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Analysis of cellular immune response in cutaneous leishmaniasis Syrian patients

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Abstract: Leishmaniasis comprises a spectrum of diseases caused by an obligate intracellular parasite *Leishmania*. There is a wide range of clinical presentations which are dependent on the different species of the parasite as well as immune response offered by the host. This study was done to analyze quantitatively the cellular immune host response by demonstrating various T cell subsets in new and old cutaneous leishmaniasis (CL) patients using flow cytometric immunophenotype, and some cytokines levels were evaluated by enzyme-linked immunoabsorbent assay in patients with new or old CL. Blood samples were collected from CL patients visiting the Dermatology Hospital in Damascus, after confirming of their infection microscopically. Patient's samples were divided into two groups according to legion's age, the first group containing new infections (1-3 months, 28 patient), and the second one including old infections (6-9 months, 30 patient). In result, immunophenotyping showed increasing in CD4 and CD8 T-cells proportion through infection progression. As well, an obvious increasing in CD4CD25 regulatory T-cells proportion was observed in old patients in comparison with early stages of infection and non-infected persons. In addition, higher interleukin (IL)-4 level was observed in patients with new lesions, whereas old and cured subjects produced IFN-gamma and IL-12 at elevated levels. In conclusion, we tried in this study to determine the immune parameters provide protective immunity and responsible for cure.

Keywords : Cutaneous leishmaniasis, cellular immune host response, immunophenotyping, Cytokines.

Introduction:

Leishmaniasis comprises a spectrum of diseases caused by an obligate intracellular parasite Leishmania. There is a wide range of clinical presentations which are dependent on the different species of the parasite as well as immune response offered by the host [1]. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis worldwide, representing 50–75% of all new cases [1-3]. In the Old World, it can be caused by *L. major* and *L. tropica*. Both species can produce large numbers of skin ulcers on the exposed parts of the body, and the infection site is usually localized to the site in which the sand fly bite occurs [4]. *L. major* is often self-curing and L. tropica is recalcitrant to treatment [5]. In Syria, both *L. tropica* and *L. major* are endemic and transmission will continue. In September 2012, a cutaneous leishmaniasis outbreak began among Syrian refugees in Lebanon. For 948 patients, *L. tropica* in 85% and *L. major* in 15% of patients were identified (http://wwwnc.cdc.gov/eid/article/20/10/14-0288_article). Leishmaniasis is characterized by a spectrum of disease phenotypes that corresponds to the strength of the host's cells-mediated immune response. World research is focused on the host's immune response in all type of leishmaniasis for a better understanding of host

parasite interaction, evasion mechanisms of the parasites and the effective elimination of the parasite by the host. It may help in better understanding of the disease and improvement on treatment modalities [6].

Both susceptible and resistant phenotypes exist within human populations [1]. The resistance is conferred by T-helper type-1 (Th1) cells while the susceptibility is conferred by T-helper type-2 (Th2) cells [7]. Interleukin-12 (IL-12) produced by macrophages and dendritic cells and interferon-gamma (IFN-γ) produced by natural killer cells (NK), and previously activated T cells, promote the development of Th1 cells, whereas IL-4 induces the development of Th2 cells. The Th1 subpopulation, important for induction of leishmaniasis resistance, produce IFN-γ and tumour necrosis factor-alpha (TNF-α) which play an important role in cellular immune responses against intracellular pathogens by activating macrophages for intracellular killing of pathogens [8]. On the other hand, Th2 cells produce IL-4, IL-5, IL-10, and IL-13, and are associated with leishmaniasis susceptibility in *L. major* infection murine models [9-11]. Macrophage activation by IFN-γ has been recognized as the major mechanism of *Leishmania* killing [12]. IFN-γ activates macrophages to express inducible nitric oxide synthetase type-2 (iNOS2), the enzyme catalyzing the formation of nitric oxide which kills the intracellular amastigotes. In contrast, Th2 immune response limits the action of Th1 functions via IL-10 and IL-4, which deactivate macrophages helping intracellular parasite growth and disease progression [7]. In human, IL-10, a key macrophage deactivating cytokine, is not restricted to Th2 cells and can be produced by several types of T helper populations [13]. T-cell receptor types have also proved interesting in the immune response to *Leishmania*. In most cell-mediated responses, T-cells bearing α β receptors are much more predominant than those expressing γ δ receptors. In contrast, during the early phase of the response to CL, 30 % of the T-cells may express γ δ receptors [14]. Being a parasite, *Leishmania* ensures its own survival by modulating host immune system either by inducing immunosuppression or by promoting pro-parasitic host functions. A detailed knowledge of this host-parasite interaction would help in designing prophylactic and therapeutic strategies against this infection [7, 15].

In view of CL spreading in Syria, especially that caused by *L. tropica*, we tried in this study to determine the immune parameters provide protective immunity and responsible for cure.

Materials and Methods:

Sampling

Blood samples were collected from CL patients visiting the Dermatology Hospital in Damascus, whose infection was confirmed microscopically. Patient's samples were divided into two groups according to legion's age; the first group containing new infections (1-3 months, 28 patient), and the second one including old infections (6-9 months, 30 patient).

Flow cytometric immunophenotype

Immunophenotyping was performed according to [16]. Manufacturer's instructions (BD Biosciences) were also considered. Flow cytometric analysis was performed using a general panel of fluorescent antibodies against the following antigens typical for different cell lineages and cell types: CD3, CD4, CD7, CD8, CD16, CD19, CD25, CD45, CD56, CD57, TCR αβ, TCR γδ. All antibodies purchased from BD Biosciences. Samples analyzed on a BD FACSCalibur™ flow cytometer. Autofluorescence, viability, and isotype controls were included. Flow cytometric data acquisition and analysis conducted by BD Cellquest™ Pro software. Interpretations of flow cytometric results were according to [17].

Determination of Cytokine levels/ELISA assay:

Cytokines concentration was measured using ELISA kits for IL-4, IL-12 and IFN-γ and according to manufacturer's instructions (DRG Instruments GmbH, Germany). Each 96-well plate (8 wells/strip, 12 strips) is coated with a cytokine-specific capture antibody. 100 µl of serum samples and Standards was added to each well, then 100 µl of Incubation Buffer was added and incubated at room temperature on shaker for 2 h. detection was performed by adding 50 µl of detecting antibody conjugated with HRP (anti-IL-HRP) in 100 µl of Specimen Diluent and incubation as described above. Final, 200 µl of TMP-Substrate was added, and after 30 min of incubation 50 µl of Solution Stop was added. All steps were separated by washing with Solution Wash. The absorbance was measured at 450 nm by an ELISA reader (Huma Reader HS).

Results:

Immunophenotyping results showed increasing in $CD4^+$ and $CD8^+$ T cells proportions through infection progression, and the highest percentages were recorded in late stage of infection; ~40% and ~50% of $CD4^+$ and $CD8^+$, respectively. As well, an obvious increasing in $CD4^+CD25^+$ regulatory T cells proportion was observed in old patients (~15%) in comparison with early stage of infection and non-infected persons (healthy control) (Fig. 1). Moreover, natural killer cells (NK) percentage was relatively higher (~18%) in late stage of infection, while B cells percentages were approximately equaled (~10%) in both stages of infection and close to its percentage in healthy control (~8%).

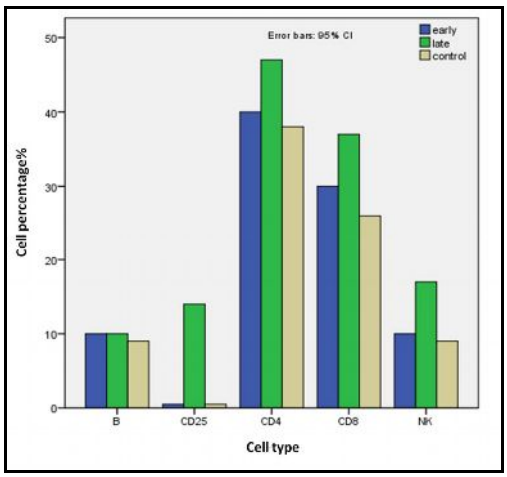

Figure 1: Illustration of cell types and its proportions as showed by immunophynotyping in both early (blue bars) and late (green bars) stages of infection in comparison with healthy control (off-white bars).

In addition, cytokines measurement showed higher concentrations of interleukin (IL)-4 in patients with new lesions (3.2 pg/ml) in comparison with those with old lesions (2 pg/ml). On the other hand, old and cured subjects produced IL-12 and IFN-γ (118 pg/ml, and 1.18 pg/ml, respectively) at elevated levels in comparison with new lesions (14 pg/ml, and 0.38 pg/ml, respectively) and healthy controls (0.1 pg/ml, and 0.2 pg/ml, respectively) (Fig. 2).

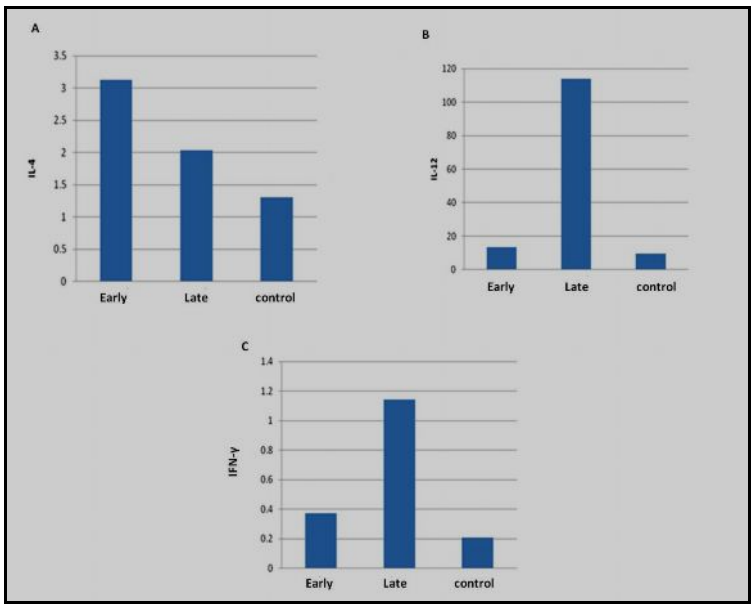

Figure 2: Illustration of cytokines concentrations (pg/ml) in both early and late stages of infection in comparison with non-infected human (healthy control). The highest concentration of IL-4 *(A)* was observed in early stage of infection, whereas IL-12 *(B)* and IFN-γ *(C)* showed the highest concentration in late stage of infection.

Discussion:

T-cell-mediated immunity plays a central role in host responses to intracellular pathogens [18]. Extensive studies with experimental models have shown that the outcome of infection is critically dependent on the activation of one of the two subsets of CD4 T cells, Th1 and Th2 [19]. Gamma interferon (IFN-γ), secreted by Th1 cells, is the most potent macrophage-activating cytokine leading to host resistance to infection with *Leishmania* parasites [20, 21], whereas interleukin-4 (IL-4), secreted by Th2 cells, is associated with down-modulation of IFN-γ-mediated macrophage activation [22, 23]. However, in human cutaneous leishmaniasis, a clear functional dichotomy in CD4 T cells has not definitely been documented. Concerning the key role of cytokines, we were interested in defining the immune response of non-healing patients and comparing it with that of individuals who had recovered from infection and patients who were newly infected. Immune response to different species of *Leishmania* have been studied mostly in subjects with active lesions and/or patients who had recovered [24-27]. There are few reports on the immune status of non-healing patients, particularly those infected with *L. major*. Treatment of patients might benefit from immunological interventions, if the role of T-cell subsets in disease and resistance is clearly clarified. Therefore, we aimed to determine parameters of protective immunity and responsible for cure in Syrian CL patients by *L. tropica.*

In infected individuals who do not develop lesions or disease, the immune response expresses a Th1 pattern that can be detected in peripheral blood cells [28]. However, the intensity of inflammation can also result in ulcerations in which the healing capacity of these cells is lost, thus favouring the immunopathology of the disease [29]. $CD4^+$ and $CD8^+$ T cells proportions showed an increasing through infection progression. As well, an obvious increasing in $CD4^+CD25^+$ regulatory T cells proportion was observed in old patients in comparison with early stage of infection and non-infected persons. The abundance of CD4+ Th1 cells may be responsible for the development of immunity and for the natural healing of lesions [30]. In biopsies of older ulcers from LCL and DL patients, there was a higher frequency of CD4+ T cells than in the early lesions and papules; these data are consistent with previous studies [31, 32]. Regulatory T cells play an important role in the regulation of immune responses and are also responsible for immunologic tolerance. $CD4^+CD25^+$ cells are found in the thymus and peripheral blood of both humans and mice [33-35]. Recently, it has been shown that cells can control a large number of infections by modulating the intensity of the effector immune response [36], which also prevents the expression of immunemediated lesions. In contrast to our result that showed an elevated percentage of $CD4^+CD25^+$ cells in late stage of infection, the levels of $CD4^+CD25^+$ cells in the peripheral blood of patients with CL were similar to those found in healthy control subjects [37], and other studies showed that $CD4^+CD25^+$ T cells represent 5%–10% of peripheral $CD4^+$ T cells in the blood [33-35]. B cells percentages were nearly equaled in both stages of infection with *L. tropica* and closed to its percentage in healthy control. Little has been reported about B cells and antibodies in CL patients. Two studies revealed that late located CL lesions had a higher frequency of B lymphocytes than early lesions. Higher production of IFN-γ is associated with increased antibody secretion by B cells, helping to contain the parasites, but IFN-γ is also associated with *Leishmania* entry into macrophages, a process that results in permanent lesions [38, 39]. One study suggested that the humoral response was not essential for the development of protective immunity in human leishmaniasis [40]. However, studies in experimental models have shown that B cell depletion leads to disease exacerbation, suggesting that B cells are necessary for the activity of the T cells that mediate lesion healing; thus, humoral responses may in fact play a role in mediating protective immunity against leishmaniasis [41].

Cytokines are central elements in the development of an immune response and have received a great deal of attention in both human and experimental leishmaniasis. Cures for leishmaniasis are related to the predominance of a Th1 response, since this leads to the production of IFN-γ and activation of parasite-infected macrophages [42]. In contrast, a Th2 response with IL-4 and IL-10 production often results in disease progression [42]. Previous efforts have focused on understanding the early events that influence the development of Th1 or Th2 cells. Cytokines measurement showed higher concentrations of IL-4 in patients with new lesions in comparison with those with old lesions. On the other hand, old and cured subjects produced IL-12 and IFN-γ at elevated levels in comparison with new lesions and healthy controls. In previous study, there is an evidence that there is existence of a T cell population producing both IFN-γ and IL-10, which could be functionally important as the regulatory subset that allows a balance between Th1 and Th2 cells in the cured VL patients [43]. IL-4 is considered to be the signature cytokine of Th-2 response. Although known to correlate with disease in murine CL but not murine VL [44].

Recent reports suggest the involvement of immune cells other than the Th1 and Th2 subsets of CD4+ T cells. Among these, CD8+ cells macrophages and NK cells play major roles. In addition, recent experimental data obtained with studies on CD4+ CD25+ T cells point to a probable regulatory function of these cells in maintaining the immune homeostasis in human leishmaniasis. In contrast to the earlier ideas that antagonistic functions of IFN-□□and IL-4 determine the outcome of protection or pathogenesis of the disease, recent studies emphasize the importance of the balance of the two regulatory cytokines IL-12 and IL-10, critical for the regulation of the immune modulation during infection, pathogenesis, and chemotherapy. To understand the nature of human infection with these parasites and to develop better chemotherapeutic and vaccine strategies, further in depth studies focused on the immune modulation in the subclinical and asymptomatic individuals needs attention. Immune status of the patients demonstrating antimony resistance and those suffering from relapse of infection should also be a major area of investigation as these patients might have developed some impairment towards protective immunity [43].

In conclusion, we tried in this study to determine the immune parameters provide protective immunity and responsible for cure after *L. tropica* infection, and to compare it with that reported in previous reports for other *Leishmania* species.

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