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ORIGINAL RESEARCH

# $CCR7^+$ Central and $CCR7^-$ Effector Memory $CD4^+$ T Cells in Human Cutaneous Leishmaniasis

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**Abstract**
*Purpose* The profile of central (=$T_{CM}$) and effector (=$T_{EM}$) memory $CD4^+$ T cell subsets and the possible role as surrogate markers of protection is studied in the volunteers with history of cutaneous leishmaniasis (HCL).
*Methods* Profile of T cell subsets based on CCR7/CD45RA expressions and phenotypic changes after soluble *Leishmania* antigen (SLA) stimulation were analyzed. Then, sorted $CD4^+CD45RO^-CD45RA^+$ naïve T, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM,}$ $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ subsets were cultured with SLA for proliferation, cytokine production and intracellular cytokine assays.
*Results* In the HCL and control volunteers, the mean frequencies of $CD4^+CD45RA^+CCR7^+$ naïve T cells and $CD4^+CD45RA^-CCR7^-$ $T_{EM}$ cells were higher than the other subsets before culture. Frequency of naïve T cells and $CD4^+CD45RA^-CCR7^+$ $T_{CM}$ cells was significantly decreased ($P$=0.01 for naïve T and $P$<0.05 for $T_{CM}$ cells) and frequency of $T_{EM}$ cells was significantly increased after SLA stimulation compared to before culture ($P$<0.001). By CFSE labeling, $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells showed more proliferation potential than $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$ cells. Stimulation of the $T_{EM}$ cells in HCL volunteers induced a significantly higher IFN-γ production ($P$=0.04) with higher number of intracellular IFN-γ positive cells ($P$=0.032) than the same cells from controls. A significantly higher number of $T_{CM}$ cells produced IL-2 in HCL volunteers compared with controls ($P$< 0.05). Most of the intracellular IFN-γ positive $T_{EM}$ cells were proliferating CFSE-dim populations ($P$<0.05).
*Conclusions* A combination of *Leishmania*-reactive IFN-γ producing $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$ and *Leishmania*-reactive IL-2 producing $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ are identified in individuals with history of CL which might play a role in protective recall immune response against *Leishmania* infection.

**Keywords** Cutaneous leishmaniasis · central memory CD4+ T cells · effector memory CD4+ T cells · CCR7 marker

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## Introduction

*Leishmania* spp are intracellular protozoan parasites of the kinetoplastida family which cause a spectrum of human diseases. It is estimated that in 98 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. Management of the disease including chemotherapy, vector and reservoir control are not fully successful in most of the endemic areas [1, 3] and to date there is no vaccine available against any form of human leishmaniasis [4–6].

The development of effector T-cell response to *L. major* infection is explored in mouse model but how memory T cells are generated and maintained in human leishmaniasis is not fully understood [7, 8]. Memory T cells rapidly initiate effector functions and kill infected cells and/or secrete Th1/Th2 cytokines [9], Heterogeneous populations of memory T lymphocytes are distinguished based on the surface markers and effector functions including cytokine secretion and proliferation capacity [10]. Human memory T cells are characterized by surface markers, including CD45RO/RA, CD27, chemokine receptors like CCR7 and adhesion molecules such as CD62L [11, 12]. Recently, central memory T cell ($T_{CM}$=$CD45RO^{+}CCR7^{+}$) and effector memory T cell ($T_{EM}$=$CD45RO^{+}CCR7^{-}$) subsets are distinguished based on the expression of CCR7 [13]. $CD45RA^{+}CCR7^{+}$ naïve T and $T_{CM}$ cells display a high capacity of proliferation without immediate effector functions. $T_{CM}$ cells home to T cell areas of secondary lymphoid organs, whereas $T_{EM}$ cells upon activation migrate to the inflamed peripheral tissues and produce Th1/Th2 cytokines. Although memory T cell subsets are studied in mouse *L. major* infection [14, 15], but the process of $T_{CM}/T_{EM}$ cells generation and maintenance and their roles in protection against human leishmaniasis remain unclear [16]. One of the challenges in leishmaniasis vaccine development is to identify the surrogate marker(s) of protection. Several first generation vaccines reached to human pragmatically but now a day development of vaccines must be done through systematic approach which requires identification of surrogate markers of protection. An appropriate population to study possible role of memory T cells in protective immunity against leishmaniasis is the individuals with history of CL (HCL) who are protected against further infection. The current study focused on this model to detect *Leishmania*-reactive memory $CD4^{+}$ T cells in peripheral blood samples and to investigate if these memory T cells play a major role in protective immunity.

## Methods

### Study Population and Ethical Considerations

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 the volunteers who were willing to sign an informed consent and donate blood samples were recruited.

In this study, 21 volunteers with history of CL (HCL) caused by either *L. major* or *L. tropica* and as control 18 healthy volunteers from non-endemic area were included. The identity of *Leishmania* species was confirmed using PCR method. Prior to blood collection, every volunteer was skin tested with leishmanin (Pasteur Institute of Iran).

**Table I** Demographic information and LST responses of included volunteers

| | HCL | Controls |
|---|---|---|
| No | 21 | 18 |
| Sex: M/F | 12/9 | 12/6 |
| Age | 36.1±5.5 | 34.9±6.7 |
| Number of lesion | 1 (1–7) | NA |
| Time between cure and sampling (months) | 54 (1–132) | NA |
| Leishmanin Skin Test (mm) | 9.0±3.81 | 0 |

Age & LST: Mean ± SD/Time and number: Median (Range)

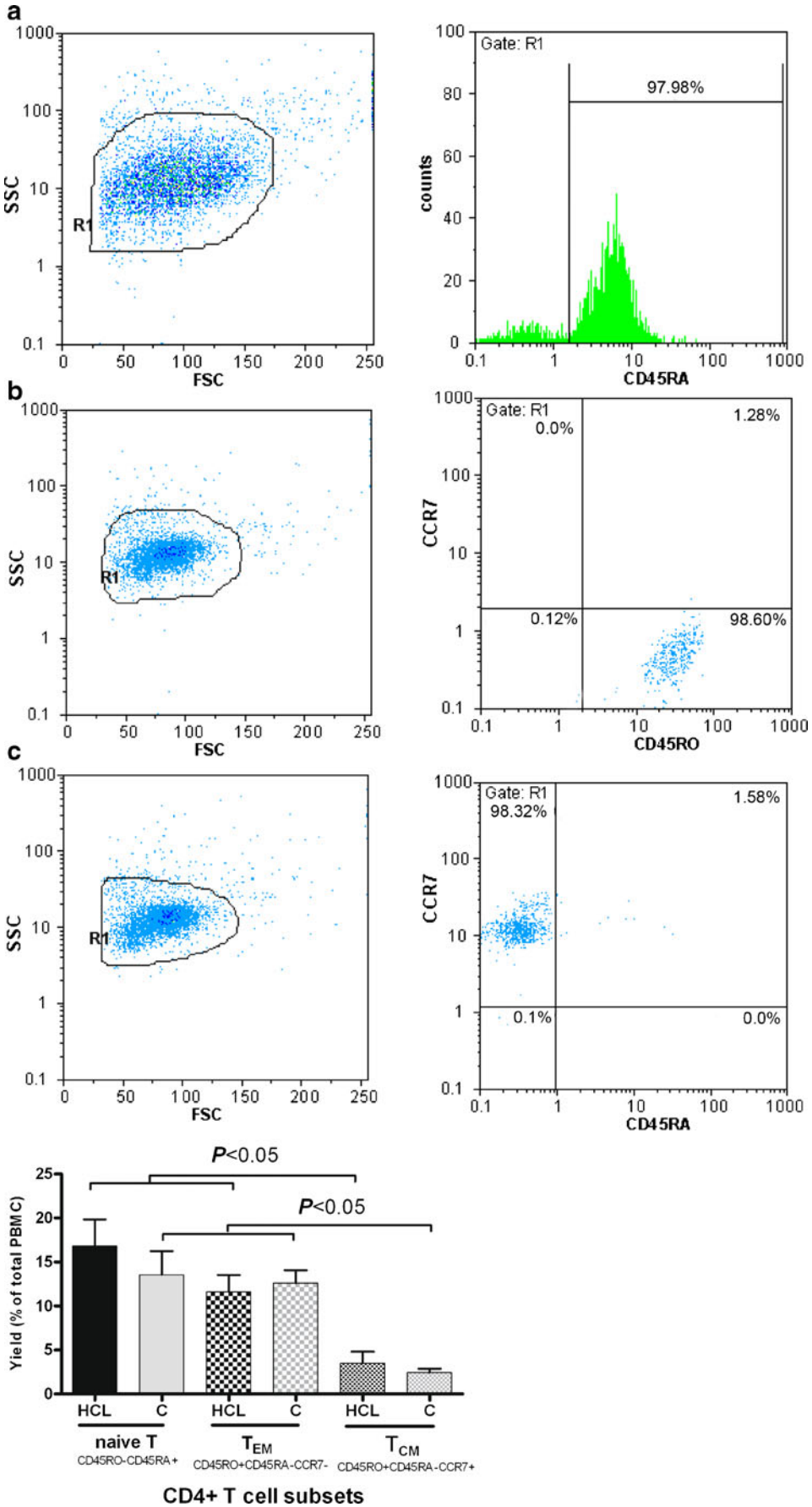
*NA* not applicable

### Parasite and Soluble *Leishmania* Antigen Preparation

*Leishmania major* (MRHO/IR/75/ER) was used in this study, promastigotes were harvested at day 5 of culture from RPMI 1640 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal calf serum (FCS), washed 3 times with PBS, pH7.2, and used for preparation of soluble *Leishmania* antigen (SLA) as previously described [17]. Briefly, 100 μl of protease inhibitor cocktail enzyme (Sigma, St. Louis, MO, USA) was added to $1\times10^{9}$ promastigotes, the parasites were freeze-thawed 10 times followed by sonication at 4 °C with two 20-sec blasts. 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.

### Total $CD4^{+}$ T Cells Purification

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using Ficoll-Hypaque


a
SSC
FSC
R1
Gate: R1
97.98%
counts
CD45RA
b
SSC
FSC
R1
Gate: R1
0.0%
1.28%
0.12%
98.60%
CCR7
CD45RO
c
SSC
FSC
R1
Gate: R1
98.32%
1.58%
0.1%
0.0%
CCR7
CD45RA
P<0.05
P<0.05
Yield (% of total PBMC)
HCL
C
naive T
CD45RO-CD45RA+
TEM
CD45RO+CD45RA-CCR7-
TCM
CD45RO+CD45RA-CCR7+
CD4+ T cell subsets

◀ **Fig. 1** CD4+ T cell subsets retrieved from PBMCs by MACS method. *Top panel*) PBMCs were isolated using Ficoll-Hypaque centrifugation method and $CD4^+$ T cell subsets were purified using MACS system. $CD4^+CD45RO^-CD45RA^+$ naïve T cells were purified from PBMC in a negative selection procedure. Biotin-conjugated mAb cocktail against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCR γδ, HLA-DR, and CD235a markers was used per $10^7$ cells followed by adding anti-biotin microbeads and isolation in a magnetic separator. With the similar procedure, for $CD4^+CD45RO^+CD45RA^-CCR7^-$ effector memory T cells, biotin-conjugated mAb cocktail against CD8, CD14, CD15, CD16, CD19, CD34, CD36, CD45RA, CD56, CD123, CD235a, TCR γδ markers and APC-conjugated mAb against CD197 (CCR7) were used and cells were isolated using anti-biotin and anti-APC conjugated microbeads in a magnetic separator. $CD4^+CD45RO^+CD45RA^-CCR7^+$ central memory T cells were purified from PBMC in a two-step procedure. Biotin-conjugated mAb cocktail against CD8, CD14, CD15, CD16, CD19, CD25, CD36, CD45RA, CD56, CD123, CD235a, TCR γδ was added per $10^7$ cells and by adding anti-biotin microbeads the cells were isolated in a magnetic separator. In the next step, PE-labeled anti CD197 (CCR7) mAb was added and using anti-PE microbeads the $T_{CM}$ cells were purified in a magnetic separator. Representative histograms show the purity of the yielded naïve T (**a**), $T_{EM}$ (**b**) and $T_{CM}$ (**c**) cells after analysis by flow cytometry using a combination of anti CD45RA, CD45RO, CCR7 conjugated mAbs. The purity of T cell subsets was more than 98 % on average. *Bottom panel*) For each volunteer the percentages of $CD4^+$ T cell subsets were calculated. Data of HCL and control volunteers were pooled separately and the mean (±SD) percentages of T cell subsets retrieved from volunteer's PBMC are shown

(Sigma, St. Louis, MO, USA) density gradient centrifugation. Total $CD4^+$ T lymphocytes were isolated by using magnetic beads system (StemCell Technologies Inc., Vancouver, BC, Canada) by positive selection using anti-CD4 coated nanoparticles. Briefly, cell suspension was prepared at a concentration of $1\times10^6$ cells/ml in a 5 ml tube in isolation buffer containing PBS plus 2 % (v/v) fetal bovine serum (FBS) and 1 mM EDTA. EasySep CD4 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 time, the supernatant was then discarded while the desired cells were remained bounded inside the tube. Purified $CD4^+$ T cells were cultured for phenotype study.

The purity of the yielded lymphocytes was more than 97 % using flow cytometry analysis and specific conjugated mAbs.

## Naïve/Memory T Cell Subsets Purification (MACS Method)

$CD4^+CD45RO^-CD45RA^+$ naïve T cells were purified from PBMC in a negative selection procedure with depletion of non-T helper cells and memory $CD4^+$ T cells. Briefly, about $5–10\times10^6$ cells were resuspended in a buffer of PBS, pH7.2, 0.5 % bovine serum albumin (BSA) and 2 mM EDTA. Then 10 μL of biotin-conjugated mAb cocktail (Miltnyi Biotec, Germany) (against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCR γδ, HLA-DR, and CD235a markers) was added per $10^7$ cells and incubated at 4 °C for 10 min. The cells were washed with buffer and then 20 μL of anti-biotin microbeads was added per $10^7$ cells and incubated at 4 °C for 15 min. The cells were washed and applied into MS column in a MiniMACS separator (Miltenyi Biotec), and flow-through containing unlabeled cells was collected as $CD4^+CD45RO^-CD45RA^+$ naïve T cells fraction.

$CD4^+CD45RO^+CD45RA^-CCR7^-$ effector memory T cells were purified from PBMC in a negative selection procedure by depletion of non-$CD4^+$ T cells, naïve and central memory $CD4^+$ T cells. The same procedure as naïve T cells was followed, except that biotin-conjugated mAb cocktail (Miltnyi Biotec) (against CD8, CD14, CD15, CD16, CD19, CD34, CD36, CD45RA, CD56, CD123, CD235a, TCR γδ markers) and APC-conjugated mAb (against CD197 [CCR7]) were used and $T_{EM}$ microbeads were conjugated to anti-biotin and anti-APC antibodies. The cells were applied into LD column in a MidiMACS separator (Miltenyi Biotec) and unlabeled cells were collected as $CD4^+CD45RO^+CD45RA^-CCR7^-$ effector memory T cells.

$CD4^+CD45RO^+CD45RA^-CCR7^+$ central memory T cells were purified from PBMC in a two-step procedure including depletion of non-$CD4^+$ T cells and $CD4^+$ naïve T cells and the subsequent positive selection of $CCR7^+$ $T_{CM}$

**Table II** Frequency of different $CD4^+$ T cell subsets based on surface markers analysis before and after total CD4+ T cell re-stimulation in vitro

| $CD4^+$ T cell subset | | $CD45RA^+CCR7^+$ (naïve T) | | $CD45RA^-CCR7^+$ ($T_{CM}$) | | $CD45RA^-CCR7^-$ ($T_{EM}$) | |
|---|---|---|---|---|---|---|---|
| SLA stimulation | | before | after | before | after | before | after |
| Mean percentage ± SD | HCL | 54.0*±9.9 | 15.4*±6.6 | 21.6*±3.5 | 8.0*±1.7 | 23.2**±4.1 | 76.2**±14.5 |
| | Controls | 58.3*±10.7 | 19.8*±5.2 | 20.8*±3.0 | 7.6*±1.2 | 19.4**±3.8 | 72.0**±14.0 |

Statistical test is used to compare the mean percentages before and after stimulation for each group of volunteers, separately.

*Significant difference $P<0.05$

**Significant difference $P<0.001$

cells. Briefly, $2\times10^7$ cells were resuspended in a buffer of PBS, pH7.2, 0.5 % BSA and 2 mM EDTA. Then, 10 μL of biotin-conjugated mAb cocktail (Miltenyi Biotec) (against CD8, CD14, CD15, CD16, CD19, CD25, CD36, CD45RA, CD56, CD123, CD235a, TCR γδ) was added per $10^7$ cells and incubated at 4 °C for 10 min. Next, 20 μL of anti-biotin microbeads was added per $10^7$ cells in the cold buffer and incubated at 4 °C for 15 min. The cells were washed and applied onto LS column in a MidiMACS separator, and then the unlabeled cells which passed through were collected and resuspended in the buffer, and 5 μL of PE-labeled anti CD197 (CCR7) mAb (Miltenyi Biotec) was added and incubated at 4 °C for 10 min. After washing the cells, 50 μL of anti-PE microbeads was added and incubated for additional 15 min at 4 °C. The cells were washed and applied onto MS column in a MiniMACS separator. The magnetically labeled cells containing $CD4^+CD45RO^+CD45RA^-CCR7^+$ central memory T cells were flushed out and collected.

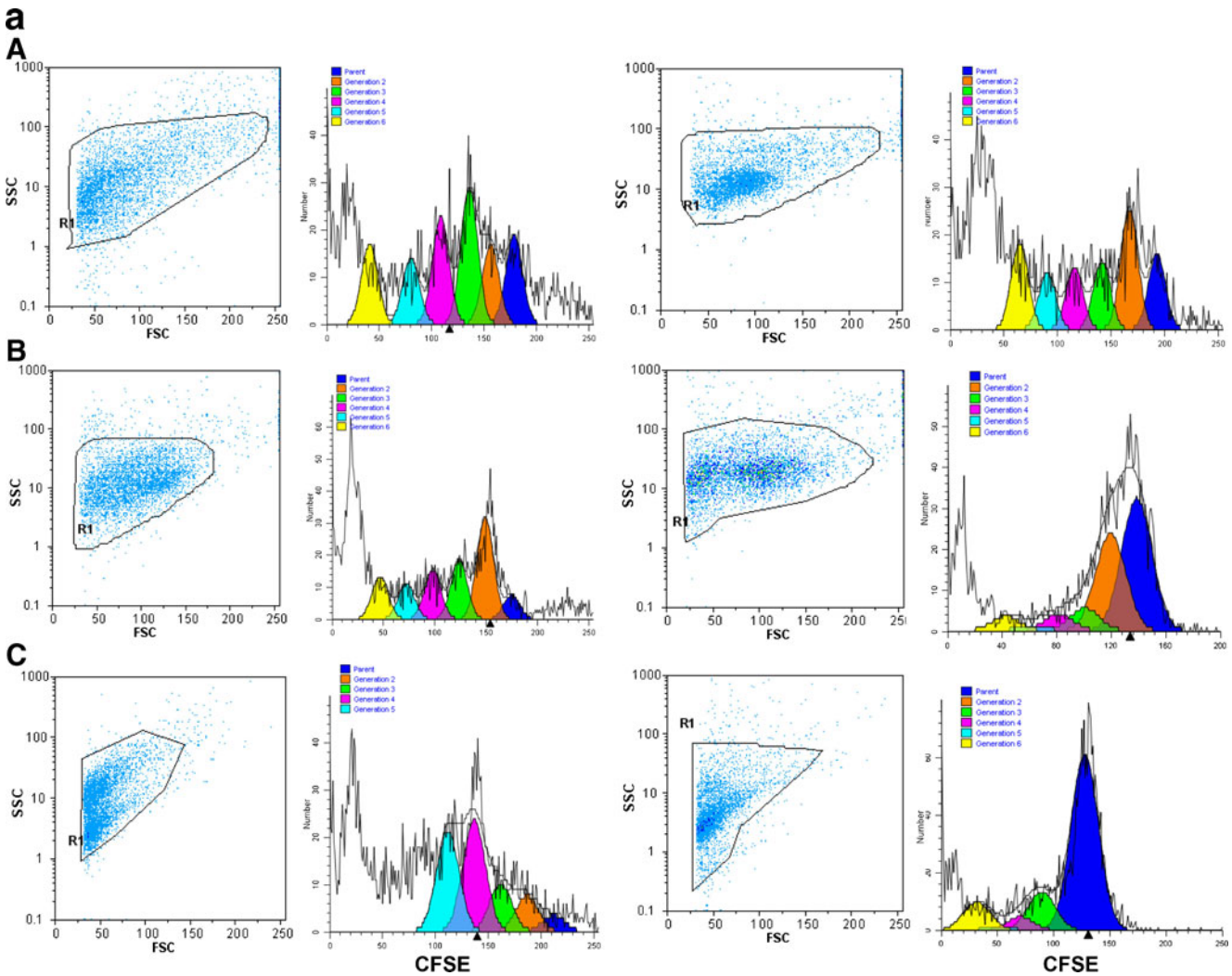



**Fig. 2** Frequency of cells in different generations of proliferation process. CFSE labeled proliferation profile of lymphocytes using ModFit software analysis. **a**) For proliferation assay, the purified $CD4^+CD45RO^-CD45RA^+$ naïve T, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$, $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells subsets were labeled using CFSE. The cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated human AB $Rh^+$ serum, 10 mM/L Hepes, 2 mML-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin with 1:10 of autologous monocytes. Cells were stimulated with either 10 μg/ml PHA or 50 μg/ml SLA. The cells were incubated at 37 °C with 5 % CO2 in humidified atmosphere, at day 5 the cells were harvested, washed and resuspended in PBS and analyzed using flow cytometry with excitation and emission filter appropriate for fluorescein. Data obtained from FloMax were then analyzed by ModFit software. Representative histograms show CFSE stained naïve (**A**), $T_{EM}$ (**B**) and $T_{CM}$ (**C**) cells as seen in ModFit analysis output (*Left column* from HCL and *right column* from control volunteers). Each peak on the histograms corresponds to the one generation of daughter cells divided from parent cells on the right. The percentage of cells in each division is reported on the software output. **b**) The daughter cell generations yielded from the parent cells were obtained from the fluorescent intensity by using the software. The numerical values for proportions of the cells at each cell generation were calculated for each sample. The data of frequency of T cell subsets for HCL and control volunteers were pooled separately and are represented as the mean percentage in each generation. *Error bars* show ± SD

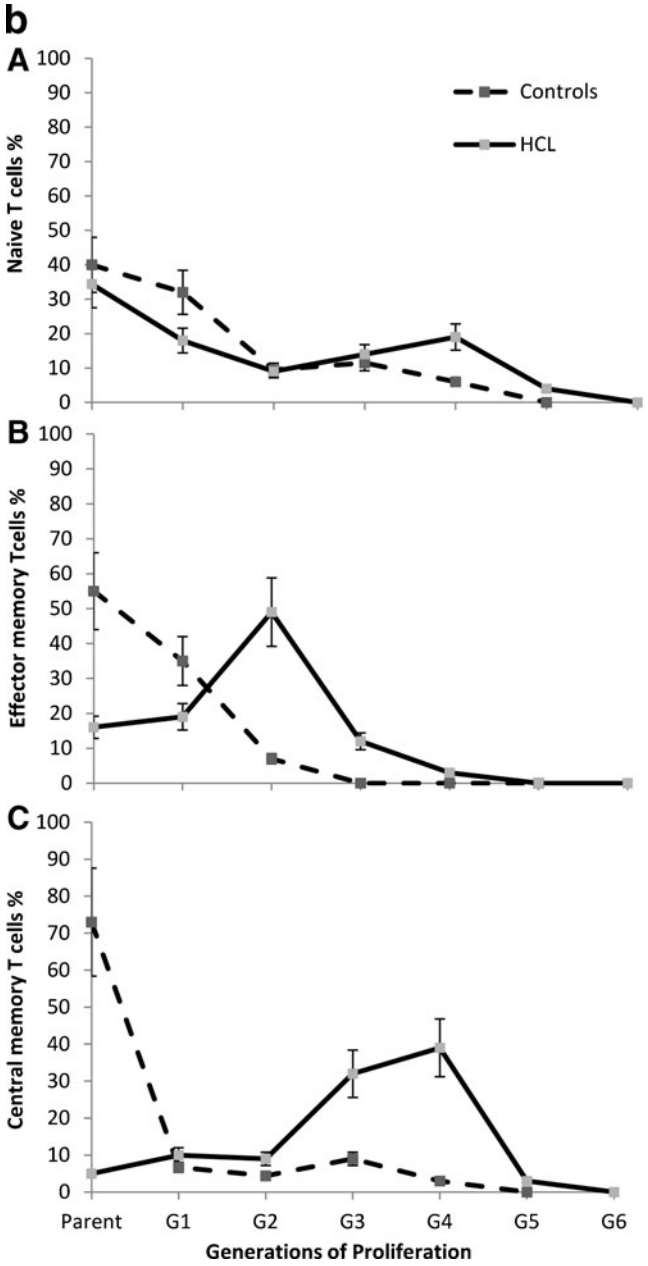



**Fig. 2** (continued)

The purity of the yielded lymphocytes was more than 98 % using flow cytometry analysis and specific conjugated mAbs.

## $CD14^+$ Cell Isolation and Monocyte Derived Macrophage (MDM) Production

Autologous $CD14^+CD16^-$ monocytes were isolated from 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 $5\times10^6$ cells/ml in PBS plus 2 % (v/v) FBS and 1 mM EDTA. EasySep monocyte enrichment cocktail Abs was added at 5 μl/ml and the cells were incubated at 4 °C for 10 min. Magnetic microparticles were added at 5 μl/ml and the cells were incubated at 4 °C for 5 min. The tube was placed into the magnet at RT for 2.5 min. The desired unbounded fraction was transferred into a new tube. The purity of the yielded monocytes was more than 96 % using flow cytometry analysis and specific conjugated mAbs. MDM was produced by resuspention of $CD14^+CD16^-$ monocytes in 10 ml of cRPMI 1640 in 75 $cm^2$ flask (Nunc, Roskilde, Denmark). After 1 h of incubation at 37 °C in 5 % $CO_2$, non-adherent cells were discarded by with pre-warmed medium. Then 10 ml of new medium supplemented with 10 % FCS was added to the flask and incubated at 37 °C in 5 % $CO_2$ for 6 days and the medium was replaced every 2 days. The adherent macrophages were removed gently by scraper and washed and used as antigen presenting cells.

## CFSE Labeling and Proliferation Assay

For proliferation assay, part of the isolated $CD4^+$ T cell subsets were labeled using Carboxy Fluorescein Diacetate Succinimidyl Ester (CFSE), as previously described [18]. Briefly, cells were resuspended in pre warmed PBS plus 0.1 % BSA at a final concentration of $1\times10^6$ cells/ml, and 2 μl of 5 mM stock CellTrace CFSE solution (Molecular Probes, USA) was added per 1 mL of the cells (final concentration: 10 μM). Tubes were incubated at 37 °C for 10 min. Staining was quenched by adding 5 ml of ice-cold RPMI 1640 plus 10 % FBS for 5 min on ice. The cells were washed 3 times at 300×g, 7 min. and in vitro cell culture with autologous monocytes was done (see below). At day 5 of culture the cells were harvested, washed and resuspended in PBS plus 0.5 mM EDTA. The cells were incubated at 37 °C for 5 min and then centrifuged for 5 min, 300×g, 20 °C. The cells were resuspended in PBS and analyzed using flow cytometry with excitation and emission filter appropriate for fluorescein. The data was analyzed using ModFit LT software (Verity Software House, USA). The daughter cell generations yielded from the parent cells were obtained from the fluorescent intensity by using the software. The numerical values for proportions of the cells at each cell generation were calculated for each sample.

## T Lymphocyte Culture

The purified total $CD4^+$ T cells, $CD4^+CD45RO^-CD45RA^+$ naïve T, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$, and $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells, with or without CFSE labeling, were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated human AB $Rh^+$ serum, 10 mM/L Hepes, 2 mML-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco Invitrogen,

Carlsbad, CA, USA). T lymphocytes were adjusted at $0.5\text{-}1\times10^6$ cells/ml, mixed with 1:10 of autologous MDM, and cultured in U bottomed 96-well plates (Nunc, Roskilde, Denmark) in the presence of either 10 μg/ml PHA (Sigma, St. Louis, MO. USA) or 50 μg/ml SLA in a final volume of 200 μl. The cells were incubated at 37 °C with 5 % CO2 in humidified atmosphere, 72 h for cytokine assay or 5 days for CFSE proliferation assay. Total $CD4^+$ T cells were incubated for 5 days for phenotypic analysis.

## ICS Assay

A portion of the purified T cell subsets was used for intracellular cytokine staining (ICS) assay after SLA stimulation. The cells were stimulated with PMA (Sigma, St. Louis, MO. USA) 50 ng/ml plus ionomycin calcium (Sigma) 500 ng/ml and incubated at 37 °C, 5 % $C0_2$ for 5–6 h. For blocking, 25 μM/ml monensin (Sigma) was added during the last 4–5 h of culture. The cells were harvested and washed 2 times using PBS, pH 7.2, plus 0.1 % BSA. The cells were permeabilized using BD Cytofix/Cytoperm kit according to the manufacturer's instruction (BD Biosciences, San Jose, CA, USA) and then the cells were stained using anti-human IFN-γ or IL-2 antibodies (BD Biosciences). Finally, the cells were washed 2 times with Perm/wash buffer and resuspended in PBS, pH7.2, plus 1 % BSA and analyzed using flow cytometry while isotype matched negative controls were used. A minimum of 50,000 events were acquired for each sample.

In part of the experiment, purified CFSE-labeled $T_{EM}$ cells were harvested from SLA culture and restimulated with PMA plus ionomycin and stained for the presence of intracellular IFN-γ. Proliferating and non-proliferating populations of $T_{EM}$ cells were gated based on fluorescence intensity of FL1.

## Monoclonal Antibodies and Flow Cytometry

For flow cytometry analysis the following monoclonal antibodies and isotype matched controls were used: FITC- or PE-conjugated mouse anti-human IFN-γ, PE-conjugated rat anti-human IL-2, PE-Cy5-conjugated mouse anti-human CD45RA, PE-conjugated rat anti-human CD197 (CCR7) PECy5-

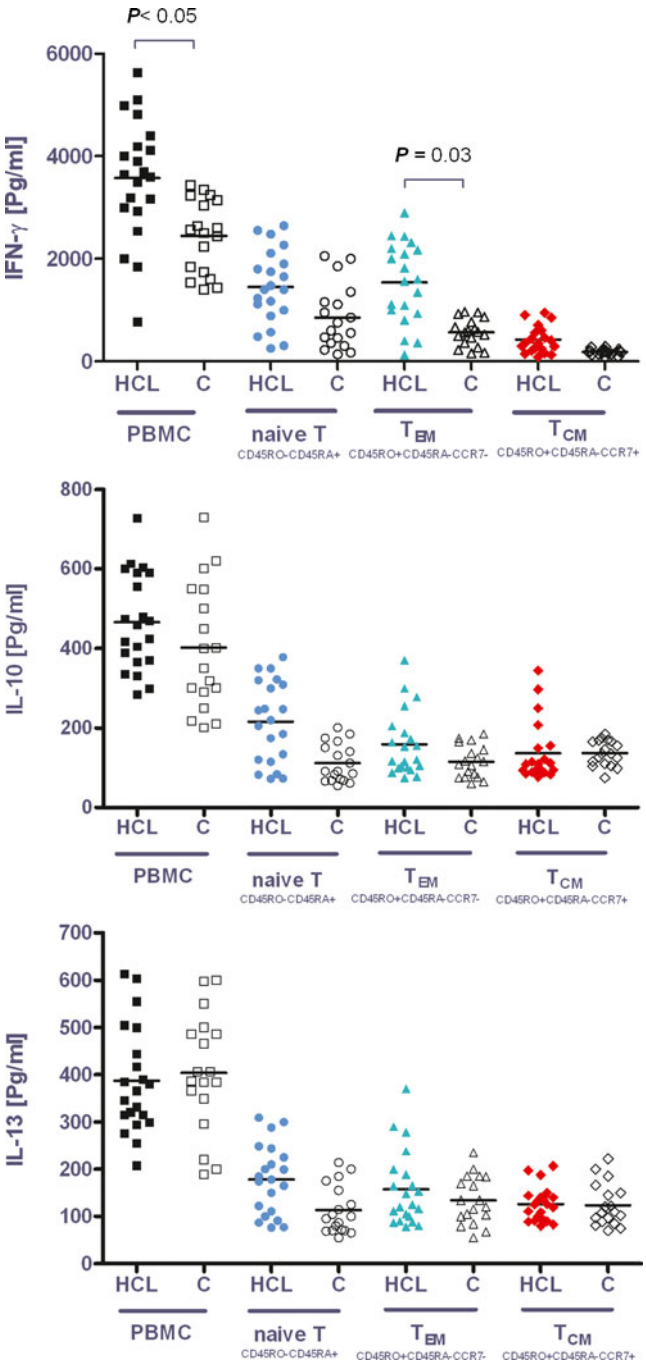



**Fig. 3** Cytokines production of sorted CD4+ T cell subsets. The purified $CD4^+CD45RO^-CD45RA^+$ naïve T, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$, $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells subsets were adjusted at $0.5\text{-}1\times10^6$ cells/ml, mixed with 1:10 of autologous MDM and cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated human AB $Rh^+$ serum, 10 mM/L Hepes, 2 mML-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin in the presence of either 10 μg/ml PHA or 50 μg/ml SLA. The cells were incubated at 37 °C with 5 % CO2, and after 72 h, culture supernatants were collected and the level of IL-5, IL-10, IL-13 and IFN-γ were titrated by ELISA method. Plates were coated with 100 μl of anti-IL-5/IL-10/IL-13/IFN-γ mAb in PBS, pH7.4, and incubated at 4 °C for overnight. After blocking the wells using 200 μl PBS plus 0.05 % Tween 20 and 0.1 % BSA, 100 μl of culture supernatants were added to each well. Biotin labeled mAb in incubation buffer and then streptavidin-HRP were used at 100 μl/well. The reaction was developed using 100 μl of TMB substrate by incubation of plates at RT for 30 min and stopped with 100 μl of 0.5 M $H_2SO_4$ solution. The plates were washed 5 times after each step of incubation using PBS plus 0.05 % Tween 20. The mean ODs of wells at 450 nm were compared with the standard curves prepared using recombinant IL-10, IL-13 and IFN-γ. The concentrations of cytokines in PBMC, naive T, $T_{EM}$ and $T_{CM}$ cell cultures are shown for both HCL and control groups. *Horizontal lines* show mean levels

conjugated mouse $IgG_2b$, PE-conjugated rat $IgG_{2\alpha}$, and PE-conjugated mouse IgG1 (BD Biosciences, Pharmingen, USA), FITC-conjugated mouse IgG1 (DakoCytomation, Denmark) and FITC-conjugated mouse anti-human CD4 (IQ Products, Netherlands). The cells were stained using 10 μl of antibodies per $1\times10^5$ cells at 4 °C for 30 min in the dark and washed 2 times by centrifugation at 300×g for 7 min. In surface staining the cells were resuspended in 1–2 ml of 1 % paraformaldehyde before running on the flow cytometer. A minimum of 50,000 events were acquired for each sample. The cells were analyzed using Partec flow cytometer (DAKO cytomation) while isotype matched negative controls were used to set the threshold of autofluorescence. FACS data analysis was performed using FloMax software (DAKO cytomation).

### ELISA Cytokine Assay

The culture supernatants of PBMC and $CD4^+$ T cell subsets were collected at 72 h and the level of IL-5, IL-10, IL-13 and IFN-γ were titrated using ELISA method (Mabtech, Stockholm, Sweden). Briefly, the plates were coated with 100 μl of anti-IFN-γ/IL-5/IL-10/IL-13 mAb in PBS, pH7.4, and incubated at 4 °C for overnight. After blocking the wells using 200 μl of PBS plus 0.05 % (v/v) Tween 20 and 0.1 % (w/v) BSA for 2 h at RT, 100 μl of culture supernatants were added and the plates were incubated for 2 h at RT. Next, 100 μl of biotin labeled mAb was added to each well and incubated at RT for 1 h and as enzyme 100 μl of streptavidin-horse reddish peroxidase (-HRP) was added and the plates were incubated for additional 1 h at RT. The reaction was developed using 100 μl of 3,3′,5,5′-tetramethyl benzidine (TMB) substrate by incubation of the plate at RT for 30 min. The reaction was stopped using 100 μl of 0.5 M $H_2SO_4$ solution per well. The plates were washed 5 times after each step of incubation using PBS plus 0.05 % (v/v) Tween 20. The plates were read at 450 nm using a microplate reader (BioTek, Winooski, VT, USA). The mean optical densities (ODs) of the wells were compared with the standard curves prepared using recombinant IL-5, IL-10, IL-13 and IFN-γ. The cytokine levels represent the differences between the ODs of the tests and the background wells. The detection limit of the assays was 4 pg/ml for IL-5, 0.5 pg/ml for IL-10, 5 pg/ml for IL-13 and 2 pg/ml for IFN-γ.

### Statistical Analysis

Non-parametric tests of Mann–Whitney, Kruskal-Wallis and Dunn's post-test for paired comparisons were used for statistical analysis of the data using SPSS version 16 (SPSS Inc., USA) and GraphPad Prism version 5.01 (GraphPad Software Inc., USA) softwares. Nonparametric tests were used because the samples did not follow a Gaussian distribution. *P* value of <0.05 was considered significant.

## Results

### LST Results

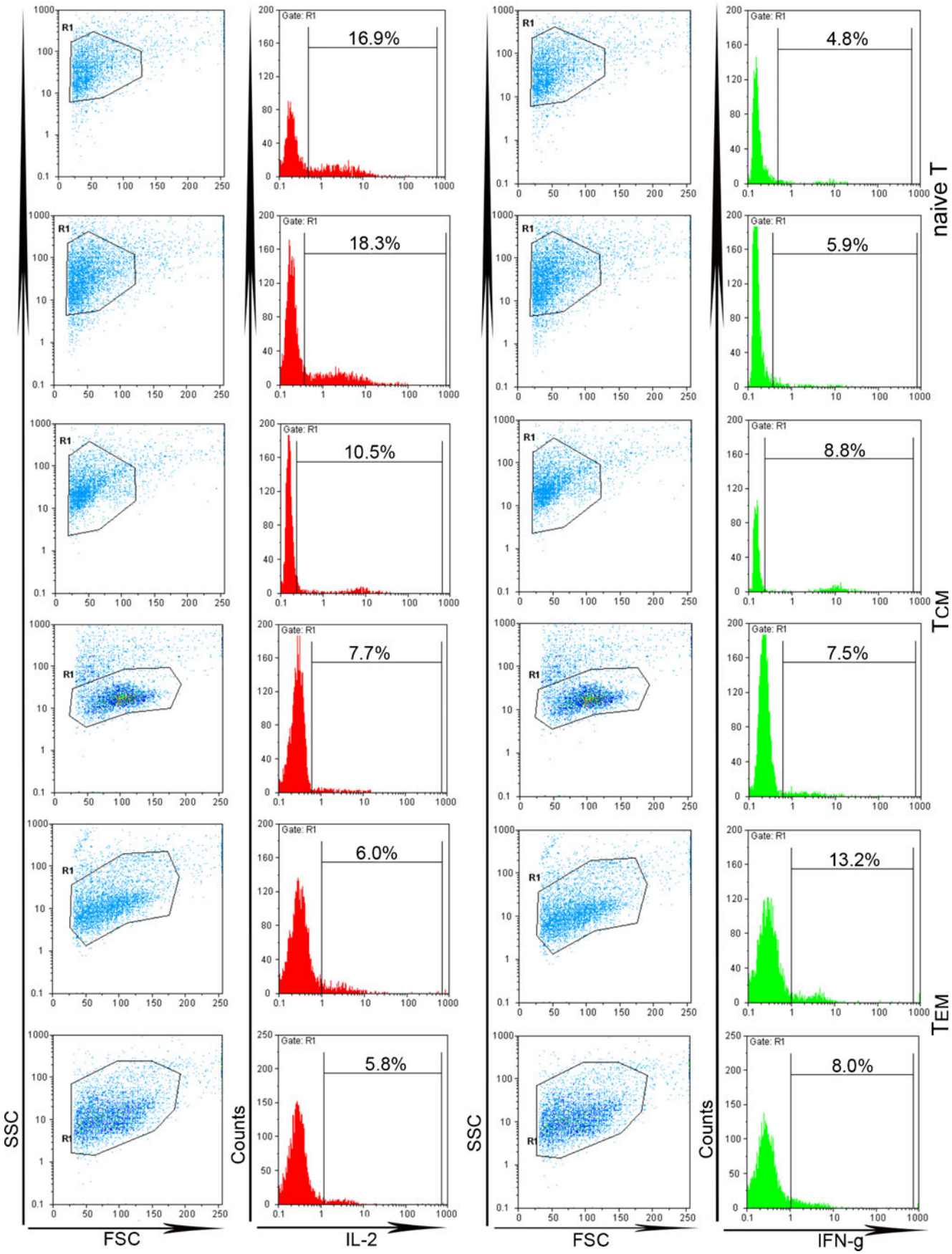
Basic demographic information of the volunteers and leishmanin skin test (LST) results are presented in Table I. The mean diameter of LST was 9.0±3.81 mm in the volunteers with HCL; healthy controls showed no response to LST (0 mm).

### Frequencies of $CD4^+$ T Cell Subsets and Alterations after ag Re-stimulation

$CD4^+CD45RO^-CD45RA^+$ naïve T, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$ and $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells were purified from PBMC by MACS system using mAbs. The mean (±SD) percentages of T cell subsets retrieved from volunteer's PBMC are shown in Fig. 1. Frequency of retrieved naïve T and $T_{EM}$ cells was significantly more than $T_{CM}$ subset in both HCL volunteers and controls ($P<0.05$).

Following total $CD4^+$ T cell isolation, frequency of different T cell subsets based on CCR7 and CD45RA expression before and after in vitro SLA stimulation was analyzed for each sample by using flow cytometry. Data of the volunteers with HCL and healthy controls were pooled separately and are outlined in Table II. In the volunteers with HCL and healthy controls, the mean frequencies of $CD4^+CD45RA^+CCR7^+$ naïve T cells and $CD4^+CD45RA^-CCR7^-$ $T_{EM}$ cells were higher than other subsets before culture. Frequency of $CD4^+CD45RA^+CCR7^+$ naïve T cells and $CD4^+CD45RA^-CCR7^+$ $T_{CM}$ cells in the volunteers with HCL and control volunteers was significantly decreased at day 5 of culture with SLA ($P=0.01$ for naïve T and $P<0.05$ for $T_{CM}$ cells, respectively). In both HCL and control volunteers,

**Fig. 4** Intracellular IL-2/IFN-γ production by sorted CD4+ T cell ▶ subsets. A portion of the purified $CD4^+CD45RO^-CD45RA^+$ naïve T, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$, $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cell subsets was used for intracellular cytokine staining (ICS) assay after SLA stimulation. The cells were stimulated with PMA 50 ng/ml plus ionomycin calcium 500 ng/ml for 5–6 h. For blocking, 25 μM/ml monensin was added during the last 4–5 h of culture. The cells were harvested, washed and permeabilized using BD Cytofix/Cytoperm solutions then stained using anti-human IFN-γ or IL-2 antibodies. Finally, the cells were washed with Perm/wash buffer and resuspended in PBS plus 1 % BSA and analyzed using flow cytometry while isotype matched negative controls were used. A minimum of 50,000 events were acquired for each sample. **a** The cells were gated based on SCC and FSC profile and threshold was selected by using isotype matched control Abs. The panel shows representative flow cytometry plots showing intracellular IL-2 and IFN-γ positive fractions of gated populations of naïve T, $T_{EM}$ and $T_{CM}$ cells in HCL (*top row* of each subset) and control (*bottom row* of each subset) volunteers. **b** Frequencies of IL-2/IFN-γ positive $CD4^+CD45RO^-CD45RA^+$ naïve T, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$, $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cell subsets were calculated from flow cytometry data for each sample. Data of HCL and control volunteers were pooled separately and are shown as mean ± SD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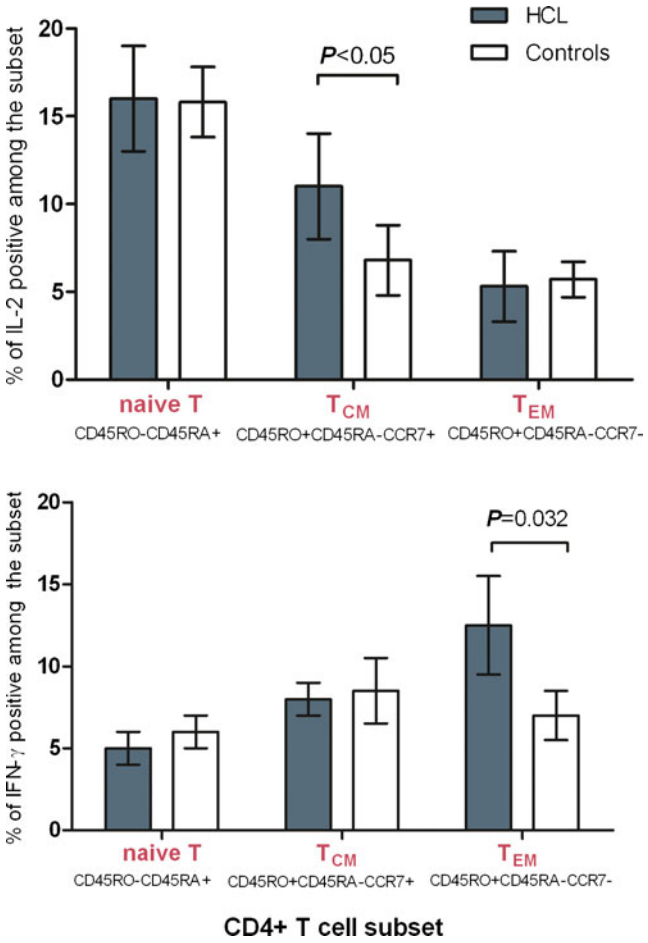


**Fig. 4** (continued)

frequency of $CD4^+CD45RA^-CCR7^-$ $T_{EM}$ cells was significantly increased after stimulation with SLA compared with the frequency of the same cells before culture ($P<0.001$). However, the alterations of the frequencies of $CD4^+$ T cell subsets after culture were similar between the volunteers with HCL and healthy controls.

## Proliferation Assay of $CD4^+$ T Cell Subsets

Purified $CD4^+$ T cell subsets were labeled with CFSE and cultured in the presence of SLA or PHA for 5 days and analyzed using flow cytometry. The proportions of the cells at each cell division in the proliferation process were calculated. Data of the volunteers with HCL and healthy controls were pooled separately and are presented in Fig. 2. Based on the results, significant difference was found between the mean number of $T_{CM}$ cells in g3 and g4 of proliferation process between HCL volunteers and healthy controls ($P<0.05$). A significantly higher ($P<0.05$) number of $T_{EM}$ cells reached g2 of proliferation process in HCL volunteers than in healthy controls.

## Cytokine Productions of Purified $CD4^+$ T Cell Subsets

PBMCs and purified $CD4^+$ T cell subsets were co-cultured with 1:10 of autologous MDM in cRPMI 1640. The cytokine levels were titrated on the supernatant collected at 72 h of SLA stimulation using ELISA method. Figure 3 shows the mean concentration of cytokines IFN-γ, IL-10 and IL-13 in volunteers' PBMC, $CD4^+CD45RO^-CD45RA^+$ naïve T, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$ and $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells cultures.

As it is shown in Fig. 3, stimulation of PBMC from HCL volunteers induced a significantly ($P<0.05$) higher IFN-γ production compared with the cells of healthy controls. Stimulation of the $CD4^+$ $T_{EM}$ cells from HCL volunteers induced a significantly ($P=0.04$) higher IFN-γ production compared with the cells of healthy controls. No significant difference was seen between $T_{CM}$ cells of HCL volunteers and healthy controls in IFN-γ production. No significant difference was seen between the naive T cells of HCL volunteers and healthy controls in IFN-γ production.

No significant difference was seen in IL-10 production of different T cell subsets between HCL volunteers and healthy controls. No significant difference was seen in IL-13 production of different T cell subsets between HCL volunteers and healthy controls (Fig. 3). The level of IL-5 was not detectable in T cell culture supernatants.

## Intracellular Cytokine Staining of $CD4^+$ T Cell Subsets

A portion of SLA stimulated $CD4^+$ T cell subsets was harvested at 72 h and stimulated with PMA plus ionomycin, and stained for intracellular IFN-γ and IL-2 and the frequency of positive cells was analyzed using flow cytometry (Fig. 4a). Results of analysis of the cells from HCL volunteers and healthy controls were pooled separately and are presented as mean number of intracellular IFN-γ and IL-2 positive $CD4^+$ T cell subsets (Fig. 4b). About 15–17 % of $CD4^+CD45RO^-CD45RA^+$ naïve T cells and 5–6 % of $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$ cells produced IL-2 intracellularly and no significant difference was seen between sorted T cells of HCL volunteers and healthy controls in the production of IL-2. About 5–12 % of $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells produced IL-2 intracellularly. Significantly ($P<0.05$) higher number of $T_{CM}$ cells produced IL-2 in HCL volunteers compared to $T_{CM}$ cells of healthy controls.

No significant difference was seen between sorted T cells of HCL and healthy controls in the intracellular production of IFN-γ. About 5–15 % of $T_{EM}$ cells produced IFN-γ and significantly ($P=0.032$) higher numbers of $T_{EM}$ cells from HCL volunteers were positive for intracellular IFN-γ than the same cells from healthy controls.

For simultaneous analysis of proliferation and intracellular cytokine assay, purified $T_{EM}$ cells were labeled with CFSE and cultured in the presence of SLA, then the cells were harvested and stimulated with PMA plus ionomycin and stained for intracellular IFN-γ. On flow cytometry analysis, histograms were divided to CFSEdim proliferating and CFSEbright non-proliferating fractions (Fig. 5). For each sample, the frequency of intracellular IFN-γ positive $T_{EM}$ cells was determined and then the data of all HCL volunteers were pooled and the mean percentage of IFN-γ positive $T_{EM}$ cells was calculated. When $T_{EM}$ cells were gated on CFSEdim populations, more than 35 % of the cells were positive for intracellular IFN-γ, while when the cells were gated on CFSEbright populations around 5 % of the cells were positive for intracellular IFN-γ. The difference in the frequency of IFN-γ positive $T_{EM}$ cells between CFSEdim and CFSEbright was statistically significant ($P<0.05$).

## Discussion

It is generally accepted that $CD4^+$ Th1 cells are mainly involved in protective immune response against *Leishmania* infection [19]. In the current study, $CD4^+$ memory T cell compartment in HCL volunteers who are assumed to be protected against further infection is studied. To our knowledge there is no report on the phenotype of memory T cell subsets based on CCR7 expression in human leishmaniasis.

Memory $CD4^+$ T cell subsets were detected on blood samples collected from volunteers whose lesions had been healed 1 month to 11 years prior to sampling. HCL volunteers were resident of endemic area with constant exposure and strong LST response (mean LST=9.0±3.81 mm) and IFN-γ production are indications of protective immune response. It is well established that memory persists for long time after initial exposure [20]. Different combinations of $T_{EM}/T_{CM}$ are shown to be important in inducing protection against secondary infections [21–25]. In malaria, it seems that $T_{EM}$ rather than $T_{CM}$ cells protect against further infection, but $CD4^+$ memory T cells are short lived and a sterile immunity is not developed [26, 27]. In viral infections, over time, $T_{EM}$ phenotype is replaced by $T_{CM}$, leading to loss of protection. In the current study in individuals with history of CL, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$ cells with significant IFN-γ secretion are detected which induce Th1 type

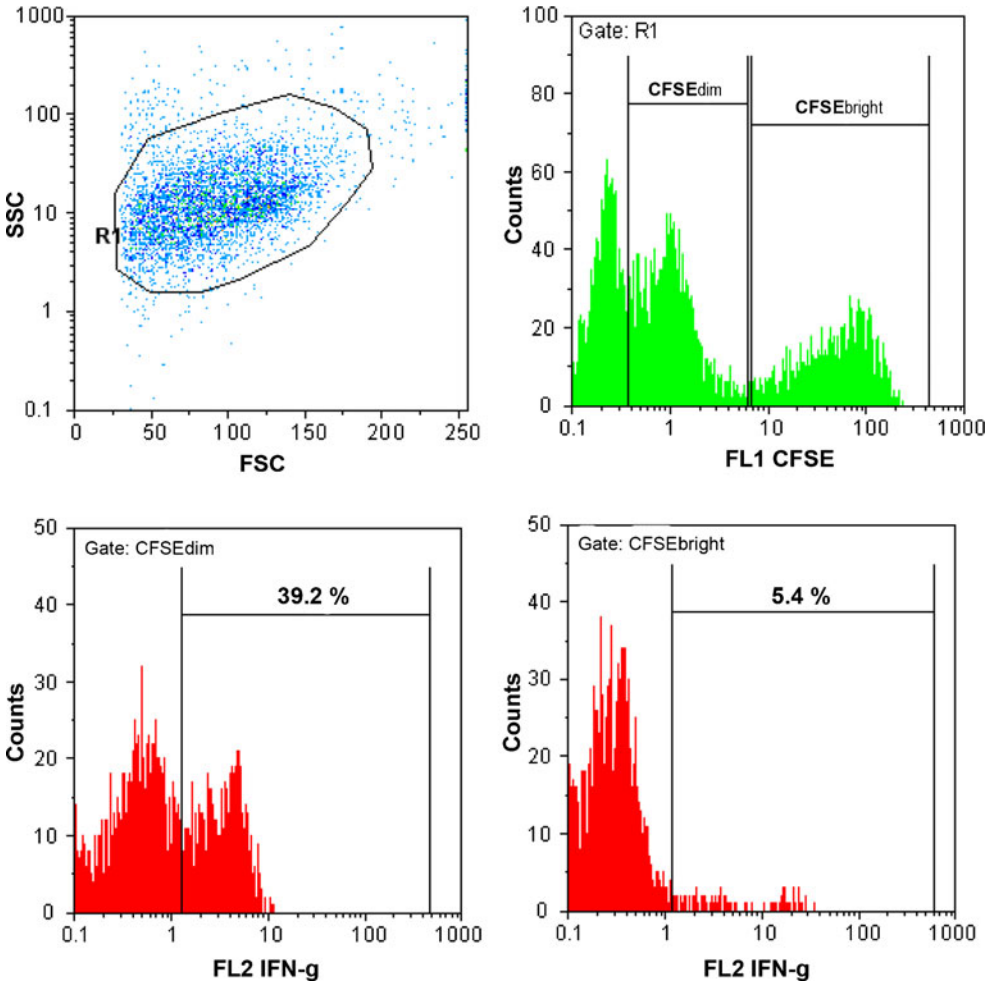


**Fig. 5** Simultaneous analysis of $T_{EM}$ proliferation and intracellular IFN-γ secretion. Purified CFSE-labeled $T_{EM}$ cells were harvested from SLA culture and stimulated with PMA plus ionomycin and stained for intracellular IFN-γ and analysed by flow cytometry. On fluorescence intensity of FL1 histograms, CFSEdim and CFSEbright fractions were gated as proliferating and non-proliferating fractions, respectively. A representative flow cytometry plot shows when the cells were gated on CFSEdim populations, 39.2 % of the cells were positive for intracellular IFN-γ production, while when the cells were gated on CFSEbright populations 5.4 % of the cells were positive for intracellular IFN-γ production. The same analysis was done for each HCL volunteer and the results were pooled

of response with immediate effector functions. We believe continuous exposure to *Leishmania* Ags through sand fly bites in residence of endemic area act as natural boosters and contributes to maintain the protection by effector population of T cells. On the other hand, IL-2 producing $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells with high proliferation potential are detected which might serve as a pool to rapidly differentiate to $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$ in case of reinfection. Increasing evidences are suggesting that $T_{CM}$ cells have a greater capacity than $T_{EM}$ cells to persist in vivo and mediate protection [20, 28].

T cell subsets in peripheral blood of the volunteers were classified by a combination of CD45RA and CCR7 markers. The phenotyping data generated by flow cytometry showed that the most frequent cell population is $CD45RA^+CCR7^+$ naïve T cells with more than half of the total $CD4^+$ T cells; similar to findings of others in healthy individuals or other infectious diseases [13, 19, 29]. The second most frequent subsets were related to $CD45RA^-CCR7^-$ $T_{EM}$ and $CD45RA^-CCR7^+$ $T_{CM}$ composing about 20–25 % of total $CD4^+$ T cells. The lower proportion of $T_{CM}$ seen in this study compared to other reports in human peripheral blood may be due to the sensitivity of chemokine receptors to physical conditions and internalization of CCR7 marker during cell preparation [30]. Variable ratios of antigen-specific memory subsets were reported in other parasitic diseases [31, 32]. In chronic Chagas disease, a relatively lower percentage of $CD45RA^+$ naive $CD4^+$ and $CD8^+$ T cells was seen than non-infected controls [31]. In *P. vivax* infection, an increase in the count of $CD4^+$ memory T cells was seen in acute infection [27]. This variability in the frequencies might be partly due to the nature of Chagas/malaria, since systemic infections are not similar to a local infection such as CL in view of availability and distribution of the antigens in host immune system.

In the present study, the proportions of $CD4^+$ T cell subsets were not significantly different between HCL volunteers and healthy controls. People continuously expose to different antigens during the lifetime and because of the burden of different pathogens that are encountered, it is not economically reasonable for the immune system to maintain a huge pool of resident memory T cells for every single antigen; Hence, total memory repertoire does not significantly increase by the age [33, 34]. In this experiment, after 5 days of SLA stimulation, purified total $CD4^+$ T cells were again analyzed for surface maker phenotypes using flow cytometry. Stimulation of $CD4^+$ T cells caused expression of CCR7 marker to fall off so that the proportions of $CD45RA^+CCR7^+$ naive T and $CD45RA^-CCR7^+$ $T_{CM}$ cells were significantly decreased and the proportion of $CD45RA^-CCR7^-$ $T_{EM}$ increased after culture. It is shown that CCR7 is rapidly lost upon antige stimulation concomitant with the differentiation of $T_{CM}$ to effector phenotype and production of effector cytokines [35].

Owing to similar frequency of the memory T cell subsets in HCL volunteers and healthy controls, in the next step $CD4^+$ T cell subsets were purified using MACS system and the functions of sorted cells were studied in vitro by proliferation analysis and cytokine assay. In MACS method, different frequencies of $CD4^+$ T cell subsets were retrieved depending on the initial cell population count and the complexity of the method. As it is expected, $CD4^+$ naïve T cells were the most frequent and $CD4^+$ $T_{CM}$ cells were the least frequent T cell subsets in total PBMC.

Data that obtained from flow cytometric analyses of CFSE labeled cells indicate that $CD4^+$ T cells from volunteers with HCL showed a high response to SLA. Both $CD4^+$ $T_{CM}$ and $CD4^+$ $T_{EM}$ subsets showed a high proliferation, but number of divisions and frequency of proliferated $CD4^+$ $T_{CM}$ was higher than $CD4^+$ $T_{EM}$ cells. Several other studies showed higher expansion potential of $T_{CM}$ in comparison with $T_{EM}$ as an in vitro characteristic of $T_{CM}$ subsets [36, 37]. It is supposed that the higher proliferation potential of $T_{CM}$ favors rapid differentiation of $T_{CM}$ pool to $T_{EM}$ cells in the case of in vivo antigenic exposure [37]. Cytokines such as IL-7 and IL-15 involve in proliferation and survival of memory $CD8^+$ T cells [38] and in memory $CD4^+$ T cells homeostasis a role for IL-7 is suggested [39, 40].

In this study, each of $CD4^+$ T cell subsets showed a different cytokine profile after stimulation with SLA. IL-10 and IL-13 as Th2 type cytokines were produced with different amounts by sorted $CD4^+$ T cell subsets but no significant difference was seen between HCL volunteers and healthy controls. IFN-$\gamma$ was the most prominent cytokine with production variations. IFN-$\gamma$ production by PBMC and $CD4^+$ $T_{EM}$ cells, and the frequency of IFN-$\gamma$ positive $CD4^+$ $T_{EM}$ cells were significantly higher in HCL volunteers compared with the healthy controls. The study of intracellular cytokine secretion of CFSE labeled cells showed that CFSEdim sub-population which was the proliferating portion of the $T_{EM}$ cells produces intracellular IFN-$\gamma$. $CD4^+$ $T_{EM}$ cells are present in the blood and migrate to the infection sites and upon restimulation produce either Th1 or Th2 effector cytokines [37]. In human leishmaniasis, several studies in Old and New world suggested that $CD4^+$ Th1 cells are the main source of IFN-$\gamma$ production while the phenotype of T cells were not determined [41–43]. The role of IFN-$\gamma$ as a hallmark of $CD4^+$ Th1 response or as a production of $CD8^+$ T cells in protection against *Leishmania* infection is documented in mouse model [44, 45] but not in human leishmaniasis [46]. In two sequential studies, we have reported that $CD8^+$ T cells [47] with memory phenotype (Microbes and Infection, 2012, in press) contribute as accessory cells to the production of IFN-$\gamma$ in CL.

Study of ICS indicates that around 10–12 % of the total $T_{CM}$ cells produced IL-2 intracellularly and the mean frequency of IL-2 positive $T_{CM}$ cells in HCL volunteers was significantly more than that of healthy controls. About 15–17 % of naive $CD4^+$ T cells also showed to be positive for IL-2 production in both HCL volunteers and healthy controls. IL-2 is the main production of naïve and $T_{CM}$ cells in human T cell repertoire and the role of IL-2 in T cell survival is known [48], IL-2 producing $T_{CM}$ might serve as a memory pool for generating $T_{EM}$ cells in new *Leishmania* infection.

In a recent publication, different T cell types including memory T cells (without subset indication) were investigated in VL due to *L. donovani* and the results showed a significantly reduced number of memory T cells in VL patients compared to the treated VL or asymptomatic individuals [19]. In animal model it is shown that two distinct sub-populations of $CD4^+$ memory T cells are present: $T_{CM}$ cells mediate long term immunity to *Leishmania* infection independent of parasite and $T_{EM}$ cells which are parasite-dependent [49] . In another study, immune cells were characterized in the liver and spleen of symptomatic/asymptomatic dogs naturally infected with *Leishmania* [50]. In the liver of asymptomatic dogs an effective immunity with granulomas around the parasites and $CD44^{low}$, $CD45RO^{hi}$ central memory and $CD44^{hi}$, $CD45RO^{hi}$ effector T cells were observed. $CD4^+$ memory T cells are studied in other infectious diseases of mouse model [51] and human [27, 29, 31, 52]. In *Plasmodium vivax* infection a significant increase in the number of $CD4^+$ memory T cells was observed in acute infection which was maintained up to 60 days post treatment [27]. Two phenotypically distinct subsets of $CD4^+$ memory T cells ($CD45RA^-CD62L^+CD11a^{dim}$ and $CD45RA^-CD62L^-CD11a^{bright}$) were reported in chronic leprosy associated with polarized Th1/Th2 immune responses [29]. In patients with intracellular fungal infection, paracoccidioidomycosis, $CD4^+CD45RO^+$ memory T cells was shown to increase compared to non-infected controls [52].

In the current study due to the limitations systemic immune response was studied by using peripheral blood while a different response might be seen at the site of infection and around the lesions. So, current data may not completely reflect the actual resident memory status around the lesion, particularly in regard of the expression of some specific surface receptors on the circulating memory T cells which are employed during migration into the skin [53].

## Conclusions

Based on the data presented here, similar pattern of $CD4^+$ memory T cell subsets are exist in peripheral blood of individuals with history of CL and healthy controls. However, $CD4^+CD45RO^+CD45RA^-CCR7^-$ $T_{EM}$ cells which showed the specific response to SLA stimulation by simultaneous proliferation and IFN-γ secretion and $CD4^+CD45RO^+CD45RA^-CCR7^+$ $T_{CM}$ cells which showed the specific response to SLA stimulation by proliferation and IL-2 secretion are identified in individuals with history of CL. Both of these *Leishmania*-reactive $T_{EM}$ and $T_{CM}$ cells seem to play roles in protective immune response against *Leishmania* infection. The current data support experimental findings in murine model suggesting that a combination of short lived pathogen-dependent $CD4^+$ $T_{EM}$ and long-lived pathogen-independent $CD4^+$ $T_{CM}$ cells contributes to the immune response against *Leishmania* infection [49].

Several questions remained to be answered concerning $T_{EM/CM}$ cells in leishmaniasis, but the present data might be used as a basis for further investigations of memory T cells in human leishmaniasis which is necessary information for vaccine development.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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