Optimization of Culture Media for *Ex vivo* T-Cell Expansion for Adoptive T-Cell Therapy

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Abstract

Background: Adoptive T-cell therapy is a promising treatment strategy for cancer immunotherapy. The ability of immunotherapy based on the adoptive cell transfer of genetically modified T cells to generate powerful clinical responses has been highlighted by recent clinical success. Techniques which are used to expand large numbers of T cells from different sources are critical in adoptive cell therapy. In this study, we evaluated the expansion, proliferation, activation of T lymphocytes, in the presence of various concentrations of interleukin-2, phytohemagglutinin (PHA), and insulin.

Materials and Methods: The effect of different supplemented culture media on T cell expansion was evaluated using MTT assay. The expression level of the Ki-67 proliferation marker was evaluated by real-time polymerase chain reaction. In addition, flow cytometry analysis was performed to access T cell subpopulations.

Results: Our results showed that supplemented culture media with an optimized concentration of PHA and interleukin-2 increased total fold expansion of T cells up to 500-fold with approximately 90% cell viability over 7 days. The quantitative assessment of Ki-67 in expanded T cells showed a significant elevation of this proliferation marker. Flow cytometry was also used to assess the proportion of CD4+ and CD8+ cells, and the main expanded population was CD3+ CD8+ cells.

Conclusions: Based on these findings, we introduced a low-cost and rapid method to support the efficient expansion of T cells for adoptive cell therapy and other *in vivo* experiments.

Keywords: Immunotherapy, interleukin-2, phytohemagglutinin, T-cell expansion

INTRODUCTION

The potential of adoptive T cell therapy (ACT) through stimulating the immune system to recognize and destroy tumor cells is emerging as a promising treatment strategy in the field of cancer immunotherapy. The results of clinical studies have shown that adoptive cell therapy using T lymphocytes from peripheral blood mononuclear cells (PBMCs), genetically engineered T lymphocytes, and tumor-infiltrating T lymphocytes, is a remarkable strategy for the treatment of various cancers.[1,2] A critical step for cell therapy using T lymphocytes, in particular chimeric antigen receptor T cells, is isolation and large-scale *ex vivo* expansion of T cells with tumor cytotoxic phenotype.[3]

In general, the frequency of tumor-infiltrating T lymphocytes and tumor-specific T cells in peripheral blood is low and leads to a reduction in the efficacy of adoptively transferred T cells. To obtain an adequate and sufficient number of T lymphocytes for infusion, *ex vivo* culture, expansion, and activation of T

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lymphocytes are required. In this approach, T lymphocyte populations are expanded to improve the functional properties of cytotoxic CD8+ cells, CD4+ cells as well as memory T cells preinfusion.[4]

Large-scale production of human T cells is a complex process that required fine-tune optimization to obtain long-term *in vivo* survival of T lymphocytes with desired phenotype and function.[5] Studies in cancer-targeting T cell therapy have often focused on the isolation and expansion of cytotoxic T cells to increase the efficiency of tumor cell killing. However, there is growing evidence for the importance of less-differentiated T memory cells as well as CD4+ T cells in therapeutic T cell products. CD4+ T cells provide a supporting role in the proliferation, activation, cytotoxic function, and recruitment of CD8+ cells to the tumor milieu as well as directly inhibiting tumor growth.[6,7]

In recent decades, many efforts have been made to optimize isolation and nonantigen-specific expansion of cytotoxic T cells for ACT. While antigen-presenting cells, especially dendritic cells, are potent initiators of T-lymphocyte activation and immune response, there are many obstacles to the utilization of these cells at clinical grade due to their long-term and costly culture process. [8,9] Furthermore, the T cell population obtained from these methods comprises a high percentage of cells that are in the final stage of differentiation, which is associated with low survival rates *in vivo*. In this regard, proliferation methods should contain substances that contribute to cell growth and activation in the shortest possible time.[10,11]

Mitogenic molecules such as phytohemagglutinin (PHA), interleukin-2 (IL-2), and concanavalin A are used widely for *in vitro* T lymphocyte stimulation and expansion.[12-14] On the other hand, the mitogenic properties of insulin, insulin receptor signaling, and insulin analogs were documented and insulin has been shown to stimulate *in vitro* T cell proliferation when supplied exogenously.[15,16]

The main aim of this study is to optimize the expansion and proliferation conditions of T lymphocytes to achieve a simple, cost-effective, and rapid method as well as a sufficient number of T lymphocytes with antitumor phenotype for adoptive cell therapy in cancer research.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cell and T-cell culture

Informed consent was obtained from five healthy donors before blood collection and the study and the use of human material were approved by the Institutional Review Boards at the Isfahan University of Medical Science (IR.MUI.RESEARCH. REC.1398.788). PBMCs were isolated from heparinized blood using the Ficoll density gradient. Cells were incubated for 24 h in complete RPMI-1640 medium (BioIdea-Iran) containing 10% fetal bovine serum (FBS) (BioIdea-Iran) at 37°C and 5% CO_2 to isolate monocytes in a T-75 cell culture flask.

Activation and expansion of T lymphocytes

Monocyte-depleted PBMC was centrifuged at 500 × g for 5 min and lymphocytes were collected and suspended in complete RPMI-1640 medium containing 1 µg/ml PHA (Sigma-Aldrich-USA), then cells were incubated for 24 h at 37°C and 5% CO_2. The cells were incubated with anti-CD3 antibody (BD Bioscience-USA) at 3.3 µg/mL final concentration for 48 h to isolate untouched mature T cells.

Isolated T cells seeded in a $0.5 × 10^3$ cells/mL cell concentration in 24 well cell culture plate and then treated with different concentrations of exogenously added IL-2 (Sigma-Aldrich-USA) (100, 200 and 300 IU/mL), PHA (0.5, 1.5 and 2 µg/mL), and insulin (Sigma-Aldrich-USA) (1 µg/mL, 2 µg/mL and 3 µg/mL) in separate triplicate groups for 7 days. From the 3rd day onward, culture media was replaced with the aforementioned-supplemented culture media every 2–3 days.

The cells were also treated using a combination of different concentrations of these reagents to examine their combined effect. From day 2, the cells were counted daily, and cell viability was assessed by Trypan Blue (Gibco, Invitrogen, CA) staining. Cell morphology and T-cell cluster formation were examined by phase-contrast microscopy.

After 7 days of treatment, MTT assay was performed to determine the cell toxicity in each treated group compared to untreated cells as a control group.

For this purpose, MTT (3-[4,5-dimethylthiazolyl -2]-2,5-diphenyl2H-tetrazolium bromide, Sigma-Aldrich, 5 mg MTT/ml phosphate buffered saline [PBS]) solution (10 µL/well) was added to the cells and incubated for 4 h in a 37°C incubator protected from light, giving rise to insoluble (purple) formazan crystals in living cells. Next, 100 µL/well of dimethylsulfoxide was added to solubilize the crystals. Absorbance (570 nm with 650 nm background correction) was measured using Microplate Reader (Eppendorf, Germany). All experiments were performed in triplicate. The cell viability (%) was calculated using the following equation:

$$Cell\ viability\,(\%) = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ control\ cells} \times 100$$

Cell proliferation assay using quantitative real-time reverse transcription polymerase chain reaction

Total RNA isolation from $1 × 10^6$ cells was performed by RNA extraction kit according to the manufacturer's instructions (Yektatajhiz-Iran) and RNA was eluted with 100 µl elution buffer and then stored at −80°C until use. The concentration of total RNA was assessed using a spectrophotometer and the quality was checked by agarose gel electrophoresis. First-strand cDNA synthesis was carried out following the manufacturer's protocol (Biofact-south Korea). Quantitative real-time polymerase chain reaction (RT-PCR) reaction was performed to evaluate the expression level of cell proliferation marker

Ki-67 and β-actin housekeeping gene using the following specific primers (designed by Primer3 and BLAST): Ki-67 forward, 5'-TCTGACCCTGATGAGAAAGCTC-3', Ki-67 reverse, 5'-TTGAGTCATCTGCGGTACTG-3' and β-actin forward, 5'-ATGTGTGACGAAGAAGCAT-CAGCC-3', and β-actin reverse, 5'-TCATCCCAGTTGGTGATAATGCCG-3'.

All qRT-PCR reactions were prepared in triplicate by mixing 10 μL of SYBR green master mix (Ampliqon kit, Denmark), 0.5 μL of each primer (10 μM), 2 μL of synthesized cDNA, and 7 μL RNase free water. ABI Applied Biosystems™ Thermal cycler (Thermo Scientific, USA) was used with the cycling conditions comprised an initial 10 min incubation at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method.

Flow cytometry
The composition of CD8+ and CD4+ cells in isolated T cells was evaluated by flow cytometry. The fluorochrome-conjugated mouse anti-human antibodies: fluorescein isothiocyanate-CD8 and phycoerythrin-CD4 were purchased from BioLegend, Inc. (San Diego, CA, USA). For surface staining, cells were washed once with PBS, incubation was conducted with antibodies in cell staining buffer (3% FBS in PBS) in dark for 25 min at 4°C. Appropriates isotype antibodies were used as negative controls. Cell fluorescence analyzed by the flow cytometry with a BD flow cytometer (BD Bioscience, USA). FlowJo (version 10, TreeStar) software was used for flow cytometric data analysis.

Statistical analysis
Statistical analyzes were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Results are shown as mean ± standard deviation. Comparison of results was carried out using the two-tailed unpaired *t*-test and one-way analysis of variance. *P* < 0.05 was considered statistically significant.
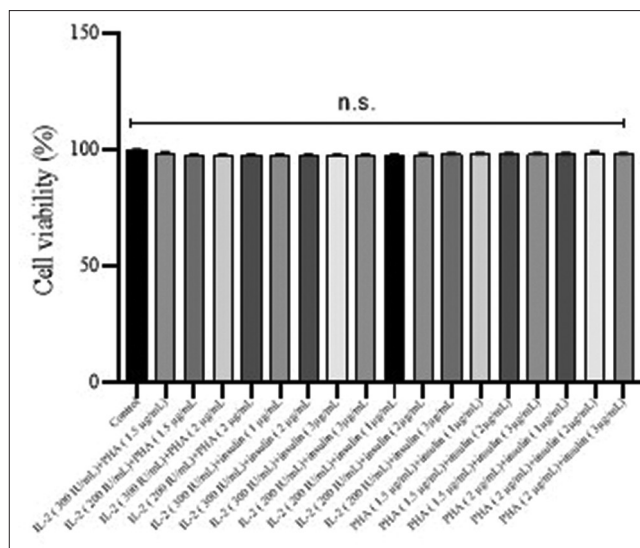
RESULTS

Cell viability assay
To quantify the viability of T cells in different culture conditions MTT assay was performed and the results indicated no significant difference in cell viability among the different groups (all data not shown) [Figure 1].

Expansion of T cells by combination of optimized concentration of interleukin-2 and phytohemagglutinin
We compared the effect of different concentrations of insulin, PHA, and IL-2 for their ability on *in vitro* T cell expansion in 7 days. The cells treated with insulin showed a low expansion rate (total fold expansion range from 0.58 ± 0.3413 to 6.2 ± 0.1794) after 7 days [Figure 2a].

In contrast, T cells expanded more when PHA and IL-2 were used separately or in combination [Figure 2b and c]. Isolated T cells showed the most prominent expansion rate with 200 and 300 IU/mL of IL-2 in combination with 1.5 μg/ml of PHA.

Figure 1: MTT assay results show no significant difference in cell viability between different groups comparing to the untreated cells (negative control; *P* > 0.05). The mean values of the cell viability of two independent experiments are plotted with mean ± standard deviation. ns: Not significant

As shown in Figure 2d, the total fold expansion of T cells in these groups was 176 ± 0.8613 and 592 ± 0.0321, respectively.

As shown in Figure 3, the addition of 300 IU/mL of IL-2 and 1.5 μg/ml of PHA to culture media efficiently activates T cells and enhances T-cell clusters formation.

Relative quantitation of proliferation marker
We evaluate the expression of proliferation marker, Ki-67 at day 7, on cultured T cells by quantitative RT-PCR. The mean value of Ki-67 relative quantitation is illustrated in Figure 4. Significantly, the level of Ki-67 mRNA expression was elevated to 79.17 ± 0.1202 and 89.16 ± 0.1732 fold in the cells expanded with IL-2 (200 IU/mL) + 1.5 μg/ml of PHA and IL-2 (300 IU/mL) + 1.5 μg/ml of PHA, respectively, compared to the untreated cells (**P* < 0.001 and ***P* < 0.001).

These results indicated that the addition of 300 IU/mL of IL-2 and 1.5 μg/ml of PHA to culture media could expand T cells effectively in a short time without any cytotoxic effect.

Phenotype of expanded T cells
Recent studies have shown that a combination of CD8+ and CD4+ T cell subpopulations can improve the efficacy of adoptive cell therapy, particularly in chimeric antigen receptors (CAR) T cell therapy. To investigate the effect of this short *in vitro* expansion time on the phenotype of expanded T cells, we examined the composition of CD8+ and CD4+ T cell subpopulations in isolated T cells. The expanded cell population possessed similar phenotypes and both CD4+ and CD8+ cells were detected under the optimized conditions. The mean percentage of CD4+ and CD8+ cells at day 7 were 24.96% ± 1.812% and 74.94% ± 2.319%, respectively [Table 1].
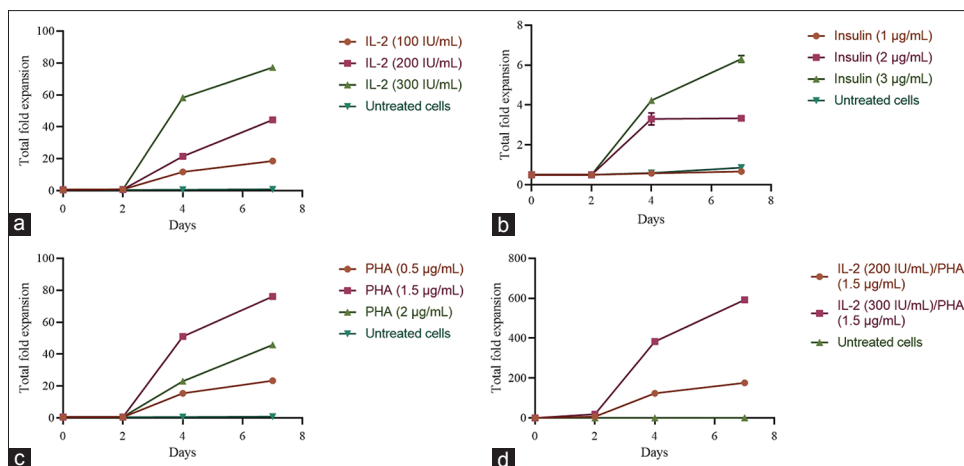
Figure 2: Optimization of culture conditions for T cells expansion. Aiming to optimize the culture condition for sufficient T cell expansion, isolated T cells were treated with different mitogen reagents for 7 days (a-d). Cell counts were determined to reflect the efficiency of T cell expansion in different culture conditions and the highest total fold expansion was observed with combination of 300 IU/mL of interleukin-2 and 1.5 μg/ml of phytohemagglutinin. Data are expressed as mean ± standard deviation ($n = 3$)
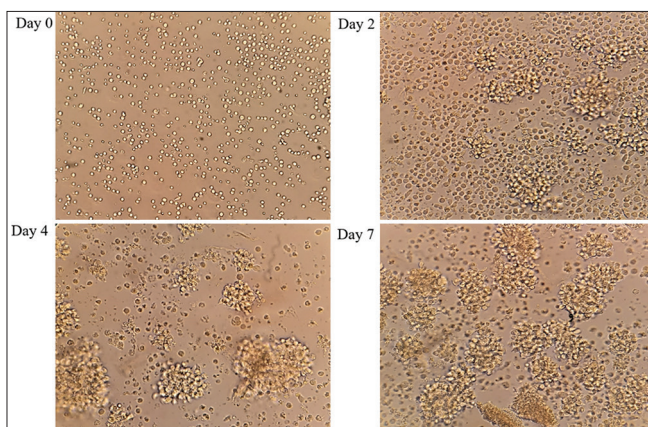
Figure 3: T-cell clusters formation. T cells effectively activated with addition of 300 IU/mL of interleukin-2 and 1.5 μg/ml of phytohemagglutinin to culture media and T-cell clusters were formed on day 2 (×400)

Table 1: Phenotype of expanded T cells in optimized culture media on day 7

Donor	CD4+	CD8+
1	23.7	75.2
2	26.2	76.8
3	24.5	73.5
4	23	77.4
5	27.4	71.8
Mean±SD	24.96±1.812	74.94±2.319

SD: Standard deviation

DISCUSSION

There have been remarkable recent advances in adoptive immunotherapy based on *ex vivo* expanded autologous or antigen-specific T cells, in particular, gene-modified T-cells expressing CARs, to treat advanced cancers.[17] ACT is a rapidly progressing arena and there is evidence for the potential of this approach as an influential treatment strategy for cancer. Since this type of treatment requires a large number of expanded T cells for re-infusion to the patient, over the last decade, the multi-step process for T-cell therapies has been refined to decrease the time of expansion and improve the quality of isolated cells.[18] The optimized protocol for the large-scale expansion of patient-derived T cells is essential to facilitate the possibilities of the therapeutic potential of ACT.[19,20] In addition, the immune-phenotype of isolated cells influences the effectiveness of treatment, and the expanded cells must be able to fight against the tumor cells,[10,21,22] so the optimization of expansion procedure to obtain high yield

while retaining the desired phenotype and function is an emerging need in T cell-based therapies. In clinical studies and commercial procedures of CAR T cells, the CD3+ T cell population is isolated from the PBMC population by anti-CD3 monoclonal antibody. This is a fast, low cost, and simple strategy in producing CAR T cells.[23]

In this report, we demonstrate that the addition of PHA and IL-2 to the cell culture medium after CD3+ T cell isolation can improve T cell growth and decrease the time of expansion. According to our results, adding insulin for 7 days had no significant effect on cell proliferation and this may be caused by the lack of insulin receptor expression on T cells. However, significant up-regulation of insulin receptor expression is observed on activated T cells, which suggests an important role of insulin signaling during T cell activation.[15] Therefore, although insulin is a suitable mitogen, its use in the expansion step does not seem to have much effect.

Furthermore, insulin in combination with PHA and IL-2 did not have a significant effect on the rate of cell proliferation induced by these reagents. Therefore, cells treated with different doses of insulin were excluded from our study.

Isolated T cells showed the most prominent expansion rate with optimized culture media. Similar results previously reported
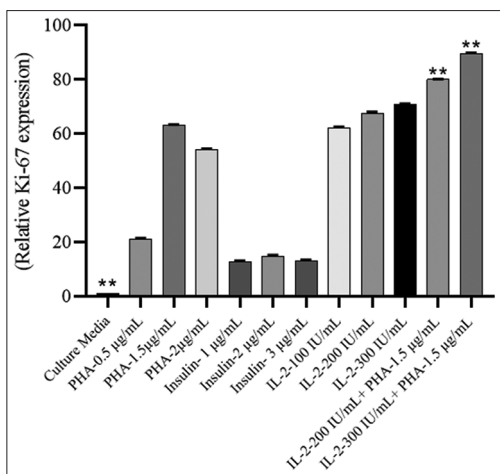
Figure 4: Quantitative real-time polymerase chain reaction results. The Ki-67 expression level was significantly increased in T cells when a combination of 300 IU/mL of interleukin-2 and 1.5 μg/ml of phytohemagglutinin was added in culture media as illustrated in the graph. The results are presented as the means ± standard deviation (*n* = 3). **$P < 0.01$)

T cell expansion in the Quantum system up to 500-fold. The Quantum® Cell Expansion System is a functionally-closed, automated, hollow-fiber bioreactor system that is able to expand T cells.[24]

Another study demonstrated 1300 mean fold expansions of T cells using the Quantum system. Given that this system conforms to the Good Manufacturing Practice, its effectiveness may be more appropriate in clinical use, however, it requires a special environment, technique, and tools.[25]

Recent studies showed that the evaluation of proliferation marker at mRNA level would provide more defined information about the proliferation state of cells.[26,27] Follow these observations, we conducted a quantitative assessment of Ki-67 as a proliferation marker using quantitative RT-PCR. Significantly, the level of Ki-67 mRNA expression was elevated in comparison with untreated cells.

On the other hand, given that studies have shown that the use of T cell populations composed of CD4+ and CD8+ cells has a synergistic effect on the response against tumor cells, optimal methods of T cell expansion should ultimately lead to the proper composition of these cells.[28]

The phenotypic composition obtained in our study is appropriate for the construction of chimeric antigen T cells using CD3+ T cell population, although, may not be suitable for other purposes. On the other hand, preclinical animal models have confirmed that memory T cells are pivotal for *in vivo* anti-tumor efficacy. Kaartinen *et al*. shown that the number of early memory T cells can be increased by reducing the amount of IL-2 as well as limiting the length of T cell expansion.[29] Since the memory phenotype in expanded cells has not been considered, it is necessary to determine the proportion of these cells in the population of expanded cell composition in future studies.

Recent work has shown that IL-2 can activate a distinctive pattern of the signaling pathway in the antigen-independent manner, which associated with strong lymphocyte-specific protein tyrosine kinase/JAK3-dependent activation of the PI3K/AKT pathway. These signaling pathways induce rapid proliferation of IL-2 activated naïve CD8+ T cells and upregulate expression of eomesodermin (Eomes), signifying the differentiation fate of primary naïve effectors into long-lived memory cells. In addition, results from previous studies indicated that IL-2–stimulated CD8 cells showed strong effector function after adoptive transfer *in vivo*.[30,31]

CONCLUSIONS

Overall, we report on our efforts to optimize the T cell expansion protocol with regard to increase the total expansion fold and activation in the shortest time. We also identified the phenotype of the cell population to determine the CD8+ and CD4+ composition of expanded T cells.

The power of native and genetically engineered T lymphocytes to mediate cancer regression in patients is a promising new approach to cancer treatment. The results from this study show that the optimization of culture media can be used as a fast, low cost, and effective method in producing cell population required in ACT studies.

Consent to participate
Informed consent was obtained from all individual participants included in the study.

Conflicts of interest
There are no conflicts of interest.

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