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Research Article

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Thymoquinone Treatment Enhances T Cell-Mediated Anti-Tumor Response *In Vitro*

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Abstract

Oil from Nigella sativa seeds ("black seed oil") has been used for health care and medical purposes for over 2000 years. The major active compound thymoquinone (TQ) has been shown to have immune regulatory effects in various diseases. Tumor microenvironment is critical for tumor development. Immune cells, particularly T cells, are central players to kill tumor cells and affect tumor progression and metastasis. The impact of TQ on human T cell-mediated anti-tumor response was investigated. Using human PBMCs, we activated T cells with anti-CD3/CD28 in vitro, and treated cells with TQ. First, we tested the impact of TQ on T cell activation, proliferation and apoptosis; second, the anti-tumor effect of T cells after treatment with TQ; last, we determined alterations in T cells gene expression at the RNA level after TQ treatment. TQ treatment increased activation and proliferation of T cells at days 2-4 and more activation-induced cell death at day 3. After 7-11 days, TQ-treated cells showed less survival than cells treated with control medium. At low TQ concentration (10uM), treatment induced more CD3+ T cell proliferation; while at 20uM and 50uM TQ showed more cytotoxic effect to T cells, and more cell death. When co-cultured with multiple types of cancer cells, TQ-treated T cells exhibited significantly enhanced anti-tumor activity, indicated by more tumor cell apoptosis and less cell expansion. Gene expression study showed upregulation of NFAT signaling pathway, including 10+ fold up-regulation of IL-2, which might explain the enhanced anti-tumor effect after TQ treatment. In summary, TQ treatment can enhance human T cell-mediated anti-tumor effect in vitro. This immunoregulatory function of black seed oil could potentially benefit patients of different cancer types.

Keywords: Thymoquinone, T Cell-Mediated, Anti-Tumor

1. Background

The tumor microenvironment (TME) plays a critical role in regulating tumor growth and death, both in primary tumors and metastatic malignancies. Alongside cancer cells, the TME comprises various non-tumor cell types, including endothelial cells, pericytes, fibroblasts, and immune cells [1]. Among these, immune cells act as central players within the TME, exerting either pro-tumor or anti-tumor effects on tumor progression and metastasis [2]. T lymphocytes (T cells), particularly CD8+ T cells, possess remarkable cytotoxic capabilities, making them potent weapons against tumor cells. The discovery of immunotherapies targeting T cells has revolutionized cancer treatment. However, the activation and differentiation status of T cells are critical factors influencing their function in cancer development and progression. Th1 cells, a subset of T helper cells, promote a proinflammatory phenotype associated with tumor suppression, while Th2 cells orchestrate an immunosuppressive phenotype that may enhance tumor progression. Various stimulation factors, arising from tumor cell secretions, regulatory mechanisms of other cell types, or external treatments, can determine whether T cells become activated or anergic, functional or dysfunctional, proliferated or exhausted. These factors have a significant impact on the outcome of cancer progression [3-5]. Therefore, understanding the stimulation and differentiation processes of immune cells is crucial for achieving effective anti-tumor immune responses.

Oil from *Nigella sativa* seeds, also known as black seed oil, has been used world-wide for culinary and medical purposes for over 2000 years. It has been shown to have regulatory functions and protective effects in many diseases, including diabetes, infections, cardiovascular disease, gastroprotection, autoimmune disease and cancers [6-13].

Many active compounds have been isolated and identified in different varieties of *Nigella sativa*. Among them, thymoquinone (TQ) stands out as the most abundant and extensively studied. TQ is a phytochemical compound that undergoes di-thymoquinone and higher oligo-condensation product formation during storage. Previous studies have demonstrated the remarkable antioxidant and anti-inflammatory effects of TQ using various experimental mod-

els. Additionally, TQ has exhibited anti-tumor properties against a wide range of malignancies, including pancreatic cancer colon cancer, renal cell carcinoma, Ehrlich ascites carcinoma lymphoma, skin cancer, stomach cancer and osteosarcoma [14-20]. The mechanisms underlying its anti-tumor effects involve the induction of apoptosis through increased expression of tumor suppressors p53 and p21WAF1 (also known as p21CIP1) in colon cancer, inhibition of anti-apoptotic protein BCL-2; down-regulation of NF-κB expression; interference with DNA synthesis and modulation of various enzymatic functions [15,18,20]. However, the precise mechanisms through which compounds derived from Nigella sativa exert their anti-tumor effects remain unclear. Therefore, gaining a more comprehensive understanding of how Nigella sativa compounds stimulate immune cell activation, proliferation, and differentiation is crucial for overcoming therapeutic challenges and developing novel interventions for potential cancer treatment.

2. Methods and Reagents

2.1 Human PBMCs activation and culture in vitro

The human PBMCs were purchased from ATCC (ATCC®PCS-800-011) and cultured in vitro with proper medium (Immunocult-XF T cell expansion medium from StEMCELL technologies, cat#10981) in 37 °C incubator with 5% CO2. PBMCs consist of all leukocytes with single nucleus in human peripheral blood, and the majority population will be lymphocytes (~50% T cells, ~10% B cells and ~7 %NK cells) and monocytes (~20%). After activated with anti-CD3 and anti-CD28 (ImmunoCult™ Human CD3/CD28 T Cell Activator from STEMCELL technologies, cat# 10971) and in the presence of human recombinant IL-2 (STEMCELL technologies, cat# NC1270616), the majority cell population will be T cells (over 70% by day 7 based on our flow cytometry check). Cells were cultured in either 12-well plate or 24-well plate for at most 2 weeks.

2.2 Thymoquinone Treatment

Thymoquinone powder is purchased from Sigma-Alorich (Lot# MKCB1381V) and dissolved in DMSO for different treatment concentration. We used three doses of Thymoquinone solution, 10uM, 20uM, 50uM, to treat human PBMCs, which not shown to be too toxic to kill the T cells based on our pilot titration experiments, the control group of cells were treated with DMSO only (which shown as 0uM).

2.3 T Cell Proliferation and Apoptosis Evaluation

Human PBMCs were activated, treated and cultured as described above. For cell proliferation evaluation, at different time point (indicated in the results), cells were stained by trypan blue and counted manually by hemocytometer. Only live and small circle cells were counted, while the adherend cells attached on the bottom were ignored (not T cells).

For cell apoptosis evaluation, cells were harvested and washed once with PBS, then stained with Guava Nexin Reagent (Lot # 2731396) following instruction, and analyzed by Guava easyCyte for apoptosis and cell death.

2.4 Tumor Killing Assay

Human PBMCs were activated, treated and cultured as described above. 5 days later, cells were harvested and washed with PBS twice before cell counting. Only live cells were counted and 10^5 were added into pre-seeded tumor cell culture medium. Tumor cells used for co-culture include breast cancer cell line (MCF-7), pancreatic cancer line (Panc-1) and liver cancer line (HUH7.5). 10^4 tumor cells were pre-seeded in 12-well plate 2 days before co-culture, and after mixed with immune cells, the cell mixture were cultured for another 2 days before harvest for analysis of cell proliferation and apoptosis.

2.5 RNA Isolation and cDNA Synthesis

Human PBMCs were activated in vitro and treated with 10 μM of TQ (Sigma Aldrich). Treated cells were then seeded into 24 well plates. On day 2, 1 ml of Il-2 added ImmunoCultTM medium (STEMCELL Technologies) was added to each well. On day 4, total RNA was isolated from each treatment using Qiagen RNeasy Total RNA Isolation Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The quantity of RNA was measured using a spectrophotometer (Thermo Scientific). 500ng of total RNA was reverse transcribed into first strand cDNA using ImProm-II Reverse Transcription System (Promega, Madison, WI) following the protocol provided by the manufacturer.

2.6 qRT-PCR and Gene Expression Studies

mRNA expression levels of NFATC-1, NFATC-5, and IL-2 were examined using realtime RT-PCR. qRT-PCR was carried out in ABI Prism 7000 Sequence Detection System (Applied Biosystems) using Luna® Universal qPCR Master Mix (New England Biolabs® Inc.). Briefly, the experimental condition for qRT-PCR is as follows: denaturation at 95°C for 2:30, followed by 40 PCR cycles of 95°C for 15 seconds and 65°C for 1:30. The primer used, and their sequences are as follows: 18s rRNA forward: 5'-AGAAACGGCTACCACATCCA-3', reverse 5'-CACCAGACTTGCCCTCCA-3'; NFATc4 forward: 5'- CTTCTCCGATGCCTCTGACG-3', reverse: 5'- CGGGGCTTGGACCATACAG-3', NFAT5 forward: 5'- GGGTCAAACGACGAGATTGTG-3', reverse: 5'- GTCCGTGGTAAGCTGAGAAAG-3'; NF-KB2 forward: 5'- GGGCCGAAAGACCTATCC-3', reverse: 5'- CAGCTCCGAGCATTGCTTG-3'; GZMB forward: 5'- CCCTGGGAAAACACTCACACA-3', reverse: 5'- GCACAACTCAATGGTACTGTCG-3'; TNF forward: 5'- GAGGCCAAGCCCTGGTAT-3', reverse: 5'- CGGGCCGATTGATCTCAGC-3'; IL-10 forward: 5'- GACTTTAAGGGTTACCTGGGTTG=-3', reverse: 5'- TCACATGCGCCTTGATGTCTG-3'. IL-2 forward: 5'-AACTCCTGTCTTGCATTGCAC-3', reverse-5'GCTCCAGTTGTAGCTGTGTTT-3'; and NF-ķB1 forward: 5'-GGTGCGGCTCATGTTTACAG-3', reverse: 5'-GATGGCGTCTGATACCACGG-3'.

A melting point dissociation curve showing a single sharp peak was

used to confirm product specificity after the completion of the PCR run. 18S ribosomal RNA (18S rRNA) was used as a housekeeping gene. The mRNA expression level of target genes was normalized against 18S rRNA. The fold change in mRNA expression of target genes, relative to control, was calculated using a comparative method based on ΔCt values (2-ΔΔCt method) [29].

3. Result

3.1 TQ Altered Human T Cell Activation and Proliferation in Vitro

To assess the impact of TQ on immune cell activation and proliferation, we conducted in vitro cultures using human PBMCs. The cells were treated with varying concentrations of TQ or control media, and subsequently, cell number and viability were analyzed at different time points. To examine the effect of treatment timing on T cell activation and proliferation, we implemented two distinct culture setups:1. Treatment during T cell activation: TQ was added to the culture medium concurrently with anti-CD3/CD28 and IL-2 stimulation (Figure 1A-D); 2. Treatment after T cell activation: TQ was introduced two days following anti-CD3/CD28 and IL-2 stimulation (Figure 1E-H). Our findings revealed that TQ treatment during T cell activation led to increased early activation and proliferation of T cells, primarily observed on days 2-3. However, after 5-7 days, cell proliferation started to decline. Conversely, TQ treatment after T cell activation predominantly exerted a cytotoxic effect, significantly reducing cell proliferation. Furthermore, we observed a dose-dependent relationship, where higher concentrations of TQ exhibited greater cytotoxic effects.

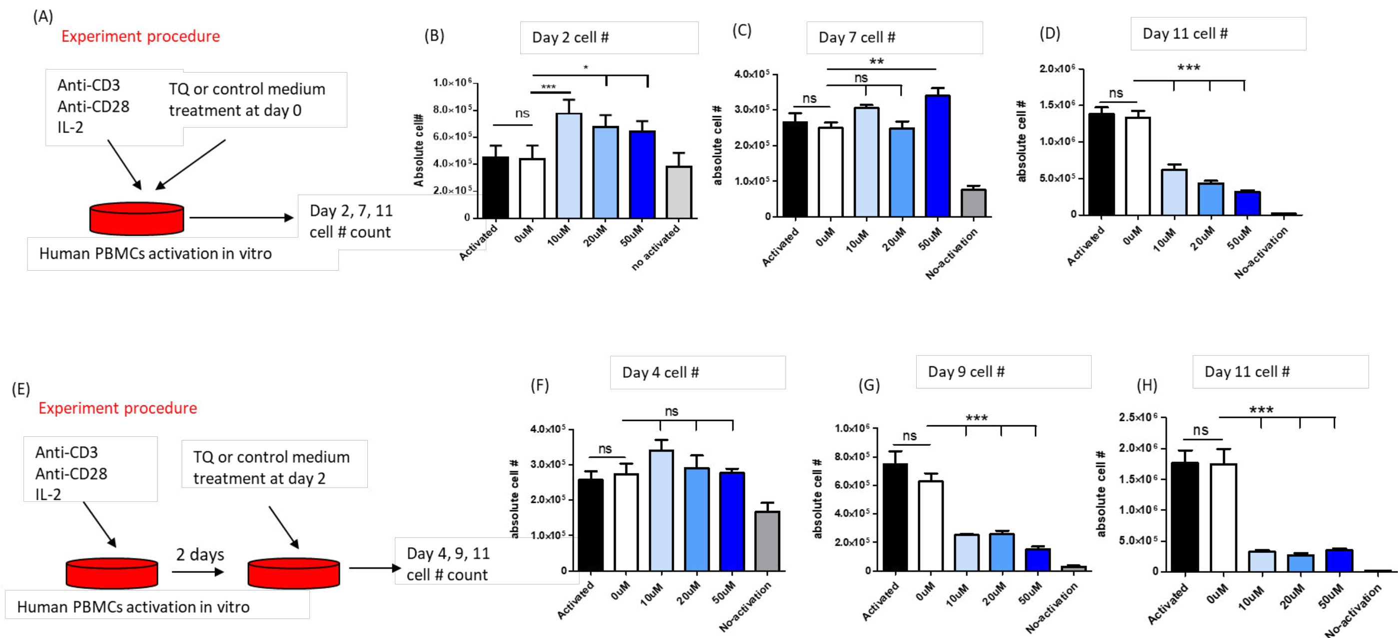

Figure 1: (A) Experiment procedure of early TQ treatment. Human PBMCs were activated with anti-CD3+anti-CD28 and IL-2 in vitro, simultaneously, cells were treated with control medium or different concentration of TQ, no activated cells without any treatment also set up as negative control. Cells were cultured for about 2 weeks to evaluate cell proliferation. (B), (C) and (D) showed cell number counted with hemocytometer at day 2, 7 and 11. Statistical significances were determined by one-way ANOVA. (E) Experiment procedure of late TQ treatment, which is similar procedure as described above, except TQ treatment 2 days after T cell activation. (F), (G) and (H) showed cell number counted with hemocytometer at day 4, 9 and 11. Statistical significances were determined by one-way ANOVA.

3.2 Low dose TQ Treatment Enhanced T Cell Activation with Lower Cytotoxic Effect

To determine the optimal TQ concentration that promotes T cell growth without inducing excessive cytotoxicity, we utilized flow cytometry to analyze the T cell population within the PBMCs five days after T cell activation and TQ treatment. Interestingly, while higher concentrations of TQ resulted in the elimination of a majority of T cells, a lower concentration of TQ (10uM) exhibited a pronounced stimulatory effect. Specifically, the lower TQ concentration significantly increased the percentage of CD3+ T cells at day 7, while demonstrating a milder cytotoxic effect compared to higher concentrations. These findings suggest that administering TQ at a low dose may potentially enhance T cell function, providing a beneficial effect. (Figure 2A-C)

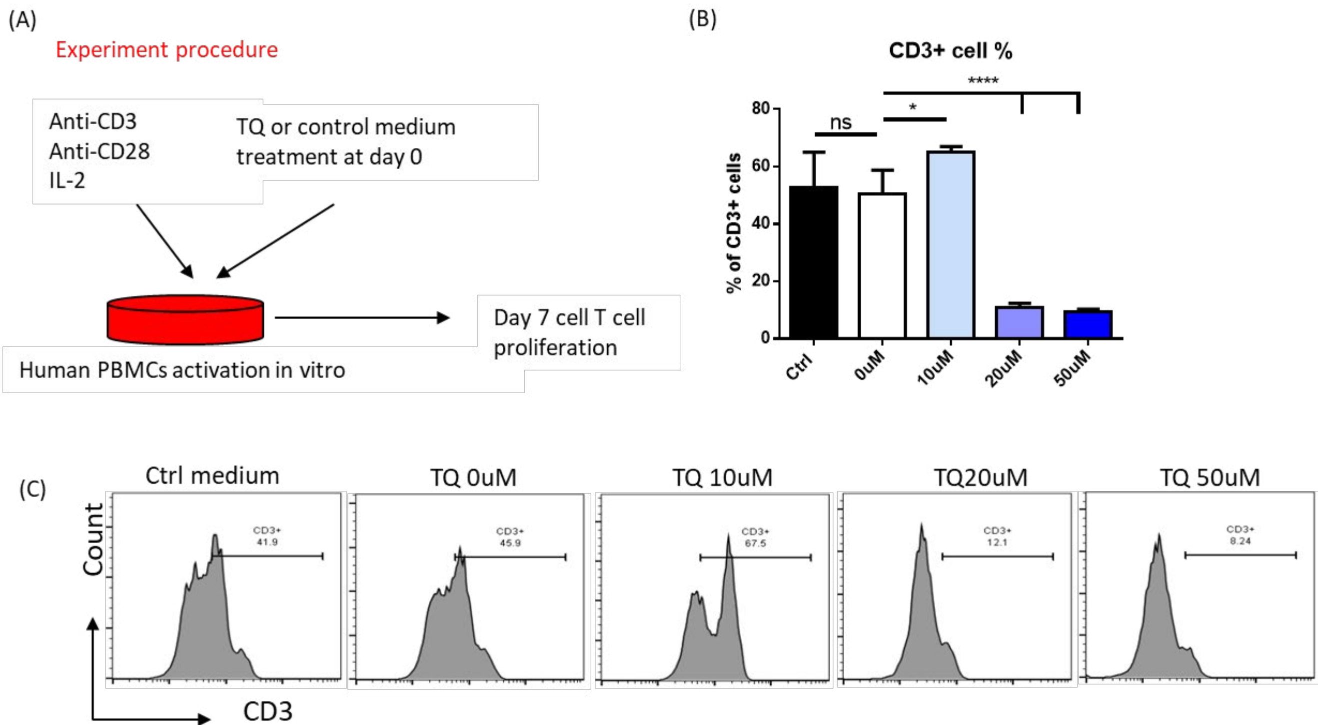

Figure 2: (A) Experiment procedure. Human PBMCs were activated with anti-CD3+anti-CD28 and IL-2 in vitro, simultaneously, cells were treated with control medium or different concentration of TQ. Cells were cultured for 7 days to evaluate CD3+ T cell proliferation. (B, C) Figure and representative picture showed T cell percentage of total cells which defined by CD3+ %. Statistical significances were determined by one-way ANOVA.

3.2 TQ Treatment Induced More T Cell Activation-Induced Cell Death

To investigate the impact of TQ on T cell activation-induced cell death in vitro, we employed the following experimental procedure: human PBMCs were activated and cultured as previously described. Subsequently, the cells were treated with either control medium or varying concentrations of TQ. After three days, the cells were stained with Annexin V and PI to assess apoptosis and cell death using flow cytometry. Our results demonstrated that compared to activated T cells without any treatment, TQ administration at day 0 led to a significant increase in cell apoptosis. Notably, this effect was particularly prominent in the late apoptosis population, as indicated by the Annexin V-positive and PI-positive cells. (Figure 3A-C)

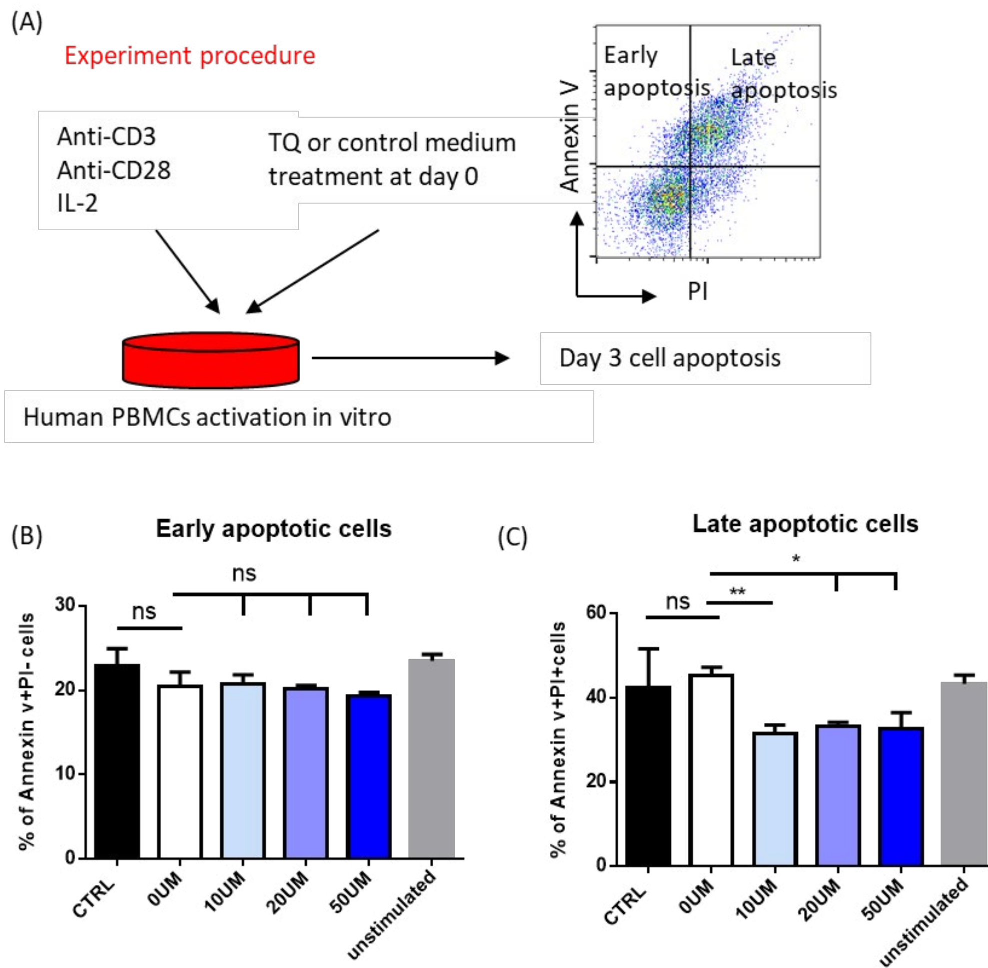

Figure 3: (A) Experiment procedure and representative picture of definition of early and late apoptosis. Human PBMCs were activated with anti-CD3+anti-CD28 and IL-2 in vitro, simultaneously, cells were treated with control medium or different concentration of TQ, no activated cells without any treatment also set up as negative control. cells were cultured for 3 days to evaluate cell apoptosis. (B) and (C) showed cell early apoptosis which defined by Annexin V+ PI- % and late apoptosis which defined by Annexin V+ PI+ %. Statistical significances were determined by one-way ANOVA.

3.3 TQ Treatment Enhanced T Cell Anti-Tumor Activity in Vitro.

To assess whether TQ treatment can modulate T cell-mediated anti-tumor effects, we conducted in vitro experiments using human PBMCs. As described previously, the cells were treated with TQ or control media. On day 5, immune cells were harvested and co-cultured with pre-seeded tumor cells. Following an additional 2 days of co-culture, we analyzed tumor cell growth and apoptosis to evaluate the anti-tumor efficacy of T cells (Figure 4A). Notably, we observed that TQ treatment during T cell activation significantly enhanced T cell-mediated anti-tumor activity when co-culture with pancreatic cancer cells Panc-1 (Figure 4B-C). We further performed similar experiments using hormone receptor positive breast cancer cells MCF7 (Figure 4D-E), and liver cancer cells HUH7.5 (Figure 4F-G). Encouragingly, consistent results were obtained across these different cancer cell lines, demonstrating that low-dose TQ treatment (10uM or 20uM) augmented the anti-tumor effects of human T cells.

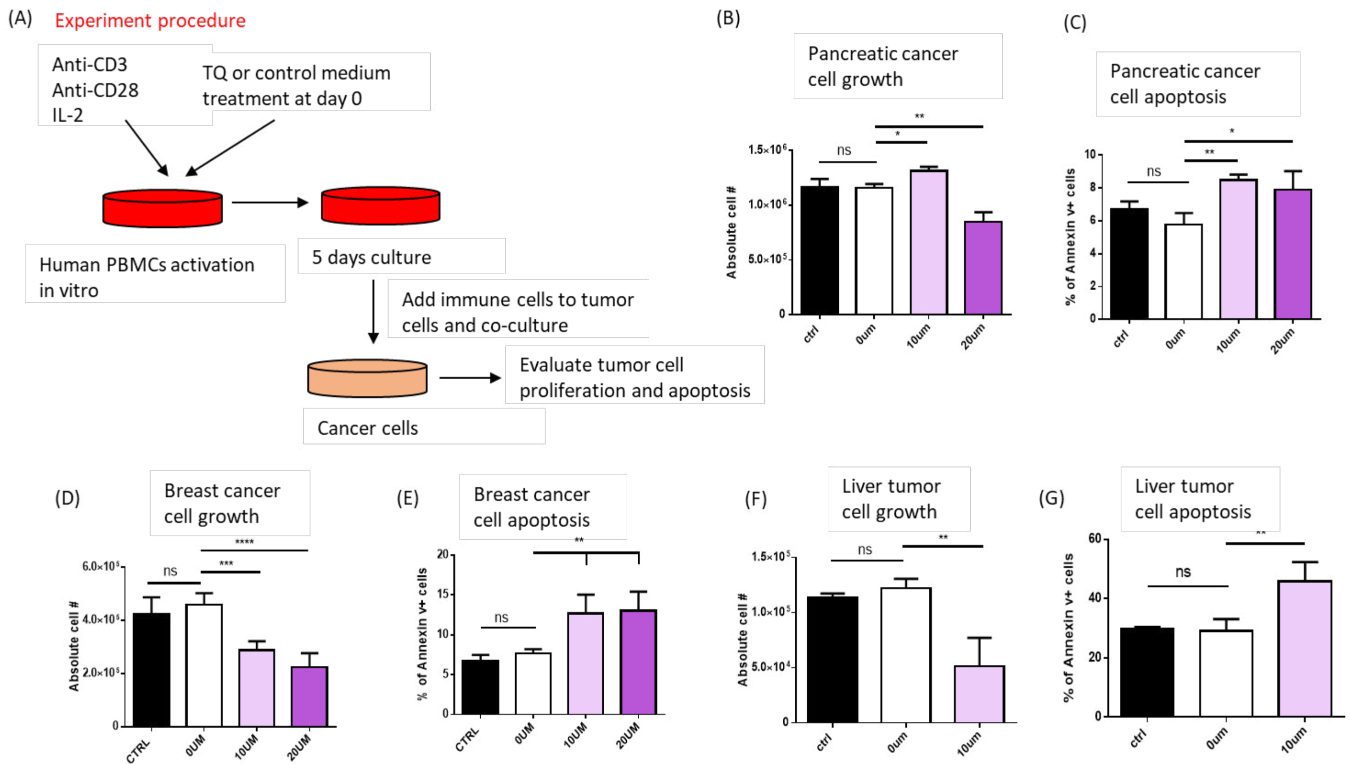

Figure 4: (A) Experiment procedure. Human PBMCs were activated with anti-CD3+anti-CD28 and IL-2 in vitro, simultaneously, cells were treated with control medium or different concentration of TQ. Cells were cultured for 5 days to allow T cell proliferation. At day 5, T cells were added to pancreatic cancer cells Panc-1 and co-culture for another 2 days. Tumor cell growth were determined by cell counting (B) and apoptosis evaluation were done by flow cytometry of Annexin V+ cells (C). (D) and (E) showed cell apoptosis evaluation with breast cancer cell, and (F) and (G) showed liver cancer cell with same procedure. Statistical significances were determined by one-way ANOVA.

3.4 TQ Treatment Altered T Cell Activation Pathway in Gene Level.

To explore the impact of TQ treatment on T cell activation at the genetic level, we extracted RNA from T cells four days after activation and treatment, followed by q-PCR analysis (Figure 5A). Our findings revealed several notable changes in gene expression. Specifically, we observed a significant decrease in NF-KB levels, as well as reduction of TNFa expression (Figure 5B-D). These results align with previous reports highlighting the anti-inflammatory effects of TQ. Moreover, we noted a substantial increase in the NFAT signaling pathway, characterized by the significant upregulation of NFATc4, NFAT5 (Figure 5E-F). Notably, with respect to downstream cytokine gene transcription, we observed a significant ten-fold increase in IL-2 expression compared to control T cells, along with a threefold increase in IL-10 expression (Figure 5G-H). Granzyme B (GZMB) expression significantly decreased, which indicated that the enhanced anti-tumor function is not through cytotoxic effect (Figure 5I).

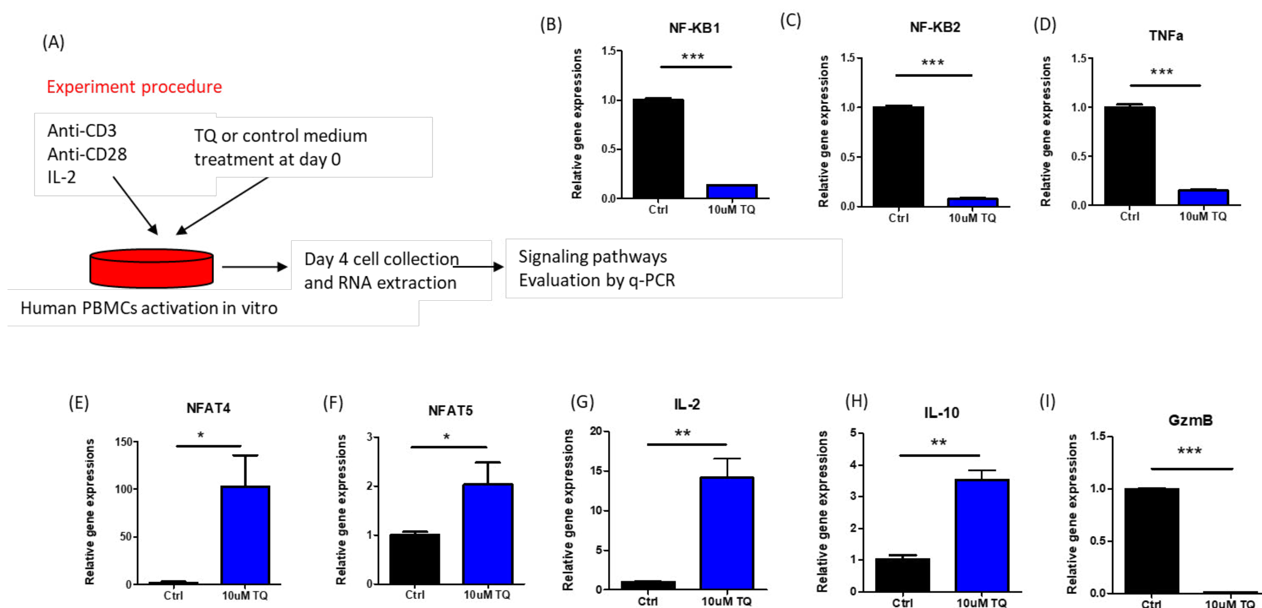

Figure 5: (A) Experiment procedure. Human PBMCs were activated with anti-CD3+anti-CD28 and IL-2 in vitro, simultaneously, cells were treated with control medium or different concentration of TQ. Cells were cultured for 4 days to allow T cell proliferation. At day 4, cells were collected and RNA was extracted for q-PCR analysis. (B)-(I) Relative gene expression change for different targets. Statistical significances were determined by student t-test.

4. Discussion

Thymoquinone (TQ), one of the predominant bioactive components found in Nigella sativa, has demonstrated significant anti-cancer properties against various malignancies. The underlying mechanisms of action involve the inhibition of cancer cell cycle progression and disruption of microtubule organization, as well as its antioxidant properties, ability to prevent chronic inflammation, inhibition of angiogenesis and metastasis, and modulation of cancer-related genes [21-23]. Researchers have also investigated the synergistic effects of TQ, such as its potential to improve chemotherapy outcomes in cancer patients [24-26]. Cancer development is a complex and chronic process that relies on the intricate interplay between cancer cells and immune cells. While certain immune cell types, including macrophages, neutrophils, and specific subsets of T cells, can promote tumor cell growth and metastasis, T cells play a crucial role in killing tumor cells and suppressing cancer development [2]. Cancer immunotherapy has exhibited remarkable success in treating various cancer types, such as melanoma and lung cancer. However, its efficacy remains limited in certain cancer types, including pancreatic cancer and liver cancer [27,28].

In our study, we investigated the therapeutic potential of TQ in enhancing human T cell-mediated anti-tumor effects. We observed that early treatment with TQ during the T cell activation phase resulted in increased T cell proliferation prior to the onset of its cytotoxic effects. Notably, these over-activated T cells demonstrated significantly enhanced tumor-killing activity in vitro when co-cultured with various cancer cell lines, including liver cancer, pancreatic cancer, and breast cancer cells. Mechanistic analysis revealed the upregulation of the NFAT signaling pathway and a substantial increase in IL-2 transcription, which could explain the transient boost in T cell proliferation and the augmented anti-tumor effects observed. These unexpected findings provide optimism for the potential of TQ to enhance immunotherapy outcomes, particularly in cancer types that exhibit resistance to immunotherapy alone. Although our study was limited to in vitro experiments, future directions include conducting in vivo animal experiments to validate our results. Given the long history of Nigella sativa use by humans, TQ holds promise as a safe compound for potential clinical trials.

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