

# The Impact of Different Cell Culture Mediums on CD8<sup>+</sup> T Cells Expansion: A Bioinformatics Study

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## Abstract

**Objective:** Different Cell Culture medias can affect the expansion of T cells. The aim of this study is to assess signaling pathways, protein interactions and genes in T cells cultured in different common T cell expansion medias to select the best candidate.

**Materials and Methods:** In this in silico observational study, with the use of bioinformatics analysis and the use of enrichment databases, gene expression profiles were investigated using microarray analysis.

**Results:** The results of this study were the joint selection of 26 upregulated genes and 59 downregulated genes that were involved in SREBP control of lipid synthesis, co-stimulatory signal during T-cell activation mitosis and chromosome dynamics, telomeres, telomerase, and cellular aging signal pathways.

**Conclusion:** Using bioinformatics analyzes, integrated and regular genes were selected as common genes *CD80*, *LST1*, *ATM* and *ITM2B* in *4-1BBL*, *Akt* inhibitor, interleukin 7 and 15 expansion media.

**Keywords:** Expansion, Microarray, TCD8<sup>+</sup> Cells

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## Introduction

T cell immunotherapy is a well-known treatment method for many infectious diseases and cancers. Over the years, extensive studies and research have been done to solve this treatment method's problems and improve the quality of treatment (1). One of the subtypes of T cells, called CD8<sup>+</sup> T cells, have a lethal property against abnormal cells and by destroying them, can restore the balanced condition in immune system. This feature has made these cells as a great therapeutic tool for many clinical complications, such as post bone marrow transplantation infections with high mortality annually (2). So far, a lot of research has been done to make the best use of these cells for treatment, which unfortunately was either very expensive or they could not produce a cost effective high quality product with adequate quantity (3). Due to the importance of T-cell therapy in immunocompromised patients and the crucial role of these cells in thymus regeneration, providing the appropriate number of proliferating cells can be really vital.

Therefore, increasing the division of CD8<sup>+</sup> cells and their optimal growth quality is very important. To do this, finding

precise molecular pathways and effective mechanisms of cell culture media affecting CD8<sup>+</sup> cells can improve CD8<sup>+</sup> cells expansion. By combining different culture media a better effect on CD8<sup>+</sup> cell quality can be achieved. Various studies have been done on CD8<sup>+</sup> cells expansion in the last few years. Each of them had their own unique theories. However, the study of several culture media's molecular mechanisms on CD8<sup>+</sup> cells expansion has not yet been elucidated (4).

Due to all the aforementioned reasons, bioinformatics is a very good way to accurately identify molecular pathways, the genes involved in them, and the function and relationship between their protein products. Over the past decade, bioinformatics, particularly gene expression profile, has played a significant role in the discovery of signaling pathways which can be used to identify the relationship between CD8<sup>+</sup> cells expansion condition media and pathways affected by these media more accurately (5).

Accordingly, this study aimed to investigate the datasets correlated with T cell expansion media with each other, classification of genes and common signaling pathways between them, and finally the genes and pathways

involved in cell division, cytoskeletal dynamics, activation and stimulation of CD8<sup>+</sup> cells.

## Methods and Materials

### Gene expression profile datasets

This original study was performed based on bioinformatics analysis by Hematopoietic Stem Cell Research Center of Shahid Beheshti University of Medical Sciences and Department of Stem Cells and Developmental Biology of Royan institute. For this study, 3 datasets from the GEO database (<https://www.ncbi.nlm.nih.gov/geo>) were selected according to the purpose of this study. GSE86284 examines CD8<sup>+</sup> lymphocyte cell expansion using 4-1BBL medium. GSE41909, using interleukin 7 and 15 medium, examines lymphocyte cells in the central memory and naive cells, and GSE98078 deals with Akt inhibitor media. Figure 1 shows the schematic pathway of bioinformatics analysis.

### Preparing gene expression profile data for additional analysis

We extracted each of these data separately from GEO database and categorized the upregulated and downregulated genes in an Excel file. At this stage, P<0.05 was selected to isolate the genes. Then, upregulated and downregulated genes were separately plotted in the venny diagram, and the intersection genes were isolated and prepared for further analysis.

### Signaling pathways analysis

We entered the common genes from the four desired modes in question separately to examine the signaling pathways in the Enrichr database (<https://amp.pharm.mssm.edu/Enrichr>). Then, through Biocarta database, we categorized the signaling pathways corresponding to the genes with high and low expression. In this section, P<0.05 was selected.

## Investigating the genes ontology

To examine the three important components, namely molecular functions, biological processes, and cellular components, we again performed the necessary evaluations from the Enrichr database in the GO subset.

## Studying the correlation between proteins

In the first part of the study, we used STRING database (<https://string-db.org>) to investigate the relationship between the protein products derived from common genes, and separately examined the upregulated and downregulated genes.

## Results

Cell division, cytoskeletal dynamics, and pathways associated with activation and stimulation of T cells were more pronounced in shared expansion media.

Gene expression profile analysis of GSE86284, GSE41909 and GSE98078 datasets showed that 26 genes upregulated and 59 genes downregulated in four groups of CD8<sup>+</sup> lymphocytes in the expansion culture medium. SREBP control of lipid synthesis, RNA polymerase III transcription, co-stimulatory signal during T-cell activation, PKC-catalyzed phosphorylation, mTOR, Rho cell motility and Rac 1 cell motility signaling pathways for upregulated genes and protein kinase A at the centrosome, T cell receptor signaling, cdc25 and chk1 regulatory, TSP-1 induced apoptosis in microvascular endothelial cell, AKAP95 role in mitosis and chromosome dynamics, telomeres, telomerase, cellular aging and immortality, and IL-7 signal transduction signaling pathways for downregulated genes were identified. Figure 2 shows the selection of genes with up/downregulated genes and that were common in culture media obtained by venny diagram and investigation of signaling pathways between common genes identified based on P value and also, Table 1 shows the most important involved and intersection genes between expansion culture media based on their expression.

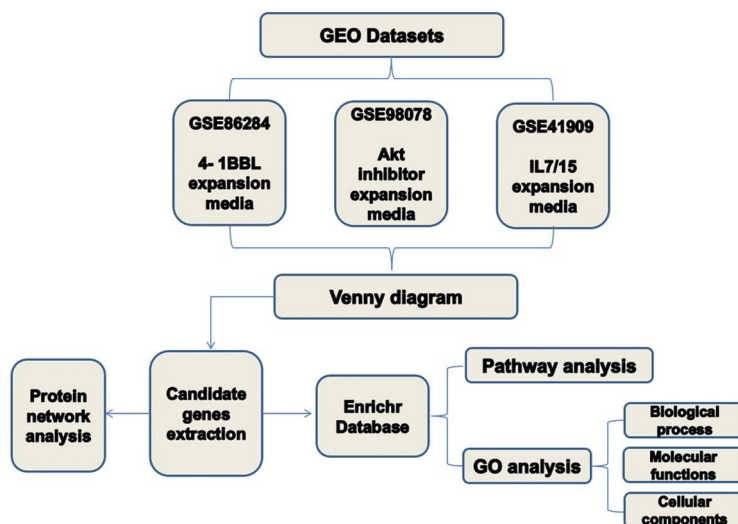
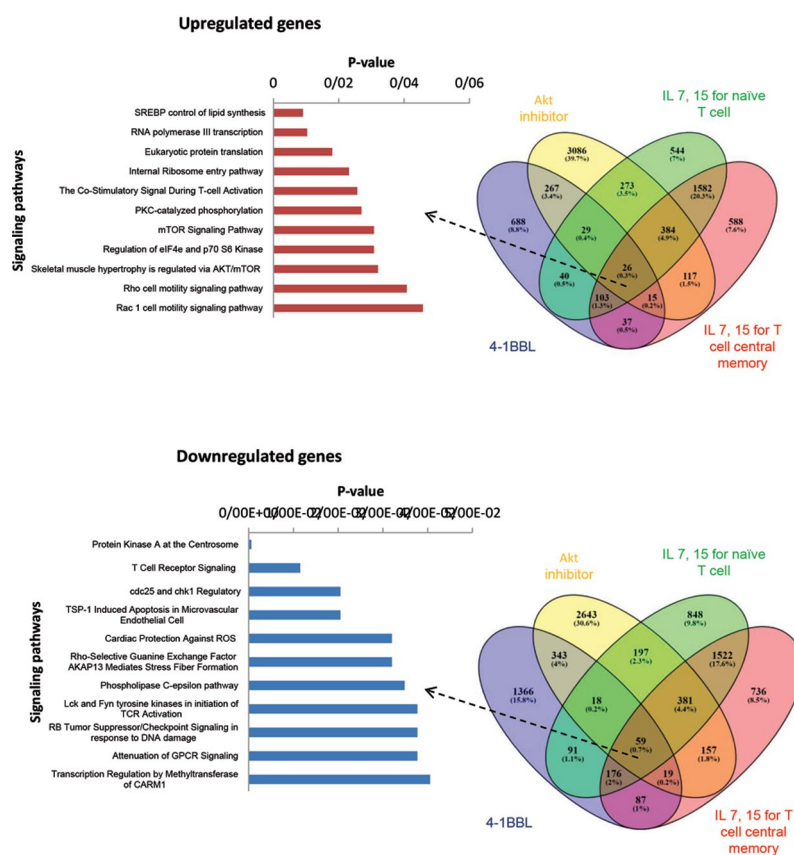


Fig.1: Schematic pathway of bioinformatics analysis.



**Fig.2:** Selection of genes with up/downregulated genes and that were common in culture media obtained by venny diagram (right). Investigation of signaling pathways between common genes identified based on P value (left).

**Table 1:** The most important involved and intersection genes between expansion culture media based on their expression

Gene symbol	Upregulated genes			
	Akt inhibitor	4-1BBL	IL 7, 15 for naïve CD8 <sup>+</sup> cells	IL 7, 15 for CD8 <sup>+</sup> central memory
<i>MBTPS2</i>	0.212	0.8626975	0.49437857	0.41288571
<i>GTF3C4</i>	0.364	0.5169238	0.55612475	0.44324705
<i>EIF4E</i>	0.195	2.0968478	0.314085	0.31697059
<i>CD80</i>	2.13	1.1257803	2.49266806	2.04126263
<i>ARHGEF5</i>	0.499	1.4043171	0.3970478	0.52683412
<i>LST1</i>	0.306	1.1870456	2.53155006	2.35809529
<i>PALLD</i>	1.12	0.853437	1.47607414	2.20898005
<i>ATP2C1</i>	0.654	0.5136197	0.48385932	0.22093148
		Downregulated genes		
<i>FYN</i>	-0.341	-0.4571853	-1.23132161	-1.42432493
<i>ATM</i>	-0.203	-0.6682416	-1.9654061	-1.50466176
<i>TNKS</i>	-0.144	-1.9316892	-0.60104772	-0.53512023
<i>USP34</i>	-0.175	-0.5955263	-0.53382035	-0.73738285
<i>TBLIX</i>	-0.375	-0.5246813	-0.74522811	-0.75279683
<i>CAPRIN2</i>	-0.269	-0.590714	-1.29518457	-0.80439361
<i>DYRK2</i>	-0.173	-0.6906726	-0.73330931	-0.86589672
<i>CLSTN1</i>	-0.2	-0.5703692	-0.38479256	-0.58479503
<i>ITM2B</i>	-0.384	-0.8688779	-0.71092999	-0.59110175

### Gene ontology analysis

This section evaluated up and downregulated genes in three parts: biological processes, molecular functions and cellular components. Negative regulation of lymphocyte proliferation (GO:0050672), negative regulation of cellular response to hypoxia (GO:1900038), release of sequestered calcium ion into cytosol by sarcoplasmic reticulum (GO:0014808), regulation of T-helper 1 cell differentiation (GO:0045625), positive regulation of interleukin-2 biosynthetic process (GO:0045086), positive regulation of T-helper 1 type immune response (GO:0002827), microtubule anchoring (GO:0034453) and positive regulation of granulocyte macrophage colony-stimulating factor production (GO:0032725) biological processes also RNA binding (GO:0003723), hydrogen-exporting ATPase activity, phosphorylative mechanism (GO:0008553), inositol trisphosphate phosphatase activity (GO:0046030), calcium-transporting ATPase activity (GO:0005388) and alpha-actinin binding (GO:0051393) molecular functions were identified in intersection of upregulated

genes. On the other hand, positive regulation of canonical Wnt signaling pathway (GO:0090263), intracellular protein transport (GO:0090316), telomere maintenance (GO:0032206) and fibroblast migration (GO:0010763), as well as biological processes and also NAD+ ADP-ribosyltransferase activity (GO:0003950), protein serine/threonine kinase activity (GO:0004674), transferase activity, transferring pentosyl groups (GO:0016763) and cAMP-dependent protein kinase activity (GO:0004691) molecular functions were observed in downregulated genes. Figure 3 shows the common genes selected from the previous step were examined in three different modes of cellular components, biological processes, and molecular functions.

### Protein- protein interactions network analysis

Examination of the protein-protein relationship revealed that there were 61 nodes and 42 edges for downregulated proteins and 125 nodes and 72 edges for upregulated proteins participated in network. Figure 4 shows the association between the upregulated proteins.

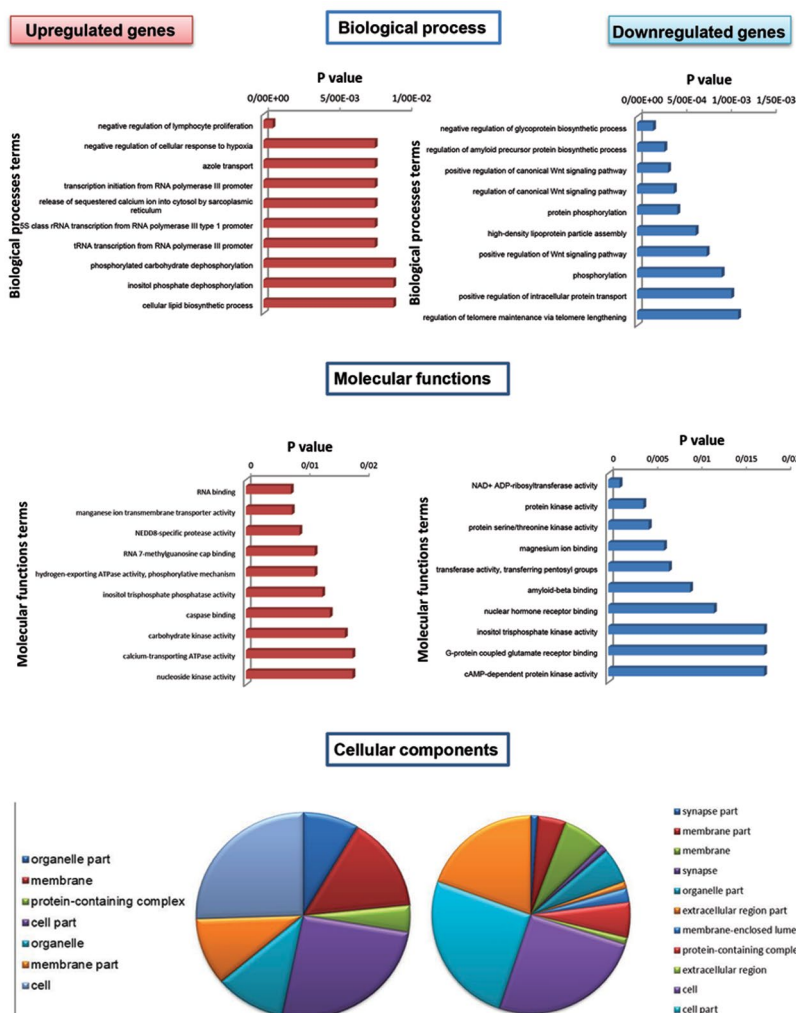
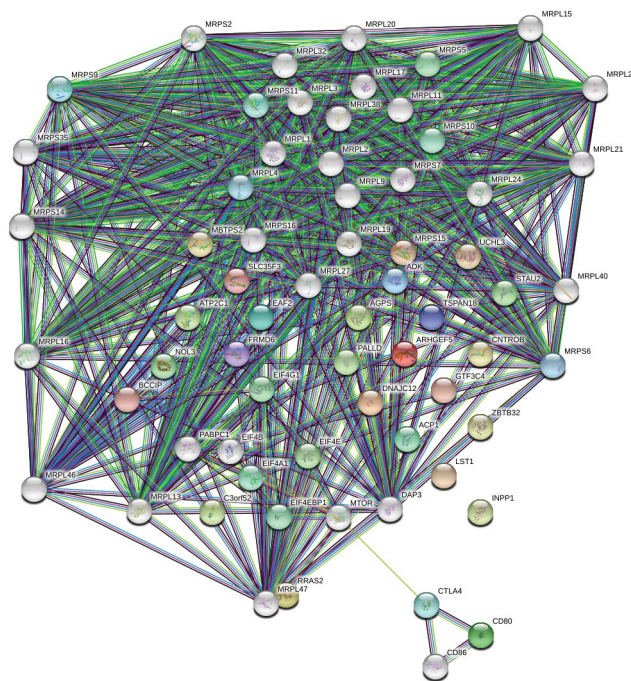


Fig.3: Common genes selected from the previous step were examined in three different modes of cellular components, biological processes, and molecular functions.



**Fig.4:** In this image, the association between the upregulated proteins was examined.

## Discussion

T cell expansion, especially CD8, division, proper and timely differentiation, and a large amount of cell memory, are of importance in various diseases related to the immune system. Finding necessary and involved regulatory elements and their role in T cell expansion can provide suitable solutions for further studies. Therefore, in this study, different pathways related to division, differentiation, cytoskeletal dynamics, and activation and stimulation of T cells, were selected more prominently.

In the first part, we examined the effect of expansion media on T cells. 4-1BBL is involved as a vital culture medium in T cell expansion. In HIV-specific T cells study, it was found that 4-1BBL, by acting on a TNF receptor factor dependent on the BIM pro-apoptotic molecule, could reduce cell apoptosis and crucial molecules. Other markers such as CD70, increased the efficiency of T cells (6). Another study proved that 4-1BBL, by increasing the transcription factors of Cyclin D2/D3, naturally increases the expression of these molecules. In turn, increased expression of these two molecules increases the expression of Cyclin E. On the other hand, the presence of 4-1BBL in the environment of T cells reduces the expression of P27<sup>kip1</sup> inhibitor and as a result of this activity, division of T cells is facilitated (7). Dealing with tumor cells is also one of the problems of today's research. A study showed that the presence of CD80 and 4-1BBL can betterly activate and stimulate cells and make them perform better against tumors (8). The production of cytokines is significant for increasing the function of T cells. In one study, production of cytokines in T cells was increased and a synergistic effect with interleukin-2 was achieved (9).

Another dataset examined in this study was the expansion medium mediated by Akt inhibitor. One of the critical roles in T cell expansion is to regulate the memory of these cells. The inhibition of Akt activates MAPK and FOXO1, which is an essential regulator of T cell memory and can play an important role in the discussion of immune compatibility. It is found in the differentiation of cells in two types of cell populations. One is useful cells with a short-lived effector cell (SLECs), and the other is memory precursor cells (MPECs). The study showed that inhibition of PI3K/Akt could play an active role in cell expansion by stimulating and strengthening their memory (10). A different study on leukemia cancer mouse model showed that inhibition of Akt in CD19CAR cells could increase the immune compatibility and memory formation in these cells, which also had significant antitumor properties (11). An almost similar study was performed to evaluate T cells' final differentiation and the formation of immune compatibility in the face of tumors on suspicious stem cells in an environment with Akt inhibitors, which showed acceptable efficacy (12).

Another dataset examined in this study is related to interleukins 15 and 7. Interleukins are important cytokines that can affect the activity of other lymphocytes.

One of the studies showed that, when IL-7 affects EBV-specific T cells, cause cell division, survival and cytotoxicity in them (13). Numerous other studies have also shown the role of restoration and induction of homeostasis in T cells by interleukin-7 (14). Another fascinating study in human immunodeficiency virus (HIV) patients showed that interleukin 7 had a significant therapeutic and recovery effect on T lymphocytes and played an important role in cell division and homeostasis (15). The relationship between expansion and CD19 CART cells was investigated using interleukins 15 and 7 in lymphoids. The results showed that these two interleukins' affect reduces apoptosis of T cells and increase memory in their stem cells (16). Various other studies have been performed on interleukin-15. Zeng et al. (17) study showed that in melanoma-mouse model, interleukin 15 had a strong synergistic effect with interleukin-21 and played a vital role in enhancing memory and expansion and division of T cells.

In another study with *mouse models* with selective deficiency in *NK cells*, it was shown that, the addition of interleukin-15 to the existing body could play an effective role in regenerating the development and memory of T cells and NK (18). In HIV infection, interleukin 15 has been shown to play a significant role in the activation and expansion of T cells at high levels (19). Another study proved that there is a correlation between dendritic cells and NK in lymphoid organs. The presence of interleukin-15 in dendritic cells increases the viability, division, and maturation of NK cells (20). Another study showed that interleukin-15 increased the homeostasis and the division of CD8 nasal cells and played an important role in expanding these cells (21).

In the next step, we shared the datasets and obtained multiple genes in different paths. Based on the literature, we selected the most relevant signaling pathways with T cell expansion and their characteristics, and based on this, we selected some of them. In contrast, we examined common genes for GO and finally isolated important genes.

Wnt signal pathway is one of the pathways identified in this study which plays a wide variety of cell roles. This pathway plays a significant role in development, cell division, and differentiation of activity. But in T cells, high and low expression of genes involved in this pathway play attractive roles in expansion. Low expression of genes in Wnt pathway accelerates the differentiation of T cells. On the other hand, low expression of genes in this pathway increases cell division and is also effective in increasing T cells' memory. Therefore, it can act as a secondary stimulant of T cells and perform better during vaccination (22). In naive T cells, the issue is different, and activation of Wnt/B catenin pathway inhibits differentiation in naive T cells. Further investigation of this pathway and its regulation can be a good option for the function of naive T cells (23). Another study showed that modulation of Wnt pathway had an acceptable effect on T cells' production with a better memory to increase immune adaptation efficiency (24).

T cells' fate depends on several pathways, such as growth factors, cytokines, cell division, and various metabolic pathways. mTOR is one of the most important pathways involved in controlling many cellular processes. When mTOR is inhibited, it can increase the tolerance of competent Th1 cells. mTOR activation is necessary for the differentiation of Th1 / 2/17 cells, and its inhibition is required for differentiation into Foxp3 regulatory T cells (25). Another study showed that temporal inhibition of leptin/mTOR pathway in the absence of interleukin-2 resulted in better differentiation of regulatory T cells, which could play a significant role in immune adaptation and autoimmune diseases (26). The hemostatic division is one of the important mechanisms in T cells to deal with different tumors. One study found that interleukin-7 and interleukin-15 indirectly affected mTOR pathway activity. Accordingly, naive T cells are involved in two important transcription factors for hemostatic division, T. bet, and eomesoderm. CD122 is included in the development of eomesoderm expression. When mTOR pathway is inhibited, T.bet and CD122 are also inhibited, but eomesoderm can act indirectly by adding interleukin-15 to the medium, ultimately increasing the memory of T cells to fight tumor cells (27). Binding proteins of sterol regulatory elements are important pathways in the production of lipids and fatty acids involved in many T cellular processes such as growth, division, post-translational mechanisms, cell motility, and tectonic activity. mTOR is directly related to SERBP and mTOR activity in the Golgi membrane and endoplasmic reticulum effectively facilitates lipid production process (28).

Cell motility is one of the most important pathways in developing and differentiation of cells from an early population. Rho and Rac1 are two vital proteins involved in cytoskeletal dynamics as well as actin filaments activity. Various studies have highlighted the importance of these two proteins with hematopoietic cells as well as lymphocytes. One of these studies showed that Rac1/2 was necessary for the initial expansion of hematopoietic cells in the bone marrow, but when they go to the spleen and the cells reach maturity, this group's protein expression decreases. One of the critical factors for clustering T cells together is the binding of T cell receptor antigen (29). NF-KB transcription factor also increases when the coupling between antigen and T cells occurs. The inhibition of PI3K activity inhibits the NF-KB pathway and reduces the clustering of T cells. Thus, increasing Rho pathway activity helps regenerate NF-KB pathway and the aggregation of T cells together, which is involved as a different pathway (30). PYGM is one of the glycogen phosphorylation isoforms with a high enzymatic role in muscle and is useful in cell division. One study showed that stimulation of T cells with interleukin-2 induced PYGM activity, which also affected Rac1. Finally, this study showed that in the presence of interleukin 2 pathways, PYGM/Rac1 has a high activity for T cell division (31). MEKK3 is a member of the MAPK family and is a serine/threonine protein, which has been continuously seen in T cells. A study showed that in a MEKK3 deficient mouse model, the immune system's response to bacterial infections is greatly reduced, and interferon-gamma, derived from interleukins 12 and 18, is not produced or is produced in minimal amounts. Besides, defects in MEKK3 cause weakness in ERK1/2, JNK, and P38 pathways. However, activation of Rac1/2 activates MEKK3 and produces cytokines (32).

One of the important data in this study was the sharing of CD80 with significant expression in each expansion culture media. CD80 is also an important co-stimulatory molecule in the immune system and response to various pathogens. Extensive studies have examined the association of CD80 with the immune system and its various pathways. CD80, 86, and 28 have high stimulatory activity in T cells and are effective in the immune response and production of interleukin-2 (33). Another study on a mouse model of lymphocytic choriomeningitis virus infection showed that the presence of CD80 and 86 were effective in boosting T cell memory and fighting a viral infection (34). An exciting study in rheumatoid arthritis showed that CD80 and 86 molecules' activity affects the differentiation and activity of B cells and the integration of blood vessels and causes the autocrine effect on T cells, which helps the strengthen of the immune system (35). Another study on HIV infection showed that the activity and presence of CD80 causes a more robust response of T cells to the virus (36). Programmed death-ligand is one of the most critical proteins on the surface of cancer cells that acts as a protector against these cells, and T cells are unable to respond to them. Therefore, if

present, CD80 molecule causes repression, and T cells can respond to these cells more effectively (37). Also, the activity of interleukin 12 and CD80 has increased the antitumor property of T cells in breast and ovarian cancer (38). A study showed that CD80 and 86 with autocrine effect for the long time, can have an acceptable effect for T cells. CD80 also has a positive impact on repairing T cell tolerance (39).

## Conclusion

Finally, it can be said that this study examines the expansion environments used on T cells, and the signaling pathways, genes, and proteins shared between these pathways play a significant role in improving the function of T cells with the approach of increasing the cell division. They had memory, immune compatibility, and, ultimately, stimulation and activation of T cells. Among these, CD80 genes were selected as essential genes in these pathways.

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## Authors' Contributions

A.J., A.B.; Participated in study design, data collection and evaluation, drafting, and statistical analysis. A.H., E.R., N.A.; Designing the project and corresponding. All authors performed editing and approving the final version of this manuscript.

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