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Original article

Phenotyping of circulating CD8$^+$ T cell subsets in human cutaneous leishmaniasis

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Abstract

Recovery from CL is usually accompanied with long-lasting protection and induction of strong immune response. The phenotypes, generation and maintenance of central (=T_{CM}) and effector (=T_{EM}) memory T cell subsets in human leishmaniasis are not well known. Profile of T cell subsets were analyzed on peripheral CD8$^+$ T cells from volunteers with history of cutaneous leishmaniasis (HCL).

In HCL and control groups, mean frequencies of CCR7$^+$CD45RA$^+$CD8$^+$ naïve and CCR7$^-$CD45RA$^-$CD8$^+$ T_{EM} cells were higher than other subsets before culture, but after stimulation with soluble *Leishmania* antigen, the frequency of naïve T cells was significantly decreased and the frequency of T_{EM} cells was significantly increased. T_{EM} phenotype composed the highest portion of proliferating Carboxy Fluorescein diacetate Succinimidyl Ester (CFSE)-dim population which was significantly higher in HCL volunteers than in control group. Stimulation of isolated CD8$^+$ memory T cells, but not naïve T cells, from HCL volunteers induced a significantly higher IFN-γ production compared with that of healthy controls. Intracellular IFN-γ staining provided the same result.

Memory population is shown to be responsible for *Leishmania*-induced IFN-γ production. *Leishmania*-reactive proliferating T_{EM} cells were identified as the most frequent subset which may play a role in recall immune response and protection against *Leishmania* infection.

Keywords: CD8$^+$ T cells; Memory T cells; Cutaneous leishmaniasis; IFN-γ

1. Introduction

It is estimated that in 88 countries mostly developing ones approximately 350 million people are at risk of acquiring leishmaniasis [1]. Two most common clinical forms of the disease Cutaneous Leishmaniasis (CL) and Visceral Leishmaniasis (VL) are mainly seen in 14 of the 22 countries of EMRO (Eastern Mediterranean Regional Office) region including Iran [2]. Cutaneous leishmaniasis is usually a self healing lesion but rarely the lesion does not heal during expected time period and might not even respond to multiple courses of therapy. It is well known that recovery from cutaneous leishmaniasis, is usually accompanied with long-lasting protection and development of strong immune response against *Leishmania* antigens which is shown by *in vivo* Leishmanin Skin Test (LST) and various *in vitro* tests [3,4]. Control measures including chemotherapy, vector and reservoir control are not successful in most of the endemic areas [1,5]. To date there is no vaccine available against any form of human leishmaniasis [6–8]. Adaptive immune response plays

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a critical role to control infections through generation of "immunological memory" which composes the basis of protection against previously encountered antigens. The memory T cells consist of $CD4^+$ and $CD8^+$ T cells which rapidly initiate effector functions and kill infected cells and/or secrete inflammatory cytokines [9]. The heterogeneous population of memory T lymphocytes is distinguished based on surface markers and/or effector functions including cytokine secretion and proliferation capacity [10]. Memory T cells in human are characterized by surface markers, including CD45RO/RA, CD27, chemokine receptors like CCR7 and adhesion molecules such as CD62L [11,12]. In lymphocyte trafficking, CCR7 is an important marker by which T cells enter secondary lymphoid tissues [13], recently base on the expression of the CCR7 central memory T cell (T_{CM} = $CD45RO^+CCR7^+$) and effector memory T cell (T_{EM} = $CD45RO^+CCR7^-$) subsets are defined [14]. Naïve ($CD45RA^+CCR7^+$) and T_{CM} cells display a high capacity of proliferation without immediate effector functions whereas T_{EM} cells produce Th1/Th2 cytokines upon *in vitro* activation. $CD45RA^+$ effector memory cells (T_{EMRA}) are the terminally differentiated phenotype of $CD8^+$ memory cells. T_{EMRA} cells are very susceptible to apoptosis and this population produces a high level of cytotoxic molecules.

A vaccine is effective when it induces long-term immunological memory [15]. Although the development of an effector T cell response to *Leishmania major* is shown in murine model of leishmaniasis [16,17], but the process of memory T cells (T_{CM}/T_{EM}) generation and maintenance in human leishmaniasis is not well known. Previously we have [18] reported that $CD8^+$ T cells is a source of IFN-γ production in individuals with history of CL. It is proposed that this population may represent a T_{CM}/T_{EM} memory phenotype, in this study the profile of circulating memory T cell subsets and IFN-γ producing population are further studied.

2. Materials and methods

2.1. Ethical considerations and study population

The proposal was reviewed by the Ethical Committees of the Center for Research and Training in Skin Diseases and Leprosy (CRTSDL) and Tehran University of Medical Sciences (TUMS). Potential candidates were informed about the study objectives and the procedure and those who were willing to sign an informed consent and donate blood samples were included.

In this study, 13 volunteers with history of CL caused by either *L. major* or *Leishmania tropica* and as control 18 healthy volunteers from non-endemic area were included. The identity of *Leishmania* species was analyzed using PCR method. Prior to blood collection, every volunteer was skin tested with leishmanin (Pasteur Institute of Iran). Leishmanin was prepared from whole promastigotes of *L. major* (strain MRHO/IR/75/ER) that were harvested from 5 to 7 days culture, fixed in thimerosal, washed in sterile pyrogen-free PBS, and resuspended at 5×10^6 promastigotes/ml in PBS containing 0.01% thimerosal [19].

2.2. Parasite and soluble Leishmania antigen preparation

L. major (MRHO/IR/75/ER) was used in this study, promastigotes were harvested from RPMI 1640 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) at day 5 of culture, washed 3 times with PBS (pH 7.2) and used for preparation of soluble *Leishmania* antigen (SLA) as previously described [20]. Briefly, 100 μl of protease inhibitor cocktail enzyme (Sigma, St. Louis, MO, USA) was added to 1×10^9 promastigotes, the parasites were freeze-thawed 10 times followed by sonication at 4 °C with two 20-sec blasts. Then the parasite suspension was centrifuged at 30,000× *g* for 20 min, the supernatant was collected and re-centrifuged at 100,000× *g* for 4 h. Protein concentration of SLA was measured using Bradford method. Finally the supernatant was sterilized using 0.22 μm membrane filter, aliquoted and stored at −20 °C until use.

2.3. $CD8^+$ T cell purification

Twenty ml of blood sample was collected from each volunteer and Peripheral Blood Mononuclear Cells (PBMCs) were isolated using Ficoll–Hypaque (Sigma, St. Louis, MO, USA) density gradient centrifugation. $CD8^+$ lymphocytes were isolated by using magnetic beads system (StemCell Technologies Inc., Vancouver, BC, Canada) by positive selection using anti-CD8 coated nanoparticles. Briefly, cell suspension was prepared at a concentration of 1×10^7 cells/ml in a 5 ml tube in isolation buffer containing PBS plus 2% (v/v) FBS and 1 mM EDTA. EasySep CD8 cocktail Abs was added at 10 μl/ml, mixed well and incubated at room temperature (RT) for 15 min. Magnetic nanoparticles were added at 5 μl/ml cells and incubated at RT for 10 min. The tube was placed into the magnet 3 times, 5 min each, the supernatant was then discarded while the desired cells were remained bounded inside the tube. A portion of the isolated $CD8^+$ T cells was directly cultured for phenotype study and the rest was labelled with CFSE and used for proliferation assay.

2.4. $CD14^+$ isolation and monocyte derived macrophage (MDM) production

Monocytes ($CD14^+CD16^-$) were isolated from autologous PBMC by negative selection according to the manufacturer's instruction (StemCell Technologies Inc., Vancouver, BC, Canada). Briefly, the cell suspension was prepared at a concentration of 5×10^6 cells/ml in isolation buffer. EasySep monocyte enrichment cocktail Abs was added at 5 μl/ml, mixed well and incubated at 4 °C for 10 min. Magnetic microparticles were added at 5 μl/ml and cells were incubated at 4 °C for 5 min. The tube was placed into the magnet, for 2.5 min at RT. The desired unbounded fraction was transferred into a new tube. The purity of the yielded lymphocytes or monocytes was more than 95% using flow cytometry analysis and specific conjugated mAb. MDM was produced by resuspending the monocytes in 10 ml of cRPMI 1640 in 75 cm^2 flask (Nunc, Roskilde, Denmark). After 1 h of incubation at 37 °C/5% CO_2,