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Evaluation of T Cell Proliferation Using CFSE Dilution Assay: A Comparison between Stimulation with PHA and Anti-CD3/Anti-CD28 Coated Beads

Sara Kashef[1], Mozhgan Moghtaderi[2], Hamid Reza Hatami[1], Mehdi Kalani[3], Soheila Alyasin[1,2], Hesamodin Nabavizadeh[1,2], and Shirin Farjadian[4]

[1] Department of Allergy and Clinical Immunology, Shiraz University of Medical Sciences, Shiraz, Iran
[2] Allergy Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[3] Prof. Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[4] Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

A decrease in T cell count or reduced T cell function can be indicative of T cell immunodeficiency. In the present study, T-cell function was assessed using Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution test after stimulation with commonly used Phytohaemagglutinin (PHA) or anti-CD3/anti-CD28 coated beads in pediatric patients with recurrent infections.

Seven infants with recurrent infections and seven sex/age-matched healthy infants were included in this study. A blood cell count, immunophenotyping, and serum immunoglobulin level were performed. The proliferation of T cells was also assessed with CFSE dilution after stimulation with PHA or anti-CD3/anti-CD28 coated beads.

This study showed increased IgA, IgG, and IgM levels in patients compared to the controls. In contrast to the controls, the immunophenotyping results showed a significant decline in the number of $CD4^+$ T cells in patients. Although there was no difference in $CD3^+$ T cell proliferation between patients and controls, the $CD4^+$ and $CD8^+$ T cell proliferation rates were significantly decreased in patients when stimulated with PHA.

As a mitogen with the potential for maximum proliferation of T cells, PHA is better able to distinguish between patients with recurrent infections and controls than anti-CD3/anti-CD28, which mimics only the TCR pathway for stimulation of T cells.

Keywords: Anti-CD3; Anti-CD28; Carboxyfluorescein diacetate succinimidyl ester; Lymphocyte transformation; Phytohaemagglutinin

INTRODUCTION

The lymphocyte transformation test (LTT) is an old test. which is still considered of the gold standard for evaluating T cell function. In this test, T cell proliferation is usually assessed by the measurement of $[^3H]$-thymidine incorporation into DNA following T cell

Corresponding Author: Shirin Farjadian, PhD; Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran. Tel/ Fax: (+98 71) 3235 1575, E-mail: farjadsh@sums.ac.ir

stimulation by a mitogen like phytohaemagglutinin (PHA). In addition, nonradioactive DNA approaches such as the incorporation of 5-bromodeoxyuridine (BrdU) and methyl-thiazolyl tetrazolium (MTT) assay can also be used.[1] Carboxyfluorescein diacetate succinimidyl ester (CFSE)-based assay is recently used to evaluate T cell proliferation in patients with primary immunodeficiency (PID).[2] Intracellular amines react with the succinimidyl ester group to generate a fluorescent signal. Then it is divided between daughter cells and continuously halves following each division as a two-fold reduction in fluorescence intensity that is easily detectable by flow cytometry. This method includes a 72-hour culture of stimulated cells by PHA mitogen in comparison to unstimulated cells.

T cell stimulation with anti-CD3 and anti-CD28 which is more close to the physiological pathway of T cell activation has lately been used for T cell proliferation assay.[3] The comparison between traditional PHA and anti-CD3/anti-CD28 to stimulate T cell proliferation has yet to be under investigation. This study was designed to compare the proliferation rates of $CD3^+$, $CD4^+$ and $CD8^+$ T cells from a group of patients with recurrent infections by CFSE dilution assay after stimulation by either PHA or anti-CD3/anti-CD28 coated beads.

MATERIALS AND METHODS

Patients and Controls

Patients were recruited from those with recurrent infections who referred to Namazi Hospital affiliated with Shiraz University of Medical Sciences, Shiraz, Iran. The study protocol was approved by our University Ethics Committee (IR.SUMS.REC.1396.S69). A total of seven infants with recurrent infections were included in this study. Seven age/sex-matched healthy infants with no acute infectious disease in the last month, and no history of any background systemic disorder prior to the blood collection were considered as the control group. After obtaining written informed consent from their parents, 6 mL blood samples were taken from each participant, 1 mL were kept in a special tube for complete blood count (CBC) and white blood cell (WBC) differential tests, 4 mL was mixed with heparin for proliferation assay and immunophenotyping, and 1 mL was kept until clotting for serum separation to analyze the levels of immunoglobulins.

CBC and Immunophenotyping

CBC and WBC differential tests were done by an automated hematology analyzer (Sysmex KX-21N, USA). A panel of fluorescent-conjugated monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ, USA) including FITC-conjugated anti-CD14 for the detection of the myeloid lineage, PE-conjugated anti-CD45 for lymphoid lineage, PerCp-conjugated anti-CD3 for T cells, APC-conjugated anti-CD4 and PE-conjugated anti-CD8 for T cell subsets, PE-conjugated anti-CD16/anti-CD56 for NK cells, and PerCP-conjugated anti-CD19 and APC-conjugated anti-CD20 for B cells were used for immunophenotyping and the results were reported in comparison to age-specific reference ranges.[4]

Evaluation of Immunoglobulin Levels

IgM, IgG, and IgA serum levels were evaluated by nephelometry (Minineph, Birmingham, UK) using commercial kits (Binding Site, Birmingham, UK) according to the manufacturer's instructions and the results were compared to the age-related normal levels.[5]

T cell Proliferation Assay

For T cell proliferation assay, peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of each patient and healthy control individual using Ficoll-Hypaque (inno-Train Diagnostik GmbH, Kronberg, Germany). A cell suspension of 1×10^6 PBMCs/mL in PBS containing 0.1% of fetal bovine serum (FBS; Gibco, Oslo, Norway) was incubated with CFSE (Invitrogen, Waltham, MA, USA) at the final concentration of 10 µM for 10 min at 37°C in dark. After removing excess dye, 3×10^5 CFSE-labeled PBMCs were seeded in each well of a 96-well flat-bottom culture plate in RPMI 1640 enriched with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 25 mM HEPES (Gibco) and stimulated with PHA-L 1 µg/mL (Gibco) or anti-CD3/anti-CD28 coated Dynabeads (DB, Gibco), bead-to-cell ratio 1:1, each in triplicate. As baseline controls, unstimulated CFSE-labeled PBMCs were also cultured in the same condition for each sample. To induce *in vitro* immunodeficiency condition, PBMCs of each control individual were treated with a combination of PHA and cyclosporine A (100 µg/mL) or a mixture of anti-CD3/anti-CD28 coated beads and cyclosporine A. Then cells were incubated for 120 hours at 37°C, in a 5% CO_2 at 90% humidity. Finally, harvested cells were stained with PerCp-labeled anti-CD3, APC-labeled anti-CD4, and PE-labeled anti-CD8 for 30 min and after

washing, cells were subjected to analysis by flow cytometry (BD FACSCalibur, San Jose, CA, USA) and data were analyzed using FlowJo 7.6.2 software.

As shown in Figure 1, lymphocytes were gated based on the forward versus side scatter (a). Then, $CD3^+$ cells were selected on the gated lymphocytes (b). Afterward, $CD4^+$ and $CD8^+$ T cells were determined on the gated $CD3^+$ cells (c). Finally, the intensity of CFSE was determined on the gated $CD3^+CD4^+$ as well as $CD3^+CD8^+$ T cells (d and e), respectively.

To determine T cell proliferation, viable $CD3^+$ cells were gated and the percentages of CFSE positive cells were determined. Proliferation in $CD4^+$ and $CD8^+$ T cells was also determined with the same method. Then the rate of T cell proliferation in each patient compared to the corresponding control (P:C) was calculated and considered normal if it was >50% of the control, low if it was between 25-50%, very low if it was 10-25%, and absent if it was <10%.[6]

Statistical Analysis

The comparison of T cell proliferation after stimulation with anti-CD3/anti-CD28 coated beads and PHA was done with the Wilcoxon test. The Mann-Whitney U test was used to compare the mean of cell proliferation between patients and controls. Statistical analyses were performed using SPSS 19 software and $p<0.05$ was considered significant.

RESULTS

The demographic and clinical data of seven children suspected of having primary immunodeficiency are presented in Table 1. The laboratory data of patients and healthy controls are summarized in (Table 2a). The results of immunophenotyping showed a notable decline in the percentage of $CD4^+$ T cells in patients compared to the controls, as the $CD4^+$ T cells percentage was less than age-specific reference ranges in seven patients (Table 2b).

The results of the CFSE dilution assay showed no significant difference in T cell proliferation after stimulation with PHA in comparison to stimulation with anti-CD3/anti-CD28 coated beads. Although the results of the CFSE assay showed no difference in $CD3^+$ T cell proliferation either after stimulation with PHA or beads, the deceased proliferation of $CD4^+$ T cells and $CD8^+$ T cells was observed in patients compared to the controls after stimulation with PHA but not after stimulation with beads (Table 3, Figure 2).

The percentage of $CD3^+$ T cell proliferation was <50% just in patient #2 after stimulation with anti-CD3/anti-CD28 coated beads, the proliferation percentage of these cells in patient #5 after stimulation with either PHA or anti-CD3/anti-CD28 coated bead were extremely higher than their corresponding controls. Poor proliferation response to PHA stimulation (<45%) was detected in $CD4^+$ T cells in patients #3 and #4 compared to their corresponding controls while this parameter was <40% in $CD8^+$ T cells just for the former patient (Table 4).

As a positive control, cyclosporine A reduced the rate of proliferation in T lymphocytes isolated from healthy individuals either after PHA or anti-CD3/anti-CD28 stimulation (Figure 3).

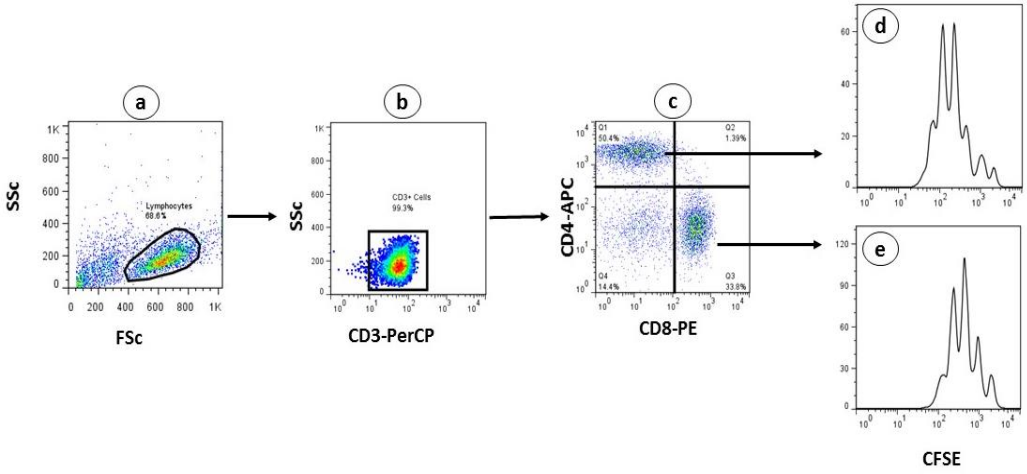

Figure 1. Flow cytometry gating strategy for determining proliferation in $CD4^+$ or $CD8^+$ T cells by CFSE dilution assay

Table 1. Demographic and clinical data of patients suspected of having primary immunodeficiency diseases

Patients	Gender	Age	Failure to thrive	Chronic diarrhea	Recurrent pneumonia	Recurrent perianal abscess	Hemolytic anemia	Recurrent skin infection
1	F	3 m	+	-	+	-	-	-
2	F	3 m	+	-	+	-	+	-
3	F	4 m	+	-	-	-	-	+
4	M	6 m	+	+	-	+	-	-
5	M	6 m	+	-	+	-	-	-
6	F	8 m	+	+	+	-	-	-
7	F	17 m	+	-	-	+	-	-

Table 2. Laboratory data of patients suspected of having primary immunodeficiency diseases compared to healthy controls (a), detailed data of each patient compared to the corresponding control for parameters with significant differences (b)

(a)

Lab data	Patients (n=7)	Healthy controls (n=7)	*p*
CBC			
WBC×10^3/mm^3	11.7±4.43	7.3±1.3	0.053
Lymphocyte/mm^3	6794.8±3765.3 (54.3±15.2%)	4344±1021 (59.3±5.8%)	0.71
Neutrophil /mm^3	4283±1751 (38.6±9.5%)	2771±512 (38.3±6.6%)	0.8
Plt.×10^3/mm^3	195.8±89.5	209.86±42.7	0.9
Immunophenotyping (%)			
$CD3^+$	63.4±18.9	66.1± 10	0.9
$CD4^+$	28±12.2	40±5.6	**0.038**
$CD8^+$	31.3±18.3	23.6± 5.3	0.53
$CD16^+$	15.6±14.1	16±9.4	1
$CD19^+$	19±13.7	17.9±7.2	1
$CD45^+$	96±2.3	96±1	0.89
$CD14^+$	4±2.3	3.9±1.1	0.9
CD11b (GM)[1]	96.3±68.7	60±38	0.46
Ig levels (mg/dL)			
IgA	1.43±0.96	0.48±0.23	**0.004**
IgG	15.4±10.3	5±2	**0.038**
IgM	1.3±0.76	0.5±0.26	**0.026**

[1] GM: Geometric Mean

(b)

	ID	CD3⁺ T%	CD4⁺ T%	CD8⁺ T%	IgA	IgG	IgM
Patients	**1**	82%^{H}	15%^{L}	62%^{H}	3.06 ^{H}	25.36 ^{H}	2.05 ^{H}
	2	48%	21%^{L}	14%	0.97 ^{H}	6.10	0.70
	3	32%^{L}	17%^{L}	11%^{L}	1.01 ^{H}	31.85 ^{H}	0.89
	4	66%	31%^{L}	34%^{H}	1.44 ^{H}	12.87 ^{H}	1.23 ^{H}
	5	79%^{H}	26%^{L}	46%^{H}	0.79	17.61 ^{H}	1.54 ^{H}
	6	57%	36%	20%)	0.38	3.08	0.25 ^{L}
	7	80%	50%	30%	2.397^{H}	11.11	2.4^{H}
Controls	**1**	52%	33%^{L}	14%	0.77	8.11	0.83
	2	71%	42%	24%^{H}	0.24	4.81	0.78
	3	67%	38%	265^{H}	0.31	4.49 ^{L}	0.52
	4	56%	36%	21%	0.38	3.54	0.45
	5	78%	50%	28%^{H}	0.26	2.42	0.13 ^{L}
	6	62%	38%	22%	0.77	7.38	0.59
	7	77%	43%	30%	0.615	4.19	0.247

L: low, H: high, compared to the normal ranges ([5,6])

Table 3. The proliferation of CD3⁺, CD4⁺ and CD8⁺ T cells in patients suspected of having primary immunodeficiencies compared to healthy controls using CFSE dilution assay: stimulation with PHA vs. anti-CD3/anti-CD28 coated Dynabeads (DB).

Proliferation%	Patients	Controls	*p1*
	CD3⁺ T cells		
PHA	53.2 ± 24.9	58.9 ± 19.6	0.53
DB	48.8 ± 18.4	58.4 ± 10.4	0.53
***p*2**	0.4	0.61	
	CD4⁺ T cells		
PHA	45.6 ± 12.8	47.9 ± 5.4	**0.002**
DB	61.6 ± 5.51	72.2 ± 4.1	0.225
***p*2**	0.063	0.398	
	CD8⁺ T cells		
PHA	67.1±16.4	87.3±5.3	**0.002**
DB	74.2 ± 9.4	80 ± 9.8	0.338
***p*2**	0.735	0.091	

p1 (*p* value): Mann-Whitney U-test between patients and control,

p2 (*p* value): Wilcoxon Signed Ranks test between PHA and DB

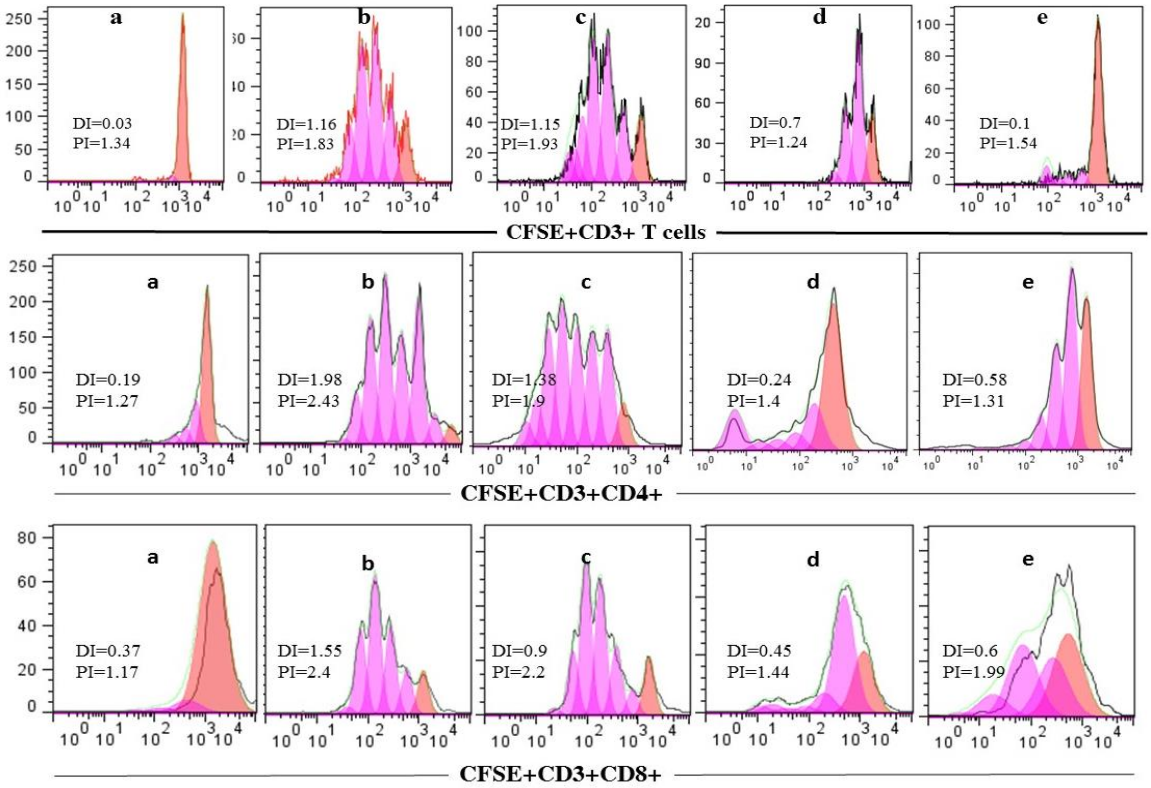

Figure 2. Representative of the proliferation of $CD3^+$ (top), $CD4^+$ (middle) and $CD8^+$ (bottom) T cells from healthy individuals under different conditions: a) unstimulated, b) treated with PHA, c) treated with anti-CD3/anti-CD28 coated beads, d) treated with the combination of PHA and cyclosporine A, e) treated with the combination of anti-CD3/anti-CD28 coated beads and cyclosporine A (DI, division index; PI, proliferation index).

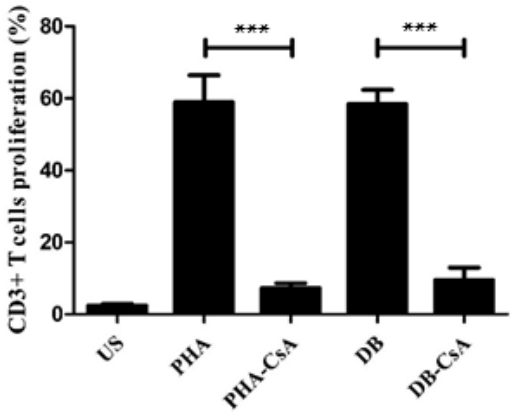

Figure 3. $CD3^+$ T cell proliferation in healthy controls under different conditions (US: unstimulated, DB: anti-CD3/anti-CD28 coated Dynabeads, CsA: cyclosporine A, ***p<0.001)

Table 4. Percentage of T cell proliferation after stimulation with PHA or anti-CD3/anti-CD28 coated beads in each patient compared to the corresponding control

		PHA			DB		
	ID	**Patient**	**Control**	**P:C %**	**Patient**	**Control**	**P:C %**
$CD3^+$ T cells	1	30.5	31.4	97%	32.0	62.2	51%
	2	29.1	51.7	56%	29.0	63.0	**46**%
	3	66.0	85.1	78%	51.5	67.7	76%
	4	40.9	81.9	50%	61.5	67.3	91%
	5	85.0	41.2	206%	68.4	39.0	175%
	6	84.9	57.4	148%	69.6	50.2	139%
	7	36.2	64.0	57%	29.7	59.6	49.8%
$CD4^+$ T cells	1	53.9	82.2	66%	75	71.20	105%
	2	57.6	71	81%	64.5	55.80	116%
	3	24.9	73.5	34%	73.50	67.00	110%
	4	31.2	76.7	41%	45.70	82.80	55%
	5	52	81.9	63%	39.70	79.80	50%
	6	44.4	70	63%	75.40	85.10	89%
	7	55.2	69.3	79%	57.1	64	89.2%
$CD8^+$ T cells	1	73.5	90	82%	72.10	66.00	109%
	2	78.1	80.3	97%	72.50	73.10	99%
	3	33.5	85	**39%**	81.90	87.51	94%
	4	57.6	81.2	71%	60.80	85.70	71%
	5	75	91.3	82%	68.00	86.50	79%
	6	77.8	94.7	82%	89.84	90.73	99%
	7	74.3	69.3	100.1%	85.9	64	134.2%

DISCUSSION

Despite all distinctions, immunodeficiency and autoimmunity diseases caused by immune dysfunction sometimes seem to have some overlaps with each other as increased immunoglobulin levels have been reported in some PID patients.[7] Accordingly, in addition to increased IgG levels in four of our patients which can be due to treatment with IVIG, IgM and IgA were also higher than normal ranges in most of our patients which might be due to recurrent infections or dysregulated B cell responses to antigens.

PIDs are frequently presented by recurrent infections and different types of cytopenia, especially anemia. In this regard, most of our patients were referred to the hospital due to recurrent pneumonia (66.7%). In a report by Azarsiz et al, also 70.5% of their patients were referred to the hospital because of different types of infections.[2]

T cell immunodeficiency can be presented by decreased T cell count or reduced T cell function. However, $CD3^+$ T cell count was normal in all of our patients, and $CD4^+$ T cell count was less than the normal range in five out of seven patients.

To analyze the T cell function of our patients, proliferation rates of total $CD3^+$ T cells, as well as each of $CD4^+$ or $CD8^+$ T cells, were assessed by CFSE dilution assay after stimulation by either PHA or anti-CD3/anti-CD28 coated beads. Our results revealed that the rates of $CD4^+$ and $CD8^+$ T cell proliferation in response to PHA stimulation were significantly lower in patients than in the control group (p=0.002 for each). However, the proliferation rates of these two T cell subsets in patients were lower than controls in response

to anti-CD3/anti-CD28 coated beads, these differences were not significant.

Using the same method, Jiang et al, investigated the proliferation of T cells in seven SCID (severe combined immunodeficiency disease) patients with DNA ligase IV deficiency. Their results showed no division peak in T cells from two patients after stimulation.[8] Roifman et al, also investigated the T cell response of SCID patients to PHA stimulation using the same technique. Contrary to different genetic defects in these patients including Omenn syndrome, ZAP70 deficiency, and IL-2Rα deficiency, a sharp decline was observed in their T cell proliferation (<10%) after PHA stimulation.[9] Lastovicka et al compared CFSE dilution, ^{3}H-thymidine incorporation, and Ki-67 expression for the evaluation of cell proliferation and introduced the first method as a selective assay. They introduced the CFSE dilution method as the low-cost and most appropriate choice in lymphocyte proliferation assay while Ki-67 was more sensitive but more expensive. Based on their results, the original^{3} H-thymidine test showed no benefit against flow cytometric methods.[10]

In an original report from Oxford University Hospital, it was shown that in a group of ten normal adults and three poor PHA responders (<45%), there was no significant difference between the percentage of T cell proliferation after stimulation with PHA and anti-CD3/anti-CD28 beads. Also, no significant difference was found when IL-2 was used in addition to the bead.[11]

In a recent report from Iran, enhanced T cell proliferation was observed in three healthy adults after stimulation with PHA compared with anti-CD2/anti-CD3/anti-CD28 coated beads.[12]

The discrepancy between our results in the case of $CD3^{+}$ T cells proliferation after stimulation with PHA and anti-CD3/anti-CD28 coated beads may be due to the difference in their signal transduction pathway. Moreover, inconsistent results of proliferation in $CD4^{+}$ and $CD8^{+}$ T cell subsets reveals that T cell proliferation assay alone is not adequate to tag a patient as having T cell dysfunction. Instead, additional tests such as cytokine production assay for $CD4^{+}$ T cells and analysis of the cytotoxic activity of $CD8^{+}$ T cells after stimulation should be assessed. In this regard, cytokine production without T cell proliferation was first reported by Evavold and Allen[13] in 1991 based on the affinity of T cell receptor to its ligand and more proved by others.[14,15]

In this study we induced *in vitro* T cell suppression by using cyclosporine A,[16] and prevention of T cell proliferation in the control group after stimulation with either PHA or anti-CD3/anti-CD28 beads confirmed the accuracy of our laboratory techniques.

According to our results, it seems that PHA as a mitogen using the maximum proliferative capacity of T cells is better able to differentiate between patients with recurrent infection and controls compared to anti-CD3/anti-CD28, which mimics only the T cell stimulation pathway through TCR. Even though, LTT assay by CFSE dilution test after PHA stimulation can be considered as an auxiliary test in the diagnosis of PID.

CONFLICT OF INTEREST

There is no conflict of interest to be declared.

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