



# Flowcytometric Assessment of Lymphocyte Subsets in Type-1 Diabetic Patients following Allograft Transplantation of Liver-derived Fetal Stem-cells

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## Abstract

**Background:** Although the pathogenesis of diabetes type 1 (T1D) is not fully elucidated. Different clusters of lymphocytes such as CD4+ and CD8+ T cells are involved in it. Moreover, the mechanism of how stem-cell therapy results in significant therapeutic outcomes in diabetes remains obscure. In the current study, we aimed to analyze lymphocyte subsets in patients with T1D before and after treatment with Liver-derived Fetal Stem-cells, and investigated the potential underlying immunological mechanism of therapeutic effects of stem-cell therapy.

**Methods:** Seventy-two patients with T1D were selected for our study and underwent allograft transplantation of liver-derived fetal stem-cells. Relative counts of peripheral blood T and B lymphocyte subsets were detected by the means of flow cytometry analysis.

**Results:** Our results demonstrated that administration of fetal liver-derived fetal stem-cells resulted in significant changes in the subpopulations of lymphocytes of the patients, more specifically, levels of CD4+, CD8+, CD16+, and CD19+ lymphocytes.

**Conclusion:** The findings of this study demonstrated that different subsets of lymphocytes significantly change following stem-cell therapy for diabetes. As it is demonstrated that immunological mechanisms are involved in pathogenesis of diabetes, these changes can suggest that therapeutic effect of stem-cell therapy for diabetes may be exerted via alternations in different lymphocyte subsets.

**Keywords:** Diabetes, Fetal stem-cell, Flow cytometry, Lymphocyte

## Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease, which results in destruction of the beta-cells in the pancreas with the consequence of insulin deficiency and hyperglycemia. However, the precise immunopathology of the disease remains obscure (1). Nonetheless, it is well established that

in the pathology of T1D, certain subpopulations of T cells, especially CD4 T cells, play important roles (2).

Since significant achievements in the field of stem-cell therapy of animals with different autoimmune diseases, this treatment modality has been

avored by scientists for the design of clinical trials all across the world (3). In several clinical trials, the remission of relapses of many diseases with auto-immune etiology including rheumatic disorders (systemic lupus erythematosus) neurological disorders (multiple sclerosis, etc.), adult and juvenile rheumatoid arthritis, systemic sclerosis, vasculitis, etc.), and hematological disorders (auto-immune hemolytic anemia, immune-mediated thrombocytopenic purpura, Evans syndrome, etc.) has been quite significant (3). Based on such evidence, stem-cell therapy for treatment of diabetes has been greatly favored in the recent years.

Modulation of the immune system is the prime responsible underlying mechanism of therapeutic effect of stem-cells in newly diagnosed type 1 diabetic patients (3). It is also demonstrated that subpopulations of lymphocytes are influenced by transplantation of stem-cells for treatment of different diseases including diabetes. One study on a Chinese cohort of T1D patients demonstrated that except CD8+ cells, the population of all lymphocytes were lower in diabetic patients in comparison with the control group, which returned to the normal levels following the transplantation (4). The current study was designed to assess the changes made to lymphocyte subpopulations because of allotransplantation of fetal-liver derived hematopoietic stem-cells for treatment of diabetes patients.

## Methods

In this study, the data of a phase 3 single-arm clinical trial designed for the assessment of efficacy of application of allotransplantation of fetal Liver-derived stem-cells in 82 patients for the treatment of T1D was analyzed and reported.

### Patients

All subjects were from Tehran (Iran) and were referred to Shariati Hospital of Tehran University of Medical Sciences in Tehran from 2010–2013. The trial population included 72 patients (44 men and 38 women) aged between 5-53 and  $19.84 \pm 9.14$  years (mean  $\pm$ SD) with diabetes duration of  $26 \pm 6.2$  month (mean  $\pm$ SD). All patients fulfilled

the diagnostic criteria set for type 1 diabetes. Exclusion criteria were acute vascular inflammation, acute thrombosis, recent retinal hemorrhage, pulmonary hypertension, corpulmonale, bone marrow malignancy, end stage diseases, infections, and signs of refractory complications.

To undergo transplantation, all patients were admitted to the hospital. On the day of admission, primary clinical examination and laboratory data (including FBS, HbA1c, fasting serum c-peptide, CBC, liver function tests, lipid profile tests and U/A) were collected and recorded. These data were recollected in the next follow up visits in the 1st, 3<sup>rd</sup>, 6<sup>th</sup>, and 12<sup>th</sup> months after the cells infusion. Each follow-up visit included a complete history taking, physical examination, and laboratory tests. In order to optimize diabetes care, each participant had access to a special 24-hour phone line physician during the first year of the follow up.

### Assessments

Peripheral blood was collected and T and B lymphocyte subset relative counts were detected by flow cytometry both before and after treatment with stem-cells (at the inclusion, and 1, 3, 6, and 12 months afterwards). The study protocol was approved by the Research Ethics Committee of Tehran University of Medical Sciences and followed the ethical guidelines of the 1975 Declaration of Helsinki and all subsequent modifications. Every patient provided a written informed consent to participate in this research.

### Stem cell preparation

Fetal liver-derived hematopoietic stem cells (HSCs) were isolated from legally aborted human fetuses aged 6–12 weeks after an informed consent was obtained from either or both of the parents. In order to detect any chromosomal abnormality and to identify the sex of the aborted fetus, karyotyping was performed for every fetal sample. Whole fetal liver was stored in Hank's balanced salt solution without calcium and magnesium (HBSS, Sigma, USA) and mechanically dissociated and homogenized. The cell suspension was then filtered through a nylon mesh for transplantation. Consequently, isolated cells were cryopreserved

with the use of 5% dimethyl sulfoxide (DMSO) in HBSS, (Wak Chemie, Germany) with a programmable freezer, and were transferred to liquid nitrogen deservers for long-term storage. Prior to the procedure, samples were thawed at 37°C and cryoprotectant was diluted by 5-milliliter normal saline. Total cell count in the prepared suspension was approximately  $35\text{-}55 \times 10^6$ , twenty percent of which were identified as hematopoietic (CD34+) stem cells.

The suspension was investigated before, during, and after processing for any possible aerobic, anaerobic and fungal contamination as well as viral infections. Rubella, Herpes Simplex Virus, Cytomegalovirus, Chlamydia, Mycoplasma Homonis, Toxoplasma Gondii and Treponema Pallidum contamination was tested. DNA/RNA extraction and polymerase chain reaction (real-time PCR) were performed for investigation of any potential viral contamination (HBV, HCV, and HIV). On receiving the results, cell samples were declared as suitable for the transplantation.

### Intervention

On the day of transplantation, each participant in the intervention group received fetal liver-derived cell suspension at the dosage of approximately  $35\text{-}55 \times 10^6$  cells ( $7\text{-}11 \times 10^6$  CD34+ HSCs) in 5 milliliter of normal saline intravenously. Participants in placebo group received 5 milliliter of normal saline intravenously.

### Flow cytometry

Whole blood was collected in the baseline and each of the following follow ups in EDTA vacutainer tubes. Cyflow reagents and consumables were utilized based on the manufacturer's protocol.

### Statistical analyses

The principal purpose of this study was to examine the overtime variations (inclusion, 1, 3, 6 and 12 months) in CD4, CD8, CD16, CD19, and CD20 following stem cell transplantation. The data were averaged across 5 follow-up sessions (inclusion, 1, 3, 6 and 12 months) and the varia-

bles were first examined for normality of distribution by Shapiro Wilks "W" statistic. Subsequently, one-way repeated measure ANOVA was used to analyze the data. Mauchly's was used to evaluate sphericity as an important assumption of repeated-measures ANOVA. Following a significant overall result, comparisons between follow-up times were made using tests of within-subjects effects (post hoc tests). In case of variables that did not have normal distribution, the Friedman non-parametric test was used to compare the differences in the variables between the five periods.

## Results

Our results demonstrated that the alternations in the number of CD4+ cells differed significantly between different assessment times ( $P=0.000$ ) (Table 1). As it is shown in figure 1, Number of CD4+ cells showed an increase in the first month following the intervention ( $P=0.003$ ). However, their number decreased in during the next two months which was not significant ( $P=0.269$ ). At this point, the number of CD4+ lymphocytes demonstrated a significantly sharp increase ( $P=0.000$ ) which reached to its peak in the 9<sup>th</sup> month when their number started to decrease to a level non-significantly higher levels in comparison with the baseline levels.

In terms of CD8+ leucocytes, although it appears that in the third month it showed an increasing trend, generally, no significant changes were observed during the whole period of the study ( $P=0.307$ ) (Fig. 1). As regards the CD4/CD8 ratio, although the changes were significant throughout the whole period of the study, as the distribution of the variables was not normal, it is not possible to pinpoint that differences between which months were significant.

As regards CD16+ leucocytes, their numbers were decreased in the first three months, and then, this number showed an increasing trend until the end of the 12 months period (Fig. 1). These changes showed significant differences in different months ( $P=0.000$ ) (Table 1).

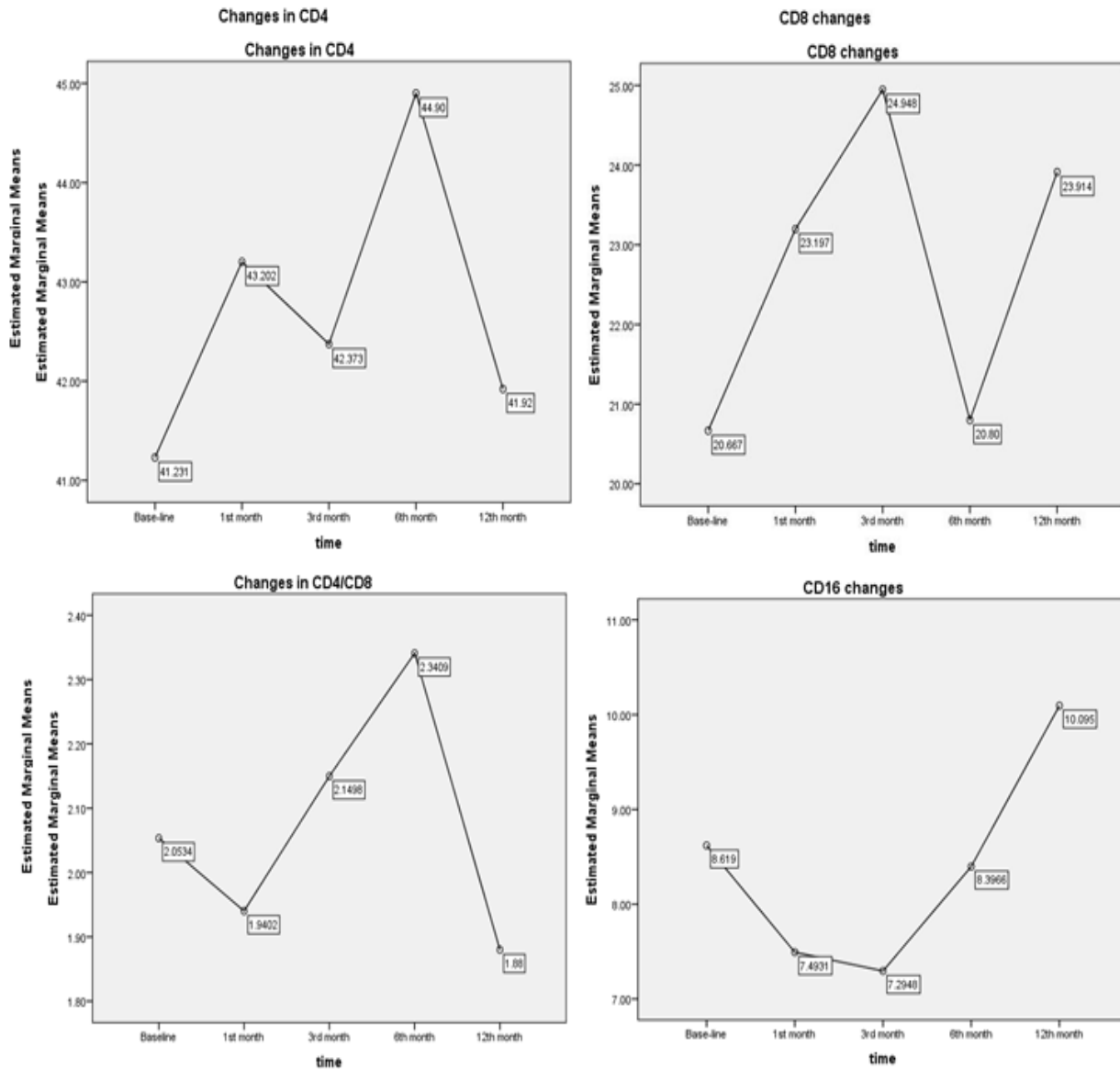


Fig. 1: Change in CD4, CD8, CD4/CD8 and CD16 during follow-up sessions

Although the number of CD16+ cells show a significant increase in the month 6 in comparison with the 3<sup>rd</sup> month values ( $P=0.015$ ), this increase was not significant in comparison with the base-line levels ( $P=0.694$ ).

Regarding CD19+ cells, as demonstrated in Fig. 1, there was a decreasing trend in the first three months following the intervention, and after the month 3, their numbers steadily increased until the 12<sup>th</sup> month. These changes were statistically significant in different months ( $P=0.000$ ) (Table 1). It should be added, however, that at the month

12, the levels of CD19+ cells were significantly higher in comparison with other months.

As regards CD20+ cells, although significant difference was observed between the levels in different months, as the distribution was not normal, it is not possible to specify difference in which months caused this significant change. In terms of CD34+ cells, similarly, although significant changes were observed, because of the distribution not being normal, no increasing or decreasing trend can be pinpointed.

**Table 1:** Changes in different flowcytometry variables during follow-up sessions

Time	Statistics	screening	1st month	3rd month	6th month	12th month	P-value
CD3	Mean	63.69	67.31	64.09	65.58	66	0.08 €
	Std. Deviation	6.79	8.1	10.9	11.84	10.74	
CD4	Mean	41.23	43.2	42.37	44.9	41.92	0.00 €
	Std. Deviation	5.59	6.01	6.46	8.11	6.03	
CD8	Mean	20.67	23.2	24.95	20.8	23.91	0.31 €
	Std. Deviation	5.45	4.51	31.01	5.46	5.6	
CD4/CD8	Mean	2.05	1.94	2.15	2.34	1.88	0.000 <sup>¥</sup>
	Mean Rank	3	2.6	3.24	3.9	2.26	
CD16	Mean	8.62	7.49	7.29	8.4	8.62	0.00 €
	Std. Deviation	4.07	4.3	3.82	4.53	4.07	
CD19	Mean	13.19	12.66	12	13.08	13.19	0.00 €
	Std. Deviation	4.35	4.87	4.65	4.86	4.35	
CD20	Mean	13.38	12.49	13.99	12.86	14.55	0.000 <sup>¥</sup>
	Mean Rank	3.21	2.86	2.29	2.89	3.75	
CD34	Mean	1.07	0.8	0.72	0.63	0.97	0.011 <sup>¥</sup>
	Mean Rank	3.32	2.97	2.88	2.46	3.37	

€. Repeated ANOVA

¥. Friedman test

## Discussion

Stem-cell therapy is considered as novel curative option for treatment of diabetes and considerable success has been achieved in terms of considerable curative outcomes. However, little is known as to the underlying therapeutic effects of stem-cell therapy for diabetes.

Stem-cell therapy may not only play a pivotal role in promotion of maturation and proliferation of lymphocytes, but also they might enhance their functional activation (5). All subpopulations of lymphocytes except CD8+ lymphocytes were low in group of Chinese patients with newly diagnosed T1D which returned to normal values following transplantation of stem-cells (4). In line with these findings, our results demonstrated an increase in the number of CD4+ lymphocytes in the first three months of the intervention followed by a steady decrease, which continued, to the end of the 12 months period.

Autoimmune destruction of pancreatic beta-cells is a cell-mediated process and involves both CD+4 and CD+8 T cells as well as macrophages (6). Regarding the precise role other subsets of

lymphocytes such as CD16+ and CD19+ cells play in the pathogenesis of diabetes, conclusive data as to their role in the disease is not yet documented although anecdotal reports demonstrate their involvement in different stages of the disease. Considering the novelty of the procedure and the fact that only a few studies have been carried out on the effect of fetal stem-cell transplantation for treatment of type 1 diabetes, there is scarcity of data about the effect of administration of these cells on the cellular immune system. Administration of autologous or allogeneic BMSCs strongly suppresses both CD4+ and CD8+ T-lymphocyte proliferation (7). However, we did not detect any significant changes in the number of CD8+ cells in the 12 months period of our follow up. Moreover, as mentioned before, the numbers of CD4+ cells increased in the first three months of our study.

Different strategies for treatment of T1D have been recently proposed with a special focus on targeting CD4 T cells through different immunomodulatory method (8).

In the pathophysiology of T1D both CD4+ and CD8+ T cells are involved. Moreover, at the onset

of diabetes, subpopulations of T-cells are altered which return to normal values during a period of a few weeks (9). This is the basis for the argument that stem-cell therapy can only be effective in early stages of the disease and it is hypothesized that it hails destruction of pancreatic beta-cells (3). In our study however, although we included diabetic patients with varied times of diagnosed diabetes, significant changes in CD4+ cells were observed in all patients.

The number of CD16<sup>+</sup> monocytes is significantly decreased in patients with diabetes-related complications (10). The level of CD14+CD16<sup>+</sup> monocyte subset can be used as an indicator of inflammation grade in diabetes. Moreover, in vivo activated blood monocytes with proinflammatory features are present in the circulation of T1D patients. Monocytes clearly have their role in balancing effector and regulatory T cell induction (11). Our finding demonstrated a significant decrease in the number of CD16<sup>+</sup> monocytes during the first three months of the intervention, which returned to normal levels after by the month 12. This finding might suggest that alternation in CD16<sup>+</sup> monocytes can also play a role in therapeutic effect of stem-cells in patients with type 1 diabetes.

In terms of CD19<sup>+</sup> cells, similar to CD16<sup>+</sup> cells, we observed a decrease during the first three months, which returned to normal levels in the following nine months. CD19<sup>+</sup> lymphocytes are demonstrated to be lower in children with newly onset T1D in comparison with those who were on insulin therapy for a period of more than one month (12). This may define a role for this subset of lymphocytes in the pathology of diabetes and might explain the therapeutic effects of stem-cell therapy for diabetes.

## Conclusion

The findings of this study indicated that following stem-cell transplantation, significant changes occur in different subsets of lymphocytes. These changes may help to shed some light on the precise immunomodulatory effect via which stem-cell therapy exerts its influence.

## Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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