4^+CD25^-$ (T_{resp}) or $CD4^+CD25^+$ (T_{reg}-enriched) cells were collected and the contents of the duplicates were pooled (200 μl in total) and stored at −70 °C to be assayed in duplicates by human cytokine ELISA kits from Bender MedSystems (GmbH, Vienna, Austria; distributed by eBioscience), according to the manufacturer's instructions. Later, IL-10 and IFN-γ were quantified by Platinum ELISA kits and TGF-β1 cytokine was quantified by Instant ELISA kit.

2.6. Intracellular cytokine assays

All mAb were from eBioscience (San Diego, CA, USA). To assay the intracellular cytokines, PMBCs in duplicates from each individual (2×10^6/ml/well) were stimulated with soluble *Leishmania* antigen (SLA; 26 μg/ml) as described previously [30] for 48 h at 37 °C (5% CO_2, 95% humidity) then monensin sodium (3 μmol/ml; Sigma, St. Louis, MO, USA) was added 4 h prior to harvesting. The cells were washed by PBS and then stained with anti-human CD4 PerCP-Cy5.5 (0.125 μg/test) mAb and fixed by Cytofix/Cytoperm fixation and permeabilization (BD Biosciences; San Diego, CA, USA). Following 20 min incubation (4 °C) and wash, the cells were treated with either anti-human IL-4 FITC (0.25 μg/test) or anti-human IL-10 PE (0.25 μg/test) or anti-human IL-17A PE (0.25 μg/test) or anti-human IFN-γ FITC (0.50 μg/test) mAb in presence of 50 μl 0.1% (w/v) saponin from quillaja bark (Sigma) for 30 min (4 °C) and then were washed with saponin and PBS. To assay $CD4^+$ T_{reg}, anti-human CD4, anti-human CD25 PE (0.125 μg/test) and anti-human *FoxP3* FITC (1.0 μg/test) mAb were treated as above with the peripheral blood samples except the cells were not stimulated with SLA. Lymphocytes were gated by plotting forward versus side scatter, followed by gating of the $CD4^+$ population. The $CD25^{high}$ were gated and the percentage of $FoxP3^+$ cells among them was determined as follows. For each sample, the percentage of $CD4^+CD25^{high}$ lymphocytes was defined from multiplication of the percentage of gated $CD4^+CD25^+$ and gated $CD4^+CD25^{high}$ cells divided by 100. The percentage of $FoxP3^+$ $CD25^{high}$ of $CD4^+$ cells was calculated by multiplication of the percentage of the above-mentioned $CD4^+CD25^{high}$ lymphocytes and the percentage of gated $FoxP3^+CD25^{high}$ cells divided by 100. Cytofluorimetric acquisitions were performed with a Partec PAS