Optimization of Culture Media for T Cell Expansion: T Cell Expansion Is a Bottle Neck in Adoptive T Cell Therapy

Ilnaz Rahimmanesh

Isfahan University of Medical Sciences

Hossein Khanahmad (✉ h_khanahmad@med.mui.ac.ir)

Isfahan University of Medical Sciences <https://orcid.org/0000-0002-5573-3557>

Research Article

Keywords: Interleukin-2, T-cell expansion, Phytohemagglutinin, Immunotherapy

Posted Date: April 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-456813/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Adoptive T cell therapy is a promising treatment strategy for cancer immunotherapy. The methods used for the expansion of high numbers of T cells are essential steps for adoptive cell therapy. In this study, we evaluated the expansion, proliferation, activation, and anti-tumor response of T lymphocytes, in presence of different concentrations of interleukin-2, phytohemagglutinin, and insulin. Our results showed that supplemented culture media with an optimized concentration of phytohemagglutinin and interleukin-2 increased total fold expansion of T cells up to 500-fold with about 90% cell viability over 7 days. The quantitative assessment of Ki-67 in expanded T cells showed a significant elevation of this proliferation marker. In addition, the proportion of CD4+ and CD8+ cells were evaluated using flow cytometry, and data showed that both cells were present in the expanded population. Finally, we assessed the activation and tumor cytotoxicity of expanded T cells against target cells. Overexpression of CD107a, as a functional marker of T cell degranulation on expanded T cells and their ability to induce cell death in tumor cells, was observed in the co-cultured experiment. Based on these data we have developed a cost-effective and rapid method to support the efficient expansion of T cells for adoptive cell therapy.

Highlight

Adoptive T cell therapy is a promising treatment strategy for cancer immunotherapy

T cell expansion is a crucial step for adoptive T cell therapy

Optimization a rapid and cost-effective method to achieve a large number of T cells is an emerging need for adoptive T cell therapy

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, 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. In order to obtain an adequate and sufficient number of T lymphocytes for infusion, *ex-vivo* culture, expansion and activation of T 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 pre-infusion [4, 5].

Large-scale production of human T cells is a complex process that required fine-tune optimization in order to obtain long-term *in-vivo* survival of T lymphocytes with desired phenotype and function [6].

Studies in cancer-targeting T cell therapy have often focused on 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 (TM) 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 [7, 8].

In recent decades, many efforts have been made to optimize isolation and non-antigen specific expansion of cytotoxic T cells for adoptive T cell therapy. 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 [9, 10]. 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 [11, 12].

Mitogenic molecules such as phytohemagglutinin (PHA), interleukin-2, and concanavalin A (Con A) are used widely for *in vitro* T lymphocyte stimulation and expansion [13–15]. On the other hand, the mitogenic properties of insulin, insulin receptor signaling, and insulin analogues were documented and insulin has been shown to stimulate *in vitro* T cell proliferation when supplied exogenously [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 anti-tumor activity for adoptive cell therapy in cancer research.

Methods

Isolation of PBMC and T-cell culture

Informed consent was obtained from five healthy donors prior to 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). Peripheral blood mononuclear cells were isolated from heparinized blood using the Ficoll density gradient. Cells were incubated for 24 hours in complete RPMI-1640 medium (Bioldea-Iran) containing 10% FBS (Bioldea-Iran) at 37 ° C and 5% CO₂ in order to isolate monocytes in a T-75 cell culture flask.

Activation and expansion of T lymphocytes

Monocyte-depleted PBMC were centrifuged at 500 × g for 5 minutes and lymphocytes were collected and suspended in complete RPMI-1640 medium containing 1 µg / ml phytohemagglutinin (Sigma-Aldrich-

USA), then cells were incubated for 24 h at 37 ° C and 5% CO₂. The cells were incubated with anti-CD3 antibody (BD Bioscience-USA) at 3.3 µg/mL final concentration for 48 h in order to isolate untouched mature T cells.

Isolated T cells seeded in a 0.5×10³ 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 IU/mL, 200 IU/mL and 300 IU/mL), phytohemagglutinin (0.5 µg/mL, 1.5 µg/mL 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 third 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 for 7 days after treatment in different groups.

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-diphenyl-2H-tetrazolium bromide, Sigma-Aldrich, 5mg MTT/ml 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 (DMSO) 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:

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

Cell proliferation assay using Quantitative real-time RT-PCR

Total RNA isolation from 1×10⁶ 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 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: 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 (FITC)-CD8 and phycoerythrin (PE)-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. Appropriate 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.

Cytotoxicity assay

To evaluate the antitumor activity of expanded T lymphocytes, MCF-7 breast cancer cell lines as target cells were co-cultured with isolated T lymphocytes in different effector-target (E:T) ratio of 1:1, 3:1, 5:1, 10:1, in 96-well cell culture plate in triplicates and incubated at 37 °C and 5% CO₂ for 24 hours.

A quantitative assessment of apoptosis was performed using FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Briefly, T lymphocytes were removed from the co-culture medium, and the supernatant was collected for further assays. Target cells were detached using 0.025% trypsin, washed twice with cold PBS, and resuspended in binding buffer. Cell staining was performed according to the manufacturer's instructions. Cell fluorescence immediately analyzed by the flow cytometry with a BD flow cytometer (BD Bioscience, USA) by accumulating up to 100,000 cells per tube and the obtained data were analyzed. The percentage cytotoxicity in the MCF-7 cells was measured in appropriate control without effector cells. Cellular debris was omitted from the analysis. In addition, MTT assay was performed to confirm the cytotoxicity power of expanded T cells.

Evaluation of T-cell degranulation

The expression level of lysosomal-associated membrane protein-1 (LAMP-1 or CD107a), as a sensitive marker for the cytotoxic activity and T-cell degranulation following stimulation, was accessed using flow cytometry. For this purpose, expanded T cells co-cultured with MCF-7 cells as target cells in 10:1 E/T ratio for 4 h. After incubation, T lymphocytes were removed from the co-culture medium and stained with PECy5-conjugated anti-CD107a (BD Bioscience, San Jose, CA) and cell fluorescence was measured by flow cytometry. FlowJo (version 10, TreeStar) software was used for flow cytometric data analysis.

Also, monocyte-depleted untreated PBMC were co-cultured with target cells in the same E:T ratio as control.

Statistical analysis

Statistical analyzes were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Results are shown as mean $\pm$ SD. Comparison of results was carried out using the two-tailed unpaired t – test and one-way ANOVA. $P < 0.05$ 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 amongst the different groups (all data not shown) (Fig. 1).

Expansion of T cells by combination of optimized concentration of IL-2 and PHA

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 (Fig 2.a).

In contrast, T cells expanded higher using PHA and IL-2 independent or in combination with each other (Fig 2.b, c). Isolated T cells showed the most prominent expansion rate with 200 and 300 IU/mL of IL-2 in combination with 1.5 μ g/ml of PHA. As shown in figure 2.d, 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 μ g/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 illustrated in Fig. 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 μ g/ml of PHA and IL-2 (300 IU/mL) + 1.5 μ g/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 μ g/ml of PHA to culture media could expand T cells effectively in a short time without any cytotoxic effect. For this reason, the aforementioned culture condition was chosen to expand T cells for further analysis in this study.

Phenotype of expanded T cells

Recent studies have shown that a combination of CD8+ and CD4+ T cell subpopulations can increase the effectiveness of adoptive cell therapy, particularly in CAR T cell therapy. According to this, we examined the composition of CD8+ and CD4+ T cell subpopulations in the isolated T cell to investigate the effect of this short *in vitro* expansion time on the phenotype of expanded T cells.

The expanded cell population possessed similar phenotypes and both CD4+ and CD8+ cells were detected under the optimized conditions and the mean percentage of CD4+ and CD8+ cells at day 7 were $24.96 \pm 1.812\%$ and $74.94 \pm 2.319\%$ respectively (Table.1).

Cytotoxic activity of isolated T cells

Annexin V/PI double staining and MTT assay were performed to analyze the impact of optimized culture media on the functional characteristics of the expanded T cells.

The cytotoxic activity was assessed against MCF-7 breast cancer target cells by flow cytometry and the induced apoptosis was analyzed in MCF-7 cells as shown in Fig. 5.A.

The percentages of apoptotic cells in case of co-culture of expanded T cells in 300 IU/mL of IL-2 and 1.5 µg/ml of PHA containing media with MCF-7 in 10:1 E/T ratio was found to be 68.5% while, no significant apoptosis was observed in control cells (0.28%). These results were well correlated with MTT data (Fig. 5.B).

CD107a Degranulation Assay

Degranulation assay detecting surface expression of CD107a as a marker for stimulation-induced granule exocytosis was performed to evaluate the degranulation and cytotoxic power of expanded T cells after the co-culture experiment. The percentages of CD107a positive cells were 75.3% in compare to monocyte-depleted untreated PBMC (0.19%). As flow cytometry histograms show in figure 6, target cell co-incubation led to a high CD107a expression in the T cell and expanded T cells can effectively stimulate in the presence of tumor cells.

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 chimeric antigen receptors (CARs), to treat advanced cancers [17]. Adoptive T cell therapy 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 adoptive T cell therapy [19]. 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 [11, 20, 21], so the optimization of expansion procedure in order 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 peripheral blood mononuclear cell population by anti-CD3 monoclonal antibody. This is a fast, low cost and simple strategy in producing CAR T cells [3].

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 [16]. Therefore, although insulin is a suitable mitogen its use in the expansion step does not seem to have much effect.

Also, 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 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 [22].

Another study demonstrated 1300 mean fold expansions of T cells using the Quantum system. Given that this system conforms to the Good Manufacturing Practice (GMP), its effectiveness may be more appropriate in clinical use, however, it requires a special environment, technique, and tools [23].

Recent studies showed that the evaluation of proliferation marker at mRNA level would provide more defined information about the proliferation state of cells [24, 25]. Follow these observations, we conducted a quantitative assessment of Ki-67 as a proliferation marker using quantitative real-time 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 [26].

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, pre-clinical animal models have confirmed that memory T cells are pivotal for *in-vivo* anti-tumor efficacy. Kaartinen and *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 [27]. 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.

In addition, the anti-tumor efficiency of expanded T cell population was assessed using a co-cultured experiment. Our results showed that isolated cells have the ability to induce cell death in tumor cells when expanded T cells exposed to them at 10:1 E/T ratio. This antitumor activity of the expanded cells was confirmed by increasing the effector cell activation marker, CD107a.

Our results are confirmed by recent studies that have shown the effect of IL-2 on T cell cytotoxic function [28, 29], but the effect of this substance in combination with PHA on cell proliferation has not been determined. 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].

Conclusion

Overall, we report on our efforts to optimize the T cell expansion protocol with regard to increase the total expansion fold 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 activation and tumor cytotoxicity of expanded T cells against target cells show that the optimized method in this study can be used as a fast, low cost, and effective method in producing cell population required in adoptive T cell therapy studies.

Declarations

Funding: This study was financially supported by grant No 198242 of the Isfahan University of Medical Science and Grant No. 970703 by the Biotechnology Development Council of the Islamic Republic of Iran and grant No. 970703 by the Biotechnology Development Council of the Islamic Republic of Iran.

Conflicts of interest: The authors declare that no conflict of interest for this study.

Ethics approval: This study and the use of human material were approved (IR.MUI.RESEARCH.REC.1398.788) by the institutional review boards at the Isfahan University of Medical science.

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

Consent for publication: Not applicable

Availability of data and material: Not applicable

Code availability: Not applicable

Author contribution statements: H.K and I.R conceived of the presented idea. I.R. developed the theoretical framework; I.R carried out the experiment and analyzed the data; I.R and H.K wrote the article. H.K revised final version of article.

References

1. Rasmussen A-M, Borelli G, Hoel HJ, Lisperud K, Gaudernack G, Kvalheim G, Aarvak T (2010) Ex vivo expansion protocol for human tumor specific T cells for adoptive T cell therapy. *J Immunol Methods* 355:52–60
2. Lewis MD, de Leenheer E, Fishman S, Siew LK, Gross G, Wong FS (2015) A reproducible method for the expansion of mouse CD8 + T lymphocytes. *J Immunol Methods* 417:134–138
3. Trickett A, Kwan YL (2003) T cell stimulation and expansion using anti-CD3/CD28 beads. *J Immunol Methods* 275:251–255
4. Gajewski TF, Schreiber H, Fu Y-X (2013) Innate and adaptive immune cells in the tumor microenvironment. *Nature immunology* 14:1014–1022
5. Maus MV, Fraietta JA, Levine BL, Kalos M, Zhao Y, June CH (2014) Adoptive immunotherapy for cancer or viruses. *Annu Rev Immunol* 32:189–225
6. Gatenby RA, Silva AS, Gillies RJ, Frieden BR (2009) Adaptive therapy. *Cancer research* 69:4894–4903
7. Ho P-C, Meeth KM, Tsui Y-C, Srivastava B, Bosenberg MW, Kaech SM (2014) Immune-based antitumor effects of BRAF inhibitors rely on signaling by CD40L and IFN γ . *Cancer research* 74:3205–3217
8. Bos R, Sherman LA (2010) CD4 + T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8 + T lymphocytes. *Cancer research* 70:8368–8377
9. Zhang DK, Cheung AS, Mooney DJ (2020) Activation and expansion of human T cells using artificial antigen-presenting cell scaffolds. *Nat Protoc* 15:773–798
10. Thomas AK, Maus MV, Shalaby WS, June CH, Riley JL (2002) A cell-based artificial antigen-presenting cell coated with anti-CD3 and CD28 antibodies enables rapid expansion and long-term growth of CD4 T lymphocytes. *Clinical Immunology* 105:259–272
11. Sato K, Kondo M, Sakuta K, Hosoi A, Noji S, Sugiura M, Yoshida Y, Kakimi K (2009) Impact of culture medium on the expansion of T cells for immunotherapy. *Cytotherapy* 11:936–946

12. Chakraborty NG, Li L, Sporn JR, Kurtzman SH, Ergin M, Mukherji B (1999) Emergence of regulatory CD4 + T cell response to repetitive stimulation with antigen-presenting cells in vitro: implications in designing antigen-presenting cell-based tumor vaccines. *J Immunol* 162:5576–5583
13. Naota H, Miyahara Y, Okumura S, Kuzushima K, Akatsuka Y, Hiasa A, Kitano S, Takahashi T, Yuta A, Majima Y (2006) Generation of peptide-specific CD8 + T cells by phytohemagglutinin-stimulated antigen-mRNA-transduced CD4 + T cells. *J Immunol Methods* 314:54–66
14. Zamani A, Vahidinia A, Ghannad MS (2009) The effect of garlic consumption on Th1/Th2 cytokines in phytohemagglutinin (PHA) activated rat spleen lymphocytes. *Phytotherapy Research: An International Journal Devoted to Pharmacological Toxicological Evaluation of Natural Product Derivatives* 23:579–581
15. Oppenheim JJ (2007) IL-2: more than a T cell growth factor. *J Immunol* 179:1413–1414
16. Fischer HJ, Sie C, Schumann E, Witte A-K, Dressel R, van den Brandt J, Reichardt HM (2017) The insulin receptor plays a critical role in T cell function and adaptive immunity. *J Immunol* 198:1910–1920
17. Ti D, Bai M, Li X, Wei J, Chen D, Wu Z, Wang Y, Han W (2020) Adaptive T cell immunotherapy in cancer. *Science China Life Sciences*:1–9
18. Torabi-Rahvar M, Aghayan HR, Ahmadbeigi N (2020) Antigen-independent killer cells prepared for adoptive immunotherapy: One source, divergent protocols, diverse nomenclature. *J Immunol Methods* 477:112690. doi:10.1016/j.jim.2019.112690
19. Disis ML, Bernhard H, Jaffee EM (2009) Use of tumour-responsive T cells as cancer treatment. *The Lancet* 373:673–683
20. Kondo M, Sakuta K, Noguchi A, Ariyoshi N, Sato K, Sato S, Hosoi A, Nakajima J, Yoshida Y, Shiraishi K (2008) Zoledronate facilitates large-scale ex vivo expansion of functional $\gamma\delta$ T cells from cancer patients for use in adoptive immunotherapy. *Cytotherapy* 10:842–856
21. Yannelli JR (1991) The preparation of effector cells for use in the adoptive cellular immunotherapy of human cancer. *J Immunol Methods* 139:1–16. doi:10.1016/0022-1759(91)90345-g
22. Nankervis B, Jones M, Vang B, Brent Rice R, Coeshott C, Beltzer J (2018) Optimizing T Cell Expansion in a Hollow-Fiber Bioreactor. *Current Stem Cell Reports* 4:46–51. doi:10.1007/s40778-018-0116-x
23. Coeshott C, Vang B, Jones M, Nankervis B (2019) Large-scale expansion and characterization of CD3 + T-cells in the Quantum® Cell Expansion System. *Journal of translational medicine* 17:258
24. Brizova H, Kalinova M, Krskova L, Mrhalova M, Kodet R (2010) A novel quantitative PCR of proliferation markers (Ki-67, topoisomerase II α , and TPX2): an immunohistochemical correlation, testing, and optimizing for mantle cell lymphoma. *Virchows Arch* 456:671–679
25. Sinn H-P, Schneeweiss A, Keller M, Schlombs K, Laible M, Seitz J, Lakis S, Veltrup E, Altevogt P, Eidt S (2017) Comparison of immunohistochemistry with PCR for assessment of ER, PR, and Ki-67 and prediction of pathological complete response in breast cancer. *BMC Cancer* 17:1–10
26. Dudley ME, Rosenberg SA (2003) Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat Rev Cancer* 3:666–675

27. Kaartinen T, Luostarinen A, Maliniemi P, Keto J, Arvas M, Belt H, Koponen J, Loskog A, Mustjoki S, Porkka K (2017) Low interleukin-2 concentration favors generation of early memory T cells over effector phenotypes during chimeric antigen receptor T-cell expansion. *Cytotherapy* 19:689–702
28. Choudhry H, Helmi N, Abdulaal WH, Zeyadi M, Zamzami MA, Wu W, Mahmoud MM, Warsi MK, Rasool M, Jamal MS (2018) Prospects of IL-2 in cancer immunotherapy. *BioMed Research International* 2018
29. Malek TR (2003) The main function of IL-2 is to promote the development of T regulatory cells. *J Leukoc Biol* 74:961–965
30. Cho J-H, Kim H-O, Kim K-S, Yang D-H, Surh CD, Sprent J (2013) Unique features of naive CD8 + T cell activation by IL-2. *J Immunol* 191:5559–5573
31. Voisinne G, Nixon BG, Melbinger A, Gasteiger G, Vergassola M, Altan-Bonnet G (2015) T cells integrate local and global cues to discriminate between structurally similar antigens. *Cell reports* 11:1208–1219

Table

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

Figures

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 $\pm$ SD and n.s.:not significant.

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 IL-2 and 1.5 µg/ml of PHA. Data are expressed as mean $\pm$ SD (n=3).

Figure 3

T-cell clusters formation. T cells effectively activated with addition of 300 IU/mL of IL-2 and 1.5 µg/ml of PHA to culture media and T-cell clusters were formed on day 2 (Magnification × 400).

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 IL-2 and 1.5 µg/ml of PHA was added in culture media as illustrated in the graph. The results are presented as the means $\pm$ SD, n = 3 (*P < 0.001).

Figure 5

(A) Annexin V-FITC/PI apoptosis assay carried out on MCF-7 cells treated with different ratio of effector to target cells. The percentage of apoptotic cells were a) 0.28% in control group, b) 3.56% in 1:1 E/T, c) 12.8% in 3:1 E/T, d) 35.8% in 5:1 E/T and 68.5% in 10:1 E/T (B) MTT assay results confirmed the cytotoxic activities of expanded T cells. All data were represented as mean $\pm$ SD from three independent experiments. All experiments were done in triplicates (n = 3).

Figure 6

Flowcytometry analysis of CD107a expression on target cell co-cultured expanded T cells. Degranulation assay was conducted using flow cytometry and degranulation marker CD107a was released by expanded

T cells in the presence of MCF-7 cells as target cells. a) CD107a expression on co-cultured monocyte-depleted untreated PBMC (0.19%) b) CD107a expression on co-cultured expanded T cells.