Tonsil-Derived Mesenchymal Stem Cells Inhibit the Proliferation of Hematological Cancer Cells through Downregulation of IL-6 Gene Expression under Hyperthermia

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Research Article

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Abstract

Stem cells are extensively being studied as promising biological therapeutic candidates in cancer treatment. In various cancer types, some studies show proliferative effects while others show inhibitory effects of MSCs on tumors. Some studies have reported that MSCs isolated from different sources display different anti-cancer properties. Tonsils are one of the secondary lymphoid organs that form an important part of the immune system and located at the mucosal interface. The relation between secondary lymphoid organs and cancer progression lead us to investigate the effect of tonsil-derived MSCs (T-MSC) on cancer treatment. Therefore, we aimed to determine the anti-tumoral effects of tonsil-derived MSCs cultured at febrile temperature on hematological cancer cell lines. We found that co-culture of K562 cells and MOLT-4 with T-MSCs significantly decreased the viable cell number post 7 days of the culture under the febrile and normal culture conditions. Besides, the T-MSC co-culture not only induced the apoptosis on K562 and MOLT-4 cells but also, induced the cell cycle arrest at G2-M phase on MOLT-4 cells. The apoptotic effect of T-MSC co-culture under febrile stimulation was confirmed by upregulation of Bax, c-myc genes for K562 cells and upregulation of Bax, p53 and c-myc genes for MOLT-4 cells in transcriptional level. Our study has contributed to highlight the effect of the cellular interaction between the T-MSCs and human hematological cancer cells during in vitro co-culture under hyperthermia for tumor progression. In the light of these results, we indicated that tonsil-derived MSCs have promising therapeutic potential for cancer therapy.

1. Introduction

Cancer is the result of abnormal and uncontrolled growth of a group of cells by overcoming the normal rules of cell division [1] and the most dangerous disease causing millions of deaths worldwide [2]. Leukemia is a universal term for blood cancer cells and develops through clonal expansion of leukemic cells in the bone marrow. Leukemia is the 15th most commonly diagnosed cancer worldwide and it ranks 11th among the causes of cancer deaths according to the study in 2018 [3]. Treatment options vary depending on the type and progression of cancer. However, treatment-related side effects, off-target effects, drug resistance and tumor recurrence risk limit the effectiveness of many therapeutic options. Therefore, it is important to develop new, effective treatments with no or low toxicity in normal cells [4].

Mesenchymal stem cells (MSC), also called mesenchymal stromal cells, are adult stem cells that have the ability to self-renew and transform into different cells. MSCs are the most widely used stem cells in clinics due to their easy culture in culture dishes, their potential to differentiate into many cells in the body, their unique immunophenotypic, tissue repair, and immune-regulatory capacity, and their capacity of beneficial growth factors and cytokine production [5]. Until today, ongoing preclinical research indicates that MSCs are suitable targets for cell therapy in various cancers. It has been reported that MSCs generally show their therapeutic potential by secreting bioactive molecules such as growth factors, cytokines and extracellular vesicles, which provide immunosuppressive, anti-apoptotic, anti-fibrotic, angiogenic and anti-inflammatory effects. It was shown that MSCs arrest cell cycle and inhibit cancer growth by inhibiting proliferation-related signaling pathways such as phosphatidylinositol 3-

kinase/protein kinase B (PI3K/AKT) [6]. However, the antitumor effects of MSCs are still controversial. In various cancer types, some studies show proliferative effects [7–11], while others show inhibitory effects of MSCs on tumors [9, 12–22].

It is also known that MSCs originated from different tissues show different characteristics [23]. Some studies have reported that MSCs isolated from different sources display different anti-cancer properties. The study about co-culture of umbilical cord blood and adipose tissue-derived MSCs with primary glioblastoma multiforme cells has shown that umbilical cord-derived-MSCs inhibit glioblastoma multiform cell growth and induce apoptosis, whereas adipose tissue-derived-MSCs promote glioblastoma multiform cell growth [24]. Besides, it was demonstrated that human bone marrow mesenchymal stem cells caused inhibition of proliferation and cell migration, induction of cell cycle arrest and apoptosis on glioma U251 cells depending on the culture period [25]. The different results reported in literature show that the types of cytokines, which is important in cancer cell death, depends on both the stem cells and the target cancer cell types.

The effect of MSCs on hematological cancers such as leukemia, lymphoma and multiple myeloma is less well known than solid cancers. In different studies, it was reported that MSCs inhibit tumor growth by suppressing the proliferation of tumor cells in hematological cancers [17, 26–28] or induce tumor growth by suppressing of apoptosis [28]. In addition, the study about culture of mouse bone marrow-derived MSCs with lymphoma, erythroleukemia, and acute lymphocytic leukemia cells indicate that it may be safe and effective in the treatment of hematological malignancies [26]. It has also been reported that adipose tissue-derived MSCs have an inhibitory effect on hematological cancer cell lines by secreting DKK-1 (dickkopf-1), which acts as a negative regulator of the WNT signaling pathway, which is known to be effective in developmental regulation and tumor formation [29]. In animal studies, it was stated that human bone marrow MSCs exhibited anti-lymphoma activity after injection into Severe Combined Immunodeficient mice (SCID) carrying common non-Hodgkin lymphoma xenografts[30]. However, in another study, it was demonstrated that adipose tissue derived MSCs support the growth of acute lymphoblastic leukemia cells in Non-obese diabetic SCID (NOD/SCID) mice [31]. In a study reported in recent years showed that long-term co-culture of bone marrow-derived MSCs with the K562 hematological cancer cell line blocked the G0/G1 phase, and also significantly induced post-apoptosis in K562 cells [32].

Hyperthermia, which occur heating at tumor site at temperatures between 40°C and 46°C for 45–60 min, is one of the therapeutic method for cancer treatment [13, 33]. The key role of fever in the immune system is related to activation of the innate immune system, such as the release of neutrophils in the periphery, the production of cytokines and nitric oxides from macrophages or dendritic cells, and the stimulation of leukocyte transport [33]. The studies investigating the effect of hyperthermia on cancer cells shows its regulatory effect on cancer cell death [34, 35] and its inhibitory effect on the proliferation of different cancer cells post stimulation with hyperthermia [36, 37]. However, researches on effect of heat on tumor cells have been limited until now. Since MSCs are significant constituents of the tumor cells, it is essential to also study the impact of hyperthermia on cancer therapy.

The cancers progression is closely associated with primary or secondary immunosuppression. The strengthening of immune system is important as a therapeutic strategy to suppress tumor progression, reactivate apoptotic mechanisms, and arrest tumor angiogenesis [38]. Tonsils are one of the secondary lymphoid organs that form an important part of the immune system and located at the mucosal interface. They are important sources for studying innate immune responses due to their ease of access and less ethical problems [39–41]. The relation between secondary lymphoid organs and cancer progression lead us to investigate the effect of tonsil-derived MSCs (T-MSC) on cancer treatment. Therefore, we focused on the tonsils which are one of the important elements of the immune system in this study. As an important marker of infection *in vivo*, we aimed to determine the anti-tumoral effects of tonsil-derived MSCs cultured at febrile temperature (40^0C) on hematological cancer cells (K562 and MOLT-4 cell lines) and also, determine the mechanism underlying the anti-tumoral activity. For that we determined the effect of T-MSCs cultured at normal (37^0C) temperature and induced at febrile (40^0C) temperature on cancer cell viability, apoptosis and cell cycle profile post co-culture with K562 human chronic myeloblastic and MOLT-4 acute lymphoblastic leukemia cell lines. We found that co-culture of K562 cells and MOLT-4 with T-MSCs significantly decreased the viable cell number post 7 days of the culture under the febrile and normal culture conditions. However, K562 cells may be resistance to hyperthermia in longer T-MSC co-culture. Besides, the T-MSC co-culture not only induced the apoptosis on K562 and MOLT-4 cells but also, induced the cell cycle arrest at G2-M phase on MOLT-4 cells. The apoptotic effect of T-MSC co-culture under febrile stimulation was confirmed by upregulation of Bax, c-myc genes for K562 cells and upregulation of Bax, p53 and c-myc genes for MOLT-4 cells in transcriptional level.

Our study has contributed to the knowledge on crosstalk between the T-MSCs and human hematological cancer cells during *in vitro* co-culture under hyperthermia for tumor progression. In the light of these results, we indicated that tonsil-derived MSCs have promising therapeutic potential for cancer therapy. However, further *in vitro* and *in vivo* studies are needed for supporting the therapeutic usage of T-MSCs.

2. Results

2.1 Confirmation of the main characteristics of tonsil-derived mesenchymal stem cells

To characterize the mesenchymal stem cells isolated from palatine tonsil tissue, the immunophenotyping was firstly performed on the cultured cells by using flow cytometry. The cells were analyzed for previously identified negative (CD34 and CD45) and positive (CD73, CD90 and CD166) cell surface CD markers. We confirmed the newly isolated mesenchymal stem cells with the findings that significant expression of positive CD markers and also, absent expression of negative markers **(Fig. 1a).**

Another characteristic property of MSCs is their multi-lineage differentiation capacity. Therefore, we evaluated the multi-lineage differentiation ability of the isolated cells with oil red O and alizarin red S. We found that the isolated T-MSCs had the differentiation ability to adipocytes and osteocytes post three

weeks of the induction with special mediums **(Fig. 1b).** All of these findings show that newly isolated cells from palatine tonsil tissue are the mesenchymal stem cells.

2.2 Febrile temperature contributes to the growth inhibitory effect of T-MSC co-culture on MOLT-4 cells as much as normal culture temperature and also, caused the resistance on K562 cells

To evaluate the effect of T-MSC co-culture on the growth of K562 and MOLT-4 cells, we counted the viable cell number of K562 and MOLT-4 cells in certain time interval (at day3, day5 and day7) post co-culture of T-MSCs. We found that the viable cell number of K562 cells was decreased at 3-fold post five days of co-culture under normal (37^0C) temperature. The febrile (40^0C) temperature reduced the viable cell number of K562 cells at 5- fold post five days of co-culture; however the reduction on viable cell number was decreased post 7 days of co-culture compared to the cell count at day5. These findings demonstrated that K562 cells developed the resistance to hyperthermia post 7 days of co-culture **(Fig. 2a).**

Furthermore, we found 2-fold decrease in the viable cell number of MOLT-4 cells post 7days of co-culture under normal culture and febrile conditions while did not find any significant changes at day 5. In the contrary to the findings on K562 cells, we did not determine the any significant effect of febrile condition on the resistance to hyperthermia on MOLT-4 cells **(Fig. 2b)**. These findings show that the febrile condition is effective on the co-culture between hematological cancer cells and T-MSCs as much as normal culture temperature in long-term (7 days). Besides, K562 cells may be resistant to hyperthermia in long-term in despite of more effectiveness on K562 cells in a short time (5 days).

2.3 Co-culture of T-MSCs induced the apoptosis and necrosis on hematological cancer cells under febrile temperature.

To elucidate the mechanism of growth inhibitory effect of T-MSC co-culture, we performed the apoptosis assay on hematological cancer cells cultured under 37^0C normal and 40^0C febrile conditions. We found that co-culture of T-MSCs slightly reduced post-apoptotic K562 cell ratio at 1,7-fold under normal culture temperature and increased the cell viability. Besides, the febrile condition did not affect the apoptosis on K562 cells at day5 in despite of reducing the viable K562 cell count shown in Figure 2A **(Fig. 3a)**. However, the apoptosis on K562 cells was induced 2,5-fold with T-MSC co-culture under febrile condition at day7 **(Fig. 3b)**. These findings are parallel with reduced cell growth on K562 cells for the results obtained from day7. It is thought that the underlying mechanism of growth inhibition on the viability of K562 cells co-cultured with T-MSCs at normal and febrile temperature conditions may be related to different cellular processes in addition to apoptosis according to the cell count results at day 5.

The co-culture of T-MSCs was found to be more inducer on apoptosis process of MOLT-4 cells in contrast with K562 post 5 and 7 days of culture. Post 5 days of co-culture, apoptotic and necrotic cell ratio in MOLT-4 cell population was significantly increased under febrile condition, whereas co-culture of T-MSCs cultured in normal condition significantly induced the necrosis on MOLT-4 cells at 3-fold **(Fig. 4a).** In addition, the longer culture (7 days) with T-MSCs induced the apoptosis and necrosis on MOLT-4 cells at

approximately 2-fold under the normal and febrile conditions. But, the necrosis was more induced on MOLT-4 cells under febrile condition compared to 37^0C temperature **(Fig. 4b)**. The findings obtained from day7 results support the growth inhibitory effect on MOLT-4 cells and also, the co-culture of T-MSC is more effective on MOLT-4 cells in the short term (5 days) under febrile condition.

2.4 Co-culture of T-MSCs induced the cell cycle arrest at S and G2-M phases on MOLT-4 cells

The cell cycle arrest on cancer cells are an important phenomena for the potential anti-tumoral effects of chemical agents or cellular therapies[46]. To investigate the effect of T-MSC co-culture on cell cycle distribution of K562 and MOLT-4 cells, we performed the cell cycle analysis post 7 days of co-culture. We found that the cell cycle distribution of K562 cells co-cultured with T-MSCs was not change for both normal and febrile conditions. The cell ratio at sub G0-G1 phase was just reduced on both two normal and febrile culture conditions at 3,7- and 2-fold, respectively **(Fig. 5a)**. The peak of sub G0-G1 phase represents the DNA fragmentation resulted by apoptosis [47]. The reduction on this phase is incompatible with our previous findings resulting the induced apoptosis on K562 cells.

In other respects, we indicated the cell cycle arrest at G2-M phase on MOLT-4 cells co-cultured with T-MSCs under the two different conditions. But the stimulation with hyperthermia more increased the arrested cell ratio (2,6-fold) compared to the culture at 37^0C (2-fold). The cell cycle arrest at G2-M phase on MOLT-4 cells could be one of the reasons of growth inhibition. Besides, the cell ratio on sub G0-G1 phase was slightly increased (1,6-fold) with febrile condition **(Fig. 5b)**. The cell cycle arrest at sub G0-G1 supports the contribution of the stimulation with hyperthermia on apoptosis on MOLT-4 cells.

2.5 K562 and MOLT-4 cells differentially expressed apoptosis and cell cycle-related genes post the co-culture with T-MSCs

The changes on the apoptosis and the cell cycle processes were validated in the gene expression level on K562 and MOLT-4 cells post co-culture of T-MSCs under 37^0C and 40^0C temperatures. We determined the expressions of the apoptotic gene Bax (Bcl2 associated x) and anti-apoptotic gene Bcl-2 (B-Cell CLL/Lymphoma 2), the cell cycle inhibitor Cdkn1a (cyclin dependent kinase inhibitor 1a; p21) and also, apoptosis and cell cycle-related downstream genes p53 (transformation-related protein 53; Trp53) and c-myc (MYC proto-oncogene). Besides, cytokine Il-6 (interleukin-6) involved in the leukemic-like phenotype and gene signature in CML mouse model[48], was also investigated. We found that the apoptotic gene Bax and anti-apoptotic gene Bcl-2 were down-regulated approximately 2-fold on K562 cells co-cultured at 37^0C while up-regulated approximately 2- and 3-fold on K562 cells co-cultured at 40^0C, respectively. The apoptotic effect of T-MSC co-culture under febrile temperature was confirmed with the up-regulated apoptotic Bax gene expression. Besides, the expression of p53 gene, which is involved in apoptosis process, was reduced 4,5-fold on K562 cells co-cultured at 37^0C, but its expression was not changed on K562 cells co-cultured at 40^0C compared to the control cells. C-myc expression was increased 2,6-fold post stimulation with hyperthermia while reduced 2-fold on K562 cells co-cultured at 37^0C **(Fig. 6a)**. C-

myc promotes the apoptosis p53-dependent or p53-independent [49]. The increased expression of c-myc with hyperthermia is consistent with apoptotic effect of the co-culture but, the stable expression of p53 showed that the role of c-myc on apoptosis may be p53-independent. In addition, the co-culture of T-MSCs did not effect on Cdkn1a (p21) gene expression at 37^0C, whereas the stimulation with hyperthermia up-regulated p21 expression 21-fold **(Fig. 6a)**. In despite of the up-regulation of the cyclin-dependent kinase inhibitor p21, we did not observe any cell cycle arrest on K562 cells co-cultured under febrile condition. Besides, Il-6 gene was slightly up-regulated on K562 cells co-cultured at 37^0C but, the heat stimulation did not affect the Il-6 expression on K562 cells co-cultured with T-MSCs compared to the control cells **(Fig. 6a)**.

For MOLT-4 cells, we found that the apoptosis related genes Bax and Bcl-2 were up-regulated 5,6- and 2–fold on MOLT-4 cells co-cultured with T-MSCs at 37^0C. However, the expression of Bax gene was increased 8-fold and also, the Bcl-2 expression was decreased on MOLT-4 cells co-cultured with T-MSCs at 40^0C compared to MOLT-4 cells co-cultured at 37^0C **(Fig. 6b)**. The apoptotic effect of T-MSC co-culture under febrile temperature was confirmed with the up-regulated apoptotic Bax gene expression and also, decreased Bcl-2 expression post hyperthermia stimulation. Besides, p53 was up-regulated post the stimulation with hyperthermia while down-regulated on MOLT-4 cells co-cultured with T-MSCs at 37^0C **(Fig. 6b)**. The knowledge about the crosstalk between p53 and apoptotic genes, resulting the regulation of apoptosis process [50] supports the upregulation of p53 and Bax genes. While the expression of c-myc did not determined on MOLT-4 cells co-cultured at 37^0C compared to the control cells, its expression was up-regulated approximately 6-fold post heat stimulation on co-cultured cells **(Fig. 6b)**. The similar changes on the expressions of p53 and c-myc show that c-myc may also contribute to p53-dependent apoptosis on MOLT-4 cells with T-MSC co-culture under heat stimulation. The cell cycle inhibitor Cdkn1a expression was significantly increased 15-fold on the cells at 37^0C, but its expression was not changed on the cells co-cultured at 40^0C compared to the control cells **(Fig. 6b)**. The p21 inhibits the cyclin-dependent kinase 2 or cyclin-dependent kinase 4 and thus, it causes the cell cycle arrest at G1, S and G2 phases [51, 52]. In the light of these literature information, it was indicated that the up-regulation of p21 on the co-cultured MOLT-4 cells provided the cell cycle arrest at S and G2-M phases under 37^0C culture conditions. The arrest on MOLT-4 cells co-cultured with T-MSCs under febrile temperature may be related to other cell cycle checkpoints. Besides, the expression of Il-6 was up-regulated 2-fold on MOLT-4 cells co-cultured at 37^0C but, we did not determine any significant changes on its expression on MOLT-4 cells co-cultured at 40^0C compared to the control cells **(Fig. 6b)**.

3. Discussion

Cancer is the one of the most important causes of morbidity and mortality worldwide [2, 17]. The side effects of chemotherapy, radiotherapy and surgical practices, which are used as the first treatment options, off-target effects or factors such as tumor recurrence have directed the researches towards the cell therapy [4, 17]. The recent studies have shown that the mesenchymal stem cells are effective therapeutic agents for various diseases, including cancer [14, 18, 53, 54]. Besides, there are many studies

showing the proliferation enhancing effects of MSCs on hematological malignancies [55] and antitumor functions and tumor growth inhibitor effects of MSCs obtained from different sources for various cancer types [56, 57].

Bone marrow is an important source that is commonly used in MSC isolation. However, the searches for new sources continue due to the difficulty of isolation process, ethical problems and the scarcity of MSC in bone marrow [58]. Therefore, tonsillar tissue obtained after tonsillectomy, which is the most frequent surgical procedure in childhood, is an alternative source of MSC since it can be obtained with a less invasive procedure. In the tonsillar microenvironment, which is also the secondary lymphoid organ, MSCs present here have potential implications for initiating and maintaining effective immunity responses [59].

In this study, we showed that MSCs obtained from the secondary lymphoid organ, the tonsil, were positive for MSCs specific cell surface antigens and negative for hematopoietic stem cell surface antigens, as well as their multi-lineage differentiation potential. It was also shown that co-cultures of TMSCs with hematological cancer cells show anti-proliferative activity in cancer cells in a time dependent manner. We also showed that febrile temperature status induces the rate of apoptosis in hematological cancer K562 and MOLT 4 cell lines. While no significant change was observed after 5 days of co-culture of K562 cells with T-MSC at normal culture temperature, a 1,4-fold increase was observed in pre-apoptotic cell number, especially at the end of the 7-day culture period. Apoptosis-inducing effect of mild hyperthermia was observed with a 2.5-fold increase in the number of pre-apoptotic cells and a 2-fold increase in the number of post-apoptotic cells under 40 degrees febrile conditions created in vitro. The apoptotic effect induced under febrile temperature was confirmed at gene expression level through the finding of 2-fold increase in pro-apoptotic Bax gene expression. The increased c-myc expression under mild hyperthermia conditions supports the apoptotic effect of co-culture with T-MSCs. However, stable expression of p53 suggests that the role of c-myc on apoptosis may be independent of p53. Pre and post apoptotic cell rates under normal temperature and febrile temperature conditions after 7-day long culture showed approximately 2-fold increase in MOLT-4 cells. Unlike K562 cells, it was observed that co-culture of MOLT 4 cells with T-MSCs under febrile temperature conditions induced apoptosis in a shorter time (5 days). Different studies conducted have shown that MSCs obtained from different sources induce apoptosis in cancer cells at different rates [17, 26]. It has also been reported that tonsil derived MSCs induced apoptosis in Head and Neck Squamous Cell Carcinoma Cells [21]. However, our study is the first study to show that tonsil-derived MSCs induced apoptosis in hematological cancer cell lines. p53 is a tumor suppressor gene that inhibits cell proliferation by stimulating apoptosis [60]. The apoptotic effect of T-MSC co-culture on MOLT4 cells under febril temperature was confirmed with the expression of decreased Bcl-2 and up-regulation of apoptotic Bax gene expression after hyperthermia stimulation. Besides, similar changes in p53 and c-myc expressions suggest that c-myc contributes to apoptosis by a p53-related mechanism in MOLT-4 cells of T-MSC co-culture under mild hyperthermia conditions.

We also performed the cell cycle analysis to determine the effects of TMSCs on cancer cell cycling ability. Under mild hyperthermia conditions, despite up-regulation of cyclin-dependent kinase inhibitor p21 in K562 cells co-cultured with T-MSCs, we did not find any significant changes in cell cycle progression.

Unlike our study, in a study conducted after the culture of K562 cells with BM- MSCs, while a significant increase was found in the percentage of G0/G1-phase (subG1), decrease was found in G1, S and G2/M stages. In addition, it was reported in the study that after 7 days of culture, K562 cells were mostly in the post-apoptotic stage [17]. The reason for the difference here is likely to be due to the difference in the source from which the MSCs were obtained. We know that it has been reported MSCs obtained from different sources can show different cellular and functional characteristics although they are cultured under the same conditions [61, 62]. The effects of MSCs on malignant tumor cells are controversial. It is thought that the changes in the data obtained from studies may be due to the heterogeneity of MSCs depending on the sources from which they were obtained, experimental changes (time, amount) of MSC applications or other unknown molecules or mechanisms [63]. However, we demonstrated 2- and 2.6-fold G2-M phase cell cycle arrest in MOLT -4 cells under normal temperature and febrile temperature conditions, respectively. It is thought that the cell cycle arrest in the G2-M phase found in MOLT-4 cells causes the growth inhibition. In addition, it was observed that the rate of cells in the sub-G0-G1 phase increased 1.6-fold at febrile temperature. This results, supports the contribution on apoptosis in MOLT-4 cells of stimulation by hyperthermia.

p21 from CDKIs causes cell cycle arrest in the G1, S and G2 by inhibiting cyclin-dependent kinase 2 or cyclin-dependent kinase 4 [51]. Expression of the cell cycle inhibitor Cdkn1a was significantly increased (15-fold) at 37°C in MOLT 4 cells co-cultured with T-MSC compared to control cells, but remained unchanged at 40°C. Accordingly, it is thought that up-regulation of p21 on co-cultured MOLT-4 cells causes cell cycle arrest at S and G2-M phases under 37°C culture conditions. Cell cycle arrest found in MOLT-4 cells co-cultured with T-MSCs under febrile temperature conditions may be related with other cell cycle control points. Gene expression level refers to transcriptional regulation; however, cross talks between proteins have many roles in cellular processes. For this reason, the cellular profile often may not be explained at transcriptional level. Ultimately, T-MSC co-culture resulted in differential expression of apoptotic and cell cycle-related genes in K562 and MOLT-4 cells. Many highlight the mechanism of changes in cellular processes and they should be examined in more detail. The mechanisms by which MSCs cause anti-proliferative effect on leukemia cells have not been fully explained. In the studies of Song et al., it was shown that the expression level of the cell cycle negative regulator p21 was up-regulated similar to our study, the pre-apoptotic cell fraction was increased and this led to cell cycle arrest [26].

Hyperthermia is a therapeutic application that raises the temperature of tumor tissue to 40°C -44°C. The key role of fever in the immune system is realized through activating the innate immune system such as the release of neutrophils in the periphery, the production of cytokines and nitric oxides from macrophages or dendritic cells and the stimulation of leukocyte transport [33]. However, an increase in body temperature of 1°C in endothermic animals causes a 10-12.5% increase in metabolic rate and results in a high metabolic activity. At the same time, uncontrolled fever has been reported to cause worse outcomes in patients with sepsis or neurological injuries [64].

P53, which is a tumor suppressive gene, controls different processes in cells against different cellular stress factors. Apart from its known cell cycle functions, P53 is also known to play a role in cell migration and cancer cell metastasis. P53 has been reported to occur against epithelial-mesenchymal transition (EMT) and cell migration. In a study by Goyal and Ta, an increase in p53 protein expression of WJ-MSC was reported under 40°C febrile temperature conditions[34]. The effect of heat stress on MSC properties has not been extensively studied. In the study conducted in 2020 by the same group, they obtained data that several adhesion-related Extracellular Matrix Protein (ECM) genes such as Vitronectin (VTN) regulated p53 expression in MSCs under heat stress at 40°C and they noted that p53 was up-regulated at 40°C [35]. In our study,, p53 expression was up-regulated at 40°C defined as mild hyperthermia condition compared to normal temperature conditions and this gene may be realated to induction of the apoptosis and suppression of the tumor proliferation in mild hyperthermia conditions in K562 and MOLT4 cells.

There are studies with positive results conducted to determine the effects of hyperthermia induced mesenchymal stem cells on cancer cell growth. In the present study, it was shown that MSC conditioned media stimulated with heat (45 minutes at 43°C) modulated the homeostatic balance of cancer cells towards cell death. It has also been found that soluble factors secreted by hyperthermia-stimulated MSCs can provide an anti-tumorigenic microenvironment and this makes the tumor cells more sensitive to chemotherapeutic therapeutic by effectively transmitting the exogenous cell death signals to the cells[65]. CMs obtained from different tissue-derived MSCs under hyperthermic conditions (at 43 ° C) were applied to human cancer cell lines and they were reported to have a negative effect on cancer cell proliferation [37, 65]. In their study, Park et al. showed that CM obtained under 45 minutes of temperature stimulation at 43°C inhibited cell growth by inducing cell cycle arrest in G2/M phase of breast cancer cells [37]. In this study, where the authors wanted to determine the effects of T-MSCs on hematological cancers at 40°C as mild febrile temperature, they showed that cell cycle arrest was induced in the G2/M phase. The apoptosis rate of K562 cells increased significantly compared to 37°C, especially during the 7-day long culture. The apoptosis was induced in MOLT 4 cells in shorter culture time compared to 37°C. A similar effect to 37°C was found on day7.

IL-6 has roles in immune regulation, regulation of cell growth, hematopoiesis and performing a wide variety of cellular functions such as oncogenesis. It is known that high expression of IL-6 in cancer cells causes cancer to progress by inhibition of the apoptosis and stimulation of the angiogenesis in cancer cells [66, 67]. The data obtained from clinical studies have been associated with the increase of IL-6 level in serum, advanced tumour stages in various solid cancers and low survival rates. Thus, it has been suggested that IL-6 inhibitors may be a potential therapeutic agent [67–69]. The role of IL6 in the pathogenesis of Chronic Myelogenous Leukemia (CML) and Chronic Lymphocytic Leukemia (CLL) and its contribution to CML progression are also known [68, 70]. It has also been shown that IL-6 blocking strategy can significantly delay CML initiation and increase survival rates [68]. Therapeutic hyperthermia used in cancer treatment causes increase in the levels of some cytokines and IL-6. Therefore, it has been reported that anticancer efficacy increased in vitro and in vivo when hyperthermia is not applied as an independent treatment, but with conventional methods such as immunotherapy, radiotherapy, chemotherapy and surgery [71].

In our study, IL-6 gene expression levels was increased 2- and 2.5-fold, respectively at 37°C in hematological cancer cells (both K562 and MOLT4) co-cultured with T-MSCSs under normal temperature. Besides, the mild hyperthermia significantly downregulated of its expression at 40°C. This downregulation has been associated with the decreased proliferation and antitumor function in cancer cells co-cultured with TMSC under mild hyperthermia conditions.

On the light of these findings, it is reported that tonsil-derived MSCs, as a new source of MSC, have antitumor function on hematological cancer cells and also, this function was correlated with hyperthermia stimulation. These findings reveal a promising therapeutical cellular application for the cancer therapy.

4. Conclusion

MSCs are widely studied in the field of cancer biology and they have extensively been studied as a potential therapeutic in cancer. However, many unknowns about the roles of stem cells in the progression and treatment of cancer are still unclear before the clinical use of stem cell researches. Especially, their effect on hematological cancers such as leukemia, lymphoma and multiple myeloma are less known than solid cancers. Although the effects of hyperthermia on cancer cells are well documented, the effect of hyperthermia on tumor stromal tissue or cells interacting with cancer cells has not been studied in detail. In this study, we have indicated that MSCs obtained from tonsillar tissue, as a new source of MSC, show antitumor properties in hematological cancer cell lines through induction of apoptosis, down-regulation of IL-6 gene expression and up-regulation of p53 expression under mild hyperthermia conditions. However, the results obtained from the studies conducted on cell lines may differ from primary cancer tissue or cells. The findings of the study, which provide important information as preliminary results, should be confirmed for the primary tumor cells. For the highlighting of the anticancer activity and the effect mechanisms of the functions needs the further investigations, particularly *in vivo*. In order to better understand the effects of MSCs on *in vivo* tumor growth, there is a need for further studies and the standardization based on the studies showing the antitumor effect. The long term safety of clinical applications of MSCs remains unclear until the detail investigations.

5. Methods

5.1. Isolation and Culture of Tonsil-Derived Mesenchymal Stem Cells

To isolate human tonsil-derived mesenchymal stem cells, the tonsil tissues were obtained from the donors who have undergone tonsillectomy with donor's consent and approval of OMU clinic research ethics committee (Decision number: 2021/347). The human tonsil tissues were washed with phosphate buffered saline (PBS; Gibco, Cat. No. 10010023) and split into 1-3 mm^3 small pieces. Then, the small pieces were digested with enzymatic solution containing 0,075% collagenase type-I (Sigma Aldrich, Cat.

No.SCR103) in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Cat. No. 41966029) at 37^0C for 30 minutes (min)[42]. Post incubation, the enzymatic activity was inactivated with 10% fetal bovine serum (FBS; Gibco, Cat. No. 10082147)-containing DMEM, and the cell suspension was centrifuged at 1.200xg for 10 min. The cell pellet was resuspended with culture medium consisting of high glucose (4.500mg/ml) DMEM supplemented with 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin (Sigma Aldrich, Cat. No. P4333) and 25µg/ml amphotericin B (Sigma Aldrich, Cat. No. A2942) [43]. Then, the cells were filtered into 100µm cell strainer (Falcon, Cat. No. 352360) and were cultured into T-25 culture flask with their medium. The next day, the adherent cells were washed with PBS following removal of the culture medium containing non-adherent cells. The culture was maintained until reaching the 80-90% confluence and then, the cells were passaged with %0.02 EDTA-containing %0.25 trypsin (Sigma Aldrich, Cat. No. T4049). T-MSCs at passage 4-5 were used in the experiments.

5.2. Characterization of T-MSC with Immunophenotyping and Differentiation Assays

The T-MSCs at passage 4 were characterized by immunophenotyping of surface antigens. T-MSCs specifically express CD106 and CD166 surface antigens in addition to classical MSC markers (positive markers: CD90, CD73, CD105 and negative markers: CD34, CD45) [44]. For immunophenotyping, T-MSCs were tyrpsinized with %0.25 trypsin/EDTA at 37^0C for 5 min and resuspend with 3% FBS in PBS. Then, the $10x10^4$ cells were labelled with Fluorescein Isothiocyanate (FITC)-conjugated anti-human CD90, Phycoerythrin (PE)-conjugated anti-human CD34 and CD45, Allophycocyanin (APC)-conjugated anti-human CD73, and CD166 markers (Biolegend, Cat. No. 328108, 343506, 304058, 323216, 344006, 305810 and 343906, respectively) at 4^0C for 30 min according to the Manufacturer's instructions. Post incubation, the cells were washed with PBS and resuspended with 200µL PBS. The cells were analyzed by flow cytometry (Cytoflex S, Beckman Coulter, Cat. No. C09766).

Moreover, T-MSCs at passage 4 were cultured with the special mediums that are specific to adipogenic and osteogenic differentiations. For adipogenic differentiation, the cells were cultured in the high glucose DMEM supplemented with 10% FBS, 1µM/L dexamethasone (Sigma Aldrich, Cat. No. D1756), 0,5mM/L 3-isobutyl-1 methylxanthine (Sigma Aldrich, Cat. No. I5879), 10µM/L insulin (Sigma Aldrich, Cat. No. I-034) and 200µM indomethacin (Sigma Aldrich, Cat. No. I7378) during 3 weeks by refresh the medium once every 3 days. The end of 3 weeks, the cells were washed with PBS in twice and then, were fixed with 4% paraformaldehyde (PFA; Sigma Aldrich, Cat. No. 158127) at RT for 30 min. Then, the cells were stained with 2% Oil red o solution (Sigma Aldrich, Cat. No. O0625) at room temperature (RT) for 1 hour. After incubation, the cellular lipid droplets were observed under the inverted microscopy following washing with PBS. For osteogenic differentiation, the cells were cultured in high glucose DMEM supplemented with 10% FBS, 1µM/L dexamethasone, 10mM/L β-glycerophosphate (Sigma Aldrich, Cat. No. G9422), 50 µg/mL ascorbic acid (Sigma Aldrich, Cat. No. A92902) during 3 weeks by refresh the medium once every 3 days. The end of 3 weeks, the cells were washed with PBS in twice and then, were fixed with 4% paraformaldehyde at RT for 30 min. Then, the cells were stained with 2% Alizarin Red S (Sigma Aldrich,

Cat. No. A5533) at room temperature (RT) for 15 min. After incubation, the extracellular matrix calcification was observed under the inverted microscopy following washing with PBS [45].

5.3. Culture Conditions of Hematological Cell Lines

From hematological malignancies, chronic myelogenous leukemia (CML) cell line K562 and acute lymphoblastic leukemia (ALL) cell line MOLT-4 were used in this study. These cell lines were provided from Yeditepe University Regenerative Biology laboratory. K562 cells were cultured in high glucose DMEM supplemented with 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin and 25µg/ml amphotericin B, and MOLT-4 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; Gibco, Cat. No. 11875093) supplemented with 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin and 25µg/ml amphotericin B. These cells were suspension cells in the culture medium and they were passaged when reaching 80-90% confluence into new culture flask following directly collection and centrifugation.

5.4. Cell Counting

To determine the effect of co-culture of T-MSCs with hematological cell lines on viable cancer cell number, we seeded $5x10^4$ T-MSCs into 6-well plates with 1ml of T-MSC culture medium. The next day, $5x10^5$ K562 and MOLT-4 cells were seeded on T-MSCs with 2ml of their specific medium. The control cells were K562 and MOLT-4 cells cultured in 1ml of T-MSC medium and 2ml of themselves medium. These cells were incubated at 37^0C and 40^0C to detect the effect of febrile condition on the co-culture. These cells were cultured for 7 days. Besides, the cells planned to be cultured at 40^0C were exposed to 40^0C for 2 hours in every day for 7 days. The viable K562 and MOLT-4 cells were counted following trypan blue (Gibco, Cat. No. 15250061) staining at day3, day5 and day7 with hemacytometer.

5.5. Apoptosis Detection Assay

Apoptosis assay was performed to determine the effect of co-culture of T-MSCs with hematological cell lines on cancer cell death. We seeded $5x10^4$ T-MSCs into 6-well plates with 1ml of T-MSC culture medium. The next day, $5x10^5$ K562 and MOLT-4 cells were seeded on T-MSCs with 2ml of their specific medium. The control K562 and MOLT-4 cells were cultured as explained above. These cells were cultured at 37^0C and febrile 40^0C (for 2 hours in every day) for 7 days. After 5 and 7 days, the cultured cells were directly collected from culture medium. Apoptosis assay was performed according to Manufacturer's instructions (Invitrogen, Cat. No. BMS500FI). Briefly, $10x10^4$ cells were collected form culture medium through centrifugation at 1.500 rpm for 5 min and then, the pellets were resuspended with 50µl of 1x binding buffer. 1µl of FITC-Annexin-V was added into every sample and the samples were incubated at RT for 10 min. Then, the cells were centrifuged and resuspended with 200µl of 1x binding buffer. 2µl of propidium iodide (PI) was added into every sample. Then, the cells were analyzed by flow cytometry.

5.6. Cell Cycle Analysis

We performed cell cycle analysis to determine the effect of co-culture of T-MSCs with hematological cell lines on cancer cell cycling by using PromoKine cell cycle analysis kit (PromoCell, Cat. No. PK-CA577-

K920). We seeded $5x10^4$ T-MSCs into 6-well plates with 1ml of T-MSC culture medium. The next day, $5x10^5$ K562 and MOLT-4 cells were seeded on T-MSCs with 2ml of their specific medium. The control K562 and MOLT-4 cells were cultured as explained above. These cells were cultured at 37^0C and febrile 40^0C (for 2 hours in every day) for 7 days. After 7 days, the cultured cells were directly collected from culture medium. The $10x10^4$ harvested cells were centrifuged at 1.500 rpm for 5 min. Then, the cell pellets were washed with 1 ml ice cold 1x cell cycle assay buffer through centrifugation. The cell pellets were fixed with 2ml of cold 70% ethanol on ice for 30 min. After fixation, the cell suspensions were centrifuged at 1.500 rpm for 5 min and the cells washed with 1ml of 1x cell cycle assay buffer. After centrifugation, the cells were resuspended with 250µl of staining solution that consists of 1,25µl enzyme A solution and 5µl nuclear dye and incubated at RT for 30 min. Then, the cells were analyzed by flow cytometry.

5.7. Gene Expression Analysis

We analyzed the expression of apoptotic (BAX, BCL-2), cell cycle related gene (CDKN1A) and cytokine-coded genes by real-time polymerase chain reaction (RT-PCR). For that, we seeded the T-MSCs and hematological cells for co-culture as explained above. Post 7 days of co-culture, the cancer cells were collected for RT-PCR. RT-PCR was performed RNA isolation and cDNA synthesis steps, respectively. RNA isolation was performed by NucleoZOL RNA purification kit (Macherey-Nagel, Cat. No. 740404.200) according to Manufacturer's instructions. 5µg of isolated RNAs were converted cDNA by using Protoscript First Strand cDNA Synthesis Kit (New England Biolabs, Cat. No. NC0319405) according to Manufacturer's instructions. Finally, the 100-150ng of cDNAs were used for each RT-PCR reaction. The information of primers used for RT-PCR were obtained from Harvard Primer Bank (shown in Table 1). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene was used as a housekeeping gene for normalization. RT-PCR was performed by Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Cat. No. K0222). Post RT-PCR assay, the relative quantification values of gene expressions were calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$).

5.8. Statistical Analysis

Results are expressed as mean ± SEM. "2-tailed Student's t test" was used to determine the level of significance. If the values had $p \leq 0.05$, results were considered statistically significant.

Declarations

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Authors' contributions MY: Conceptualization, investigation, methodology, data curation, validation, funding acquisition, project administration, resources, writing and original draft preparation. EA:

Conceptualization, investigation, methodology, data curation, validation and original draft preparation. All authors read and approved the final manuscript.

Additional Information

Ethical Approval and Consent to participate All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Clinic Research Ethics Committee of the Ondokuz Mayıs University of A (No. 2021/347). Tonsil tissues were obtained after tonsillectomy from patients who applied to the Training and Research Hospital, Department of Otorhinolaryngology, with the consent of the participant. Participant consent is reserved in order to protect the confidentiality of personal information according to the Helsinki Declaration. Informed consent was obtained from all individual participants and legal guardians included in the study. Patients signed informed consent regarding publishing their data.

Consent for publication All authors have agreed to publish this manuscript.

Availability of supporting data All data are available in the article or available from the authors upon request.

Competing interests The authors declare that they have no competing interests.

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Table 1

Table1. Human primer sequences used for RT-PCR

Genes	Forward (5’-3’)	Reverse (5’-3’)
hGAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
hBAX	TCAGGATGCGTCCACCAAGAAG	TGTGTCCACGGCGGCAATCATC
hBCL-2	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC
hC-MYC	CCTGGTGCTCCATGAGGAGAC	CAGACTCTGACCTTTTGCCAGG
hCDKN1A	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG
hp53	GAGGTTGGCTCTGACTGTACC	TCCGTCCCAGTAGATTACCAC
hIL-6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG

Figures

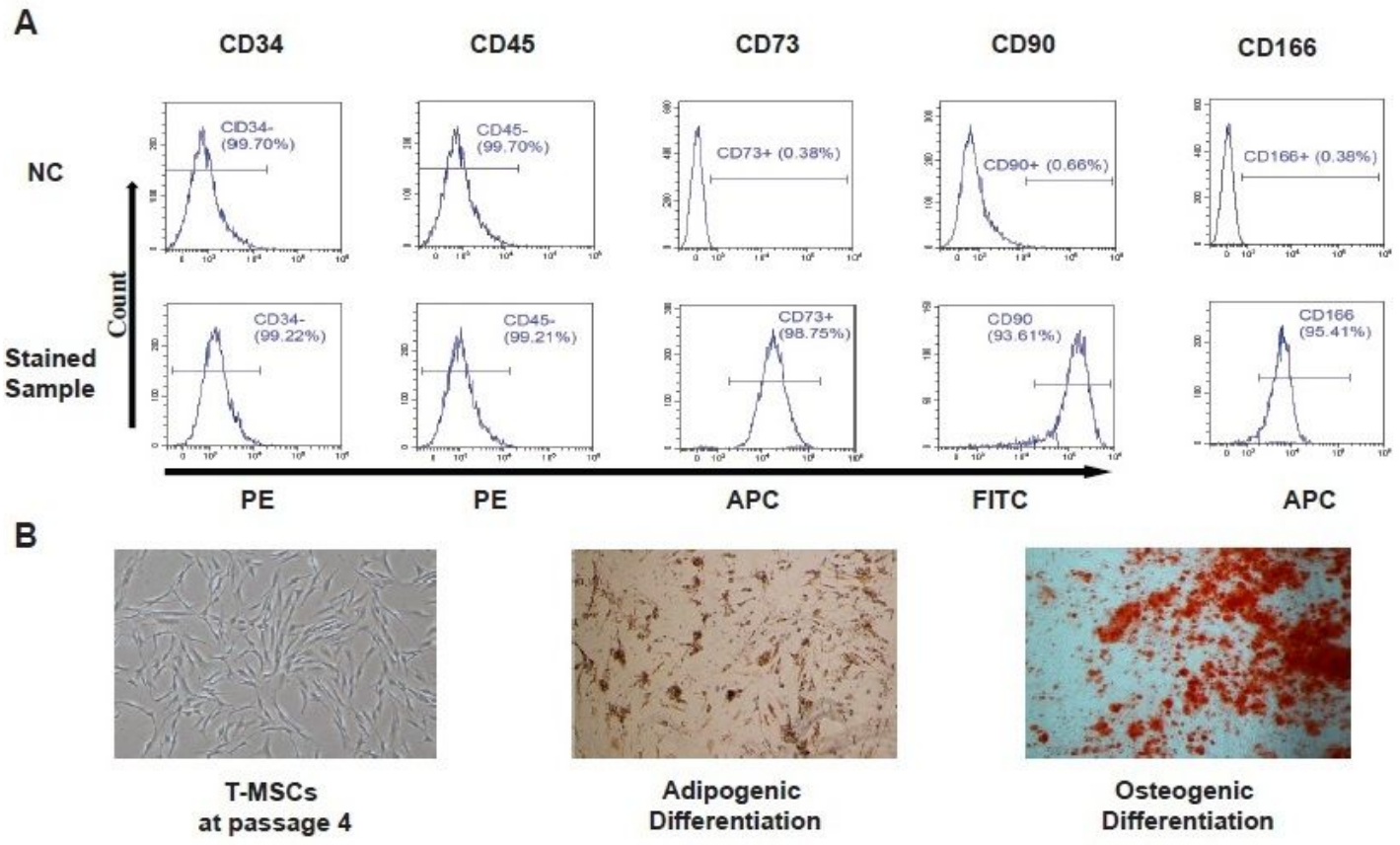

Figure 1

Characterization of newly isolated T-MSCs by determination of the cell surface CD markers and multi-lineage potential A) The expressions of previously identified CD markers on T-MSCs and B) the microscopic view of multi-lineage differentiation post adipogenic and osteogenic specific staining were presented. PE: Phycoerythrin; APC: Allophycocyanin; FITC:Fluorescein isothiocyanate.

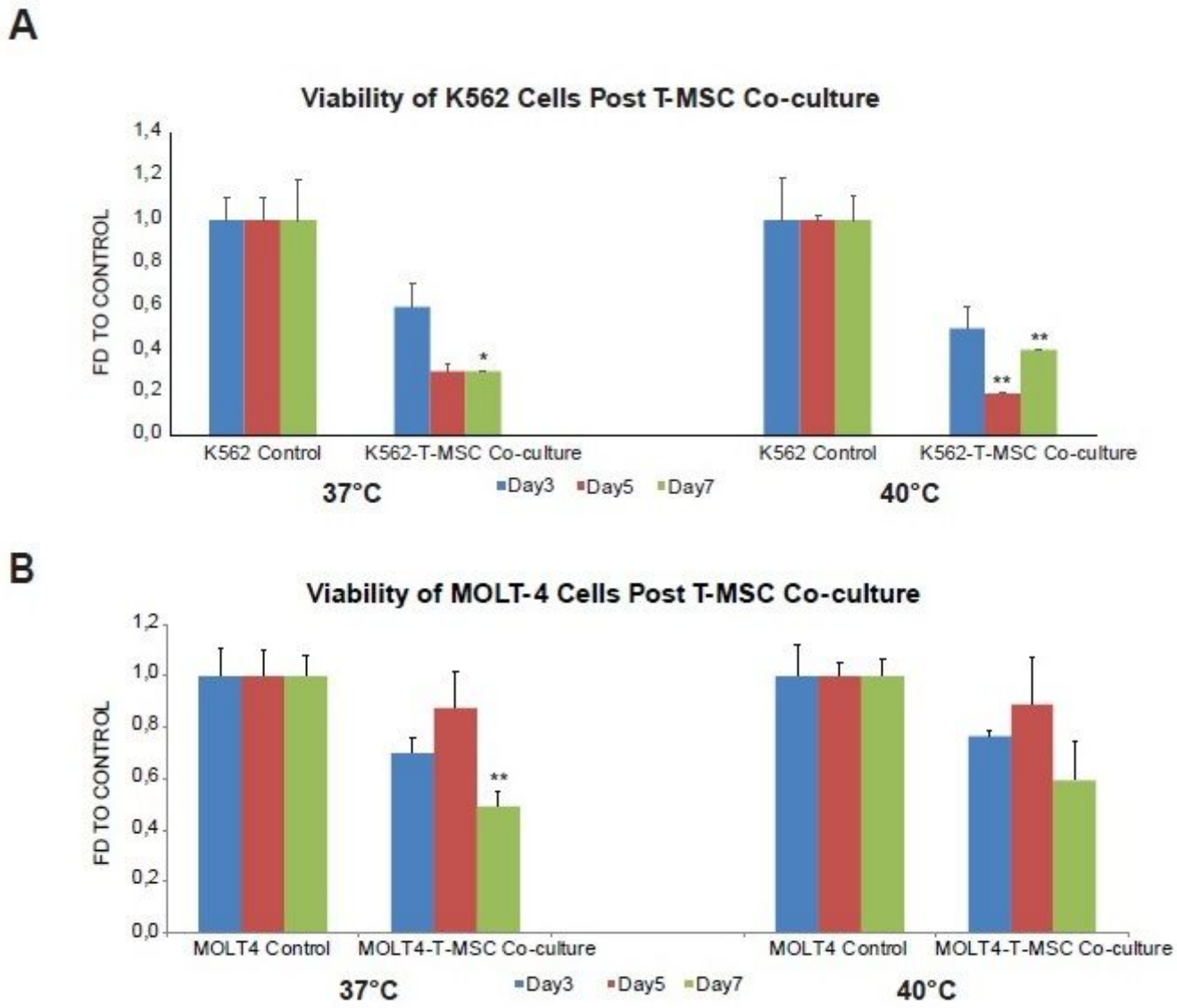

Figure 2

Viable cell count post co-culture of T-MSCs with K562 and MOLT-4 cells The growth inhibitory effect of co-culture of T-MSCs on A) K562 and B) MOLT-4 cells under the normal and febrile temperatures was shown as fold-difference values compared to the control cells. n=3. * p<0,05, ** p<0,01.

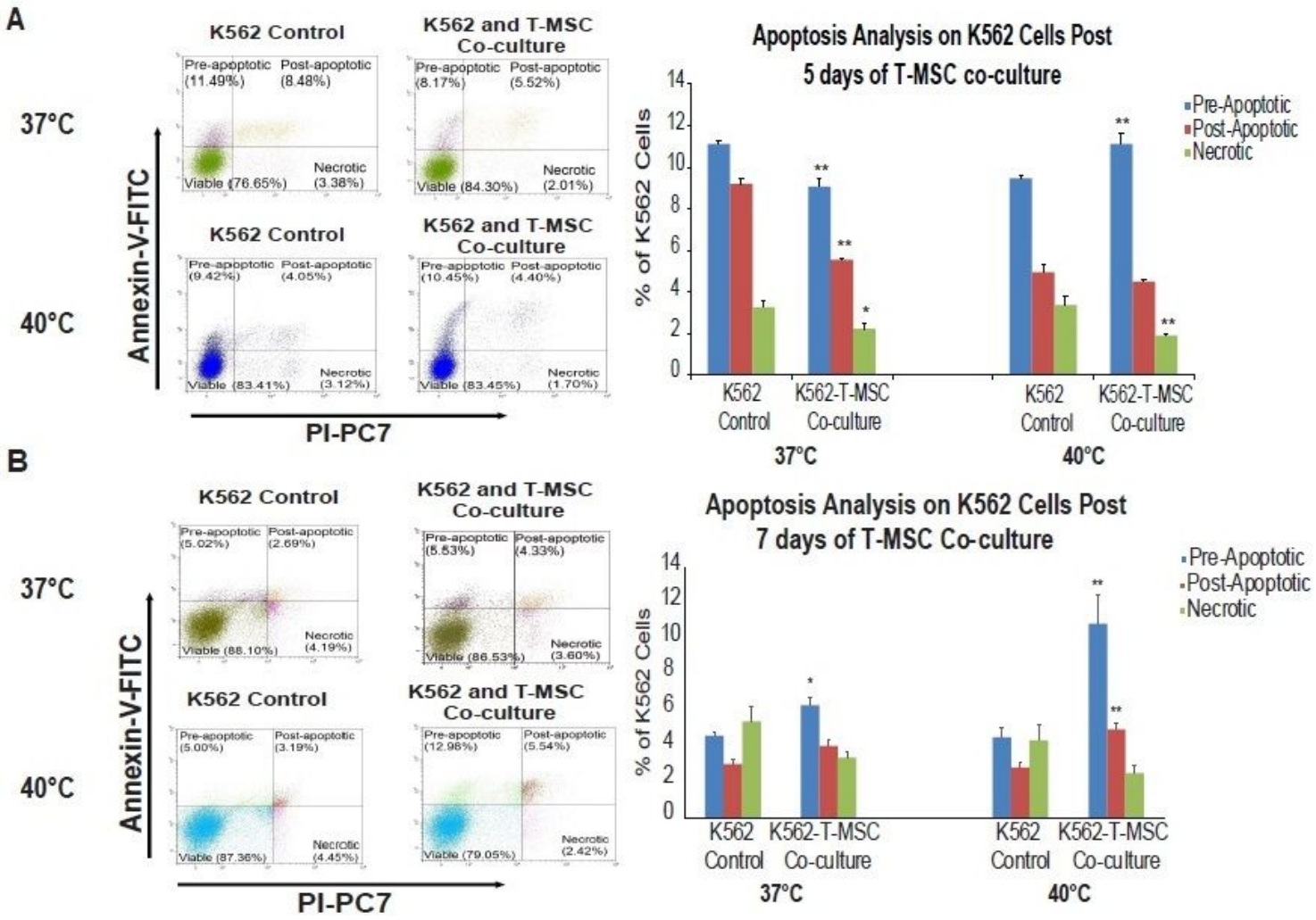

Figure 3

Apoptosis analysis post 7 days of T-MSCs co-culture on K562 cells The flow plots (left) and the quantification graphs (right) for A) 5 days and B) 7 days of co-cultured with T-MSCs under the normal and febrile conditions were shown compared to the control cells. n=4. * p<0,05, ** p<0,01.

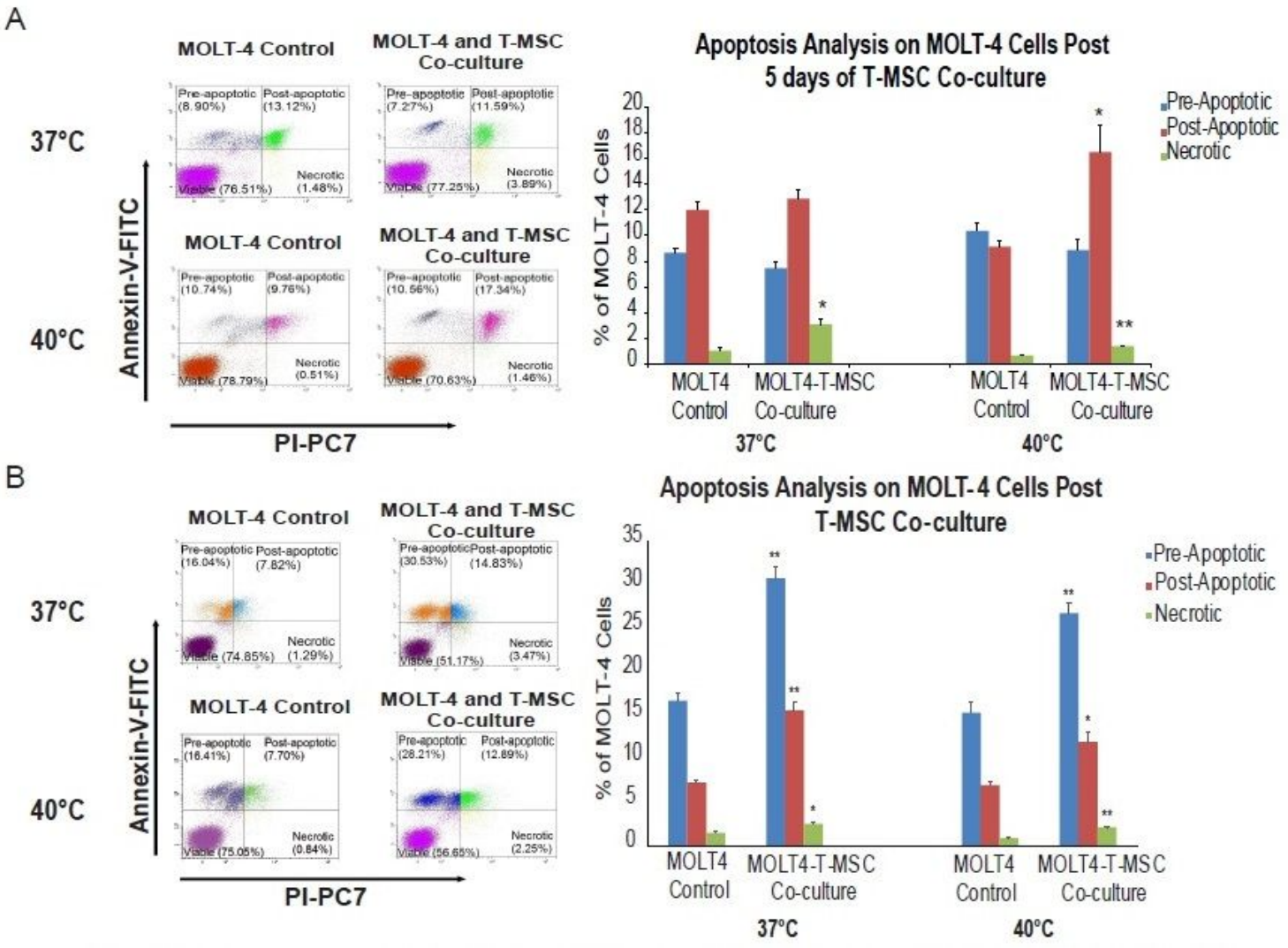

Figure 4

Apoptosis analysis post 7 days of T-MSCs co-culture on MOLT-4 cells The flow plots (left) and the quantification graphs (right) for A) 5 days and B) 7 days of co-cultured with T-MSCs under the normal and febrile conditions were shown compared to the control cells. n=4. * p<0,05, ** p<0,01.

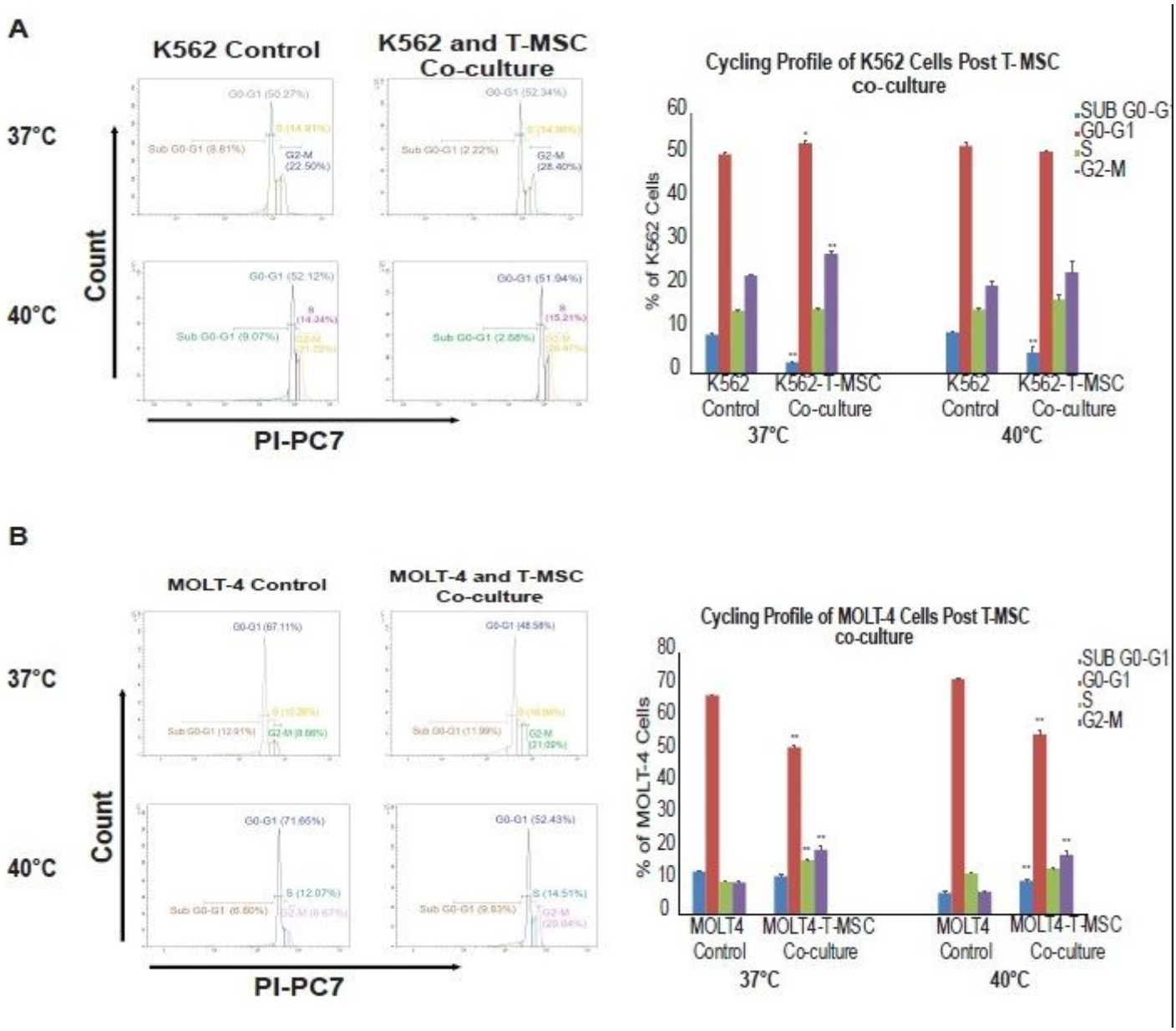

Figure 5

Cell cycle analysis post 7 days of T-MSC co-culture on K562 and MOLT-4 cells The cell cycle analysis was performed on A) K562 and B) MOLT-4 cells post 7 days of co-culture under the normal and febrile conditions. The flow plots (left) and quantification graph (right) are represented. n=4. * p<0,05, ** p<0,01.

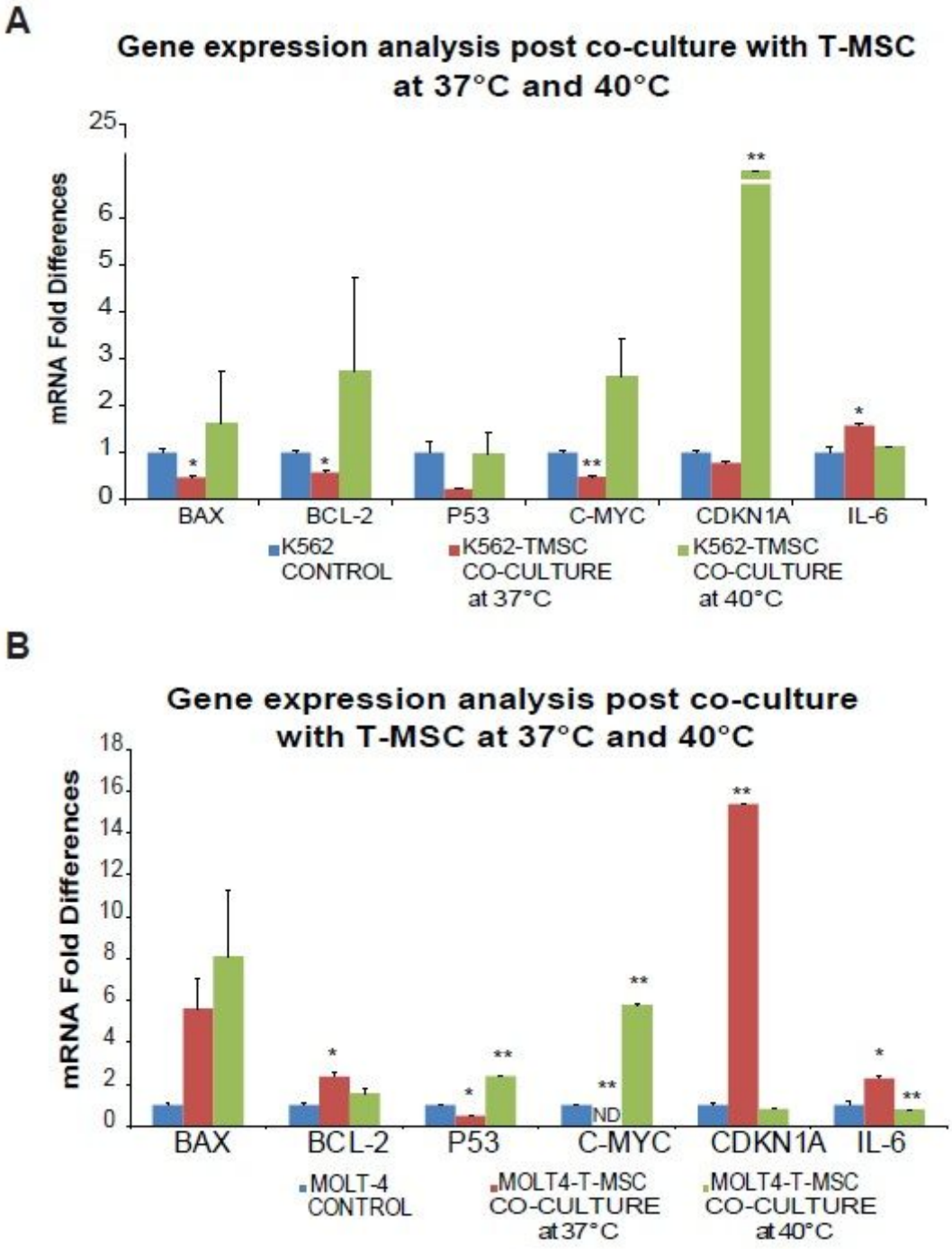

Figure 6

Gene expression analysis post T-MSCs co-culture with K562 and MOLT-4 cells The expression profiles of the apoptosis, cell cycle and cytokine- related genes were shown for A) K562 cells and B) MOLT-4 cells co-cultured under the normal and febrile conditions. The gene expressions were normalized to GAPDH and also, compared to the control samples according to 2-ΔΔCt method. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; ND: Not determined. * p<0,05, ** p<0,01.