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The Effect of Antigen Dose and Antigen Presenting Process on T Cell Stimulation: A Method for Enrichment of TB10.4 Antigen-specific T-cell Clones

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ABSTRACT

T-lymphocytes have critical functions in the immune responses against viral and intracellular bacterial infections as well as cancers. Antigen (Ag)-specific T-lymphocyte clones enriched and expanded *in vitro* are valuable tools in the study of immune responses in animal models and adoptive T-cell therapy of patients with cancer or infection.

We described a method for inducing, enriching, and replicating Ag-specific poly-clonal T-cells from BALB/c mice infected with live *Bacillus Calmette Guérin* (BCG) bacterium. During a 7-8 days procedure, T-lymphocytes were purified from immune cells of lymph nodes stimulated with immunodominant Ag of BCG, TB10.4, and expanded by interleukin -2 cytokine. We evaluated the effect of Ag doses (1, 10, and 100 µg/mL) and exposure method of Ag presenting cells (APCs) to T-cells, on T-cells' proliferation, viability, and Interferon-gamma (IFN-γ) secretion at 2, 5, and 7 days after Ag stimulation.

Increasing Ag concentration increased the average cell division, but at the highest dose of Ag (100 µg/mL), T-cell viability is decreased. Only clones induced by 10 µg/mL Ag produced a desirable amount of IFN-γ. Incubation of Ag and APCs, 24 h before T-lymphocytes addition, increased the proliferation and viability of cells. T cells are in a more favorable condition around day 5 of Ag stimulation in terms of proliferation and survival, and it is the desired time for T cell restimulation.

For optimal preparation of specific T-cells for adoptive cell transfer, optimization of Ag dose, the order of APCs and T-cells exposure with Ag, and the duration of initial Ag stimulation, as well as the time for restimulation, is essential.

Keywords: Antigens; Antigen-presenting cells; Cell- and tissue-based therapy; Clone cells; Immunotherapy; T-lymphocytes

INTRODUCTION

T lymphocyte is a key player of the immune responses in viral and intracellular bacterial infections and cancer. T cell recognizes antigen (Ag) presented by

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MHC molecules expressed on Ag presenting cells (APC), including macrophages and dendritic cells. This interaction activates Ag-specific T cells to start the clonal expansion and differentiate into effector subsets. As a result, a repertoire of Ag-specific T lymphocytes emerges that can recognize the Ag, derived from invading pathogen and generate a wide range of immune responses.[1] The existence of several Ag-specific T-cell clones, including those specific to immunoprotective and virulent Ags of the pathogen, is essential for a protective and efficient immune response.[2,3] *In vitro* isolation and expansion of Ag-specific T cell clones are essential tools for studying immune responses in cancer and infectious diseases in animal models.[4,5,6] Also, it is the main stage of cell preparation for adoptive T cell therapy (ACT) of patients with cancer or chronic infections.[4] ACT usually involves an *in vitro* process in which autologous or allogeneic T cells taken from a patient or donor are stimulated and activated by the target Ag. Then specific clones are amplified in large numbers and infused into the patient. The *in vitro* process has three foremost steps: selecting appropriate target Ag, enrichment and isolation of T-cell clones, and expansion of Ag-specific T-cell clones.[5]

To select the appropriate Ag, immunogenic, and immunodominant Ags of a pathogen are screened using experimental and bioinformatics studies. Then, those that we're able to stimulate T lymphocytes and elicit protective immune responses are used.[6] The selected Ag can be a cell surface molecule belonging to a pathogen that is produced and purified as recombinant protein and used to stimulate the T cell population.[7,8] The method used for enriching and isolating Ag-specific T-cell clones is the next challenge in this process. It performs by adding Ag to the culture medium containing APCs to uptake Ag by their receptors and present it by MHC molecules. T lymphocytes recognize presented peptides by T cell receptors (TCRs), and Ag-specific clones are stimulated and activated. The efficiency of this step depends on various factors such as the concentration of Ag (Ag dose), method and duration of APCs and T cells exposure, and repetition of Ag stimulation.[4,5,9] In the final stage of work, high doses of interleukin(IL)-2 are used to expand specific T cells; however, without Ag restimulation, these cells are relatively short-lived.

The ideal method for enriching specific T clones should have the least manipulation steps so that T cells do not get exhausted or lose their function and can persist for a long-time *in vivo*. The procedure should produce polyclonal T cells specific for Ag, to identify the target cells and allow efficient protection against pathogens.[10] This means that immunotherapy studies usually require a large number of T cells, so we need techniques that eventually produce large numbers of cells.[11] In laboratory studies performed using mouse T lymphocytes to model T cell therapy trials in humans, conventional cellular sources for T lymphocytes' isolation are the mice spleen and lymph nodes. When using splenocytes as cell sources, we must first separate mononuclear cells from other blood cells by a Ficoll density fractionation. But in the use of lymph node cells, this step is not required. On the other hand, T cells constitute up to 25% of splenocytes, but this ratio is 80% among lymph node cells.[12,13] Therefore, the efficiency and ease of purification of T lymphocytes from lymph node cells are higher than that of splenocytes.

The target Ag of the study was TB10.4 that is a highly conserved Ag and is essential for the virulence of *Mycobacterium Tuberculosis*. TB10.4 protein is an immunodominant Ag, making it an ideal target for designing immunotherapy and vaccines against *Mycobacterium Tuberculosis*.[14,15,16] In the present study, we described a method for inducing, enriching, and replicating Ag-specific clones of T cells from BALB/c mouse infected with Bacillus Calmette Guérin (BCG) bacterium. The BCG was used as a live bacterium that mimics Mycobacterium tuberculosis infection and has the *TB10.4* gene. This led to murine T-lymphocytes priming to TB10.4 Ag *in vivo*. During a one-week procedure, immune cells were isolated from the lymph nodes, and T lymphocytes were purified and stimulated with TB10.4 protein and expanded by IL-2 cytokine *in vitro*. We evaluated the effect of Ag doses (1, 10, and 100 µg/mL) and exposure method of Ag presenting cells (APCs) to T cells, on this process by analyzing the T cells' proliferation, viability, and interferon-gamma (IFN-γ) secretion. We also compared the T cells proliferation and viability at 2, 5, and 7 days after Ag stimulation. Besides, we investigated the impact of factors such as Ag dose, duration, and method of exposure of T cells and APCs on the process of induction and enrichment of specific T cell clones.

MATERIALS AND METHODS

Mice

Female BALB/c mice (6- to 8-week-old) were obtained from the Pasteur Institute's breeding stocks (Tehran, Iran). Mice were kept in appropriate pathogen-free facilities at 25±5°C with a 12-h day/night cycle at Tarbiat Modares University animal house, with cages including sterilized feed, autoclaved bedding, and water. This study was approved by the Ethics Committee of Tarbiat Modares University (IR.TMU.REC.1396.680).

TB10.4 Recombinant Protein Construction and Purification

TB10.4 was produced and purified in collaboration with a research team led by Dr. Mojtaba Sankian in Immunobiochemistry laboratory, Immunology Research Center, Bu-Ali Research Institute of Mashhad University of Medical Sciences. pET 102/D vector is a plasmid containing the *TB10.4* gene, which was transformed into competent *E. coli TOP10*. A positive transformant was chosen, and plasmid DNA was isolated and subsequently transformed into competent *E. coli BL21* (DE3).[17] The bacterium was induced by IPTG, and its lysates were loaded directly onto SDS-PAGE. The purified recombinant protein was achieved using metal affinity chromatography (Ni-nitrilotriacetic acid).

Bacillus Calmette Guérin Infection of Mice

To prime the immune system for inducing T cells specific to TB10.4 Ag *in vivo*, mice were challenged with BCG bacterium via the aerosol route using a nebulizer (Omron, Hamburg, Germany). A suspension containing 10^7 CFU BCG (Mycobacterium Bovis, vaccine strain; 1173p2, from the Pasteur Institute of Iran) in 6 mL of PBS was used in the nebulizer cup. Mice were exposed to the aerosol obtained by nebulization. Three mice were put in a chamber, the nebulizer aerosolized BCG into the chamber, and mice stood in it for 20-30 minutes before relocating to their cages.

Cell Isolation from Lymph Nodes and Spleen

21-30 days post-infection, to isolate immune cells from tissue, BCG-infected mice (n=3) were sacrificed, and superficial cervical, axillary, brachial, mesenteric, and inguinal lymph nodes (n=5-10) and spleen were removed. Lymph node cell suspension was harvested by mechanical dissociation of lymph nodes in RPMI 1640 (Biowest, France) and filtered through sterile 100 µm nylon mesh to prepare a single-cell suspension. Then splenocytes were harvested from the spleens by perfusion. Splenocytes suspension was treated with ACK (Ammonium-Chloride-Potassium) Lysing Buffer (3-5 ml) and incubated at room temperature for 3-5 minutes to omit erythrocytes. Lymph node cells and splenocytes were centrifuged at 1500 rpm 5-7 min, and the cell pellet was suspended in 1-2 mL complete cell culture medium and cells were counted, and their viability was assessed by trypan blue staining.

Purification of T Cells from Lymphoid Cells

For enrichment of T lymphocytes from the lymphoid cell population, $1\text{-}1.5\times10^8$ lymph node cells were suspended in 1-2 mL of complete cell culture medium (RPMI 1640 containing 5% FBS). The single-cell suspension was added to a pre-prepared sterilized nylon wool column (packed and stabilized with RPMI in a 5-mL syringe in 5% CO_2 and 37°C for one hour) to allow the B cells, fibroblasts, and accessory cells to adhere to the nylon wool, according to standard protocol.[18] After an incubation time of one hour at 37°C, in 5% CO_2 cell culture incubator, the column was washed with 37°C, RPMI1640. The first 15 mL that came out of the column was collected for best T cell enrichment. The collected solution was then centrifuged, and harvested T cells were counted, and suspended in RPMI 1640 medium containing 10% FBS. To evaluate the purification procedure, before and after the nylon wool purification, 1×10^5 cells were collected for flow cytometry analysis with an anti-CD3-PE antibody (eBioscience, USA). Cells viability was also assessed before and after purification by trypan blue exclusion during cell counting.

Methods of Splenocytes and T Cells Co-culture for Ag Stimulation

To stimulate T lymphocytes with TB10.4 Ag, splenocytes were used as stimulator cells or APCs. In the first method, on day -1 of T lymphocytes stimulation, splenocytes were extracted and treated with 50 µg/mL mitomycin C (2 mg, Mitonco, Korea United Pharm. Inc., Korea) for 20 minutes. After incubation time, cells were washed three times with PBS 5% FBS and cultured in a 24-well tissue culture plate (4×10^6/well) containing culture medium (RPMI

1640 medium containing 10% FBS) and Ag (10 µg/mL). After 24 hours of incubation, the culture supernatant was removed, and 1×10^6 purified T lymphocytes and fresh culture medium were added to the wells (responder cells: stimulator cells=1:4). In the second method, T lymphocytes, splenocytes (treated with mitomycin C), and Ag were simultaneously cultured on day 0.

Optimization of TB10.4 Ag Concentration

To assess T cell function and viability after stimulation with different Ag doses, purified T cells were co-culture with splenocytes as stimulator cells (APCs), and the Ag was added to wells in three concentrations, (1, 10, and 100 µg/mL) each in triplicate. The plate was incubated at 37°C in 5% CO_2 incubator for seven days. IL-2 (150 U/ml) was added on day 5 of the T cell stimulation. T cell proliferation in the presence of different concentrations of Ag was measured by Carboxy fluorescein diacetate Succinimidyl Ester (CFSE) staining method on days 5 and 7 post Ag stimulation. IFNγ concentration was also measured in the supernatant of culture. The percentage of living cells at different time points was also examined by Propidium Iodide (PI) staining.

Assessment of T Cell Proliferation

CFSE labeling was performed to evaluate the proliferative response of T cells to stimulation. After the T cell enrichment procedure, cells were washed with RPMI1640 (without FBS) and centrifuged (1700 rpm, 5 min) and resuspended at a final concentration of 10×10^6 cells in 900 µL (5% FBS) PBS or RMPI 1640 in a 15 mL conical tube. 1 µL CFSE (5 mM; Cell Trace, Invitrogen, USA) was eluted in 100 µL PBS and added to the tube and vortex for 15 seconds. After incubation of cells for 20 min at room temperature in a dark place, the staining was quenched by washing cells three times with 5 mL of 5% FBS culture medium. After the last time of centrifuging, the cell pellet was resuspended in RPMI containing 10% FBS at a final concentration of 1×10^6 cells/mL. 100 µL of cells were cultured with 100 µL (4×10^6 cells/mL) splenocytes (as stimulator cell) and Ag in 96-well plates. On days 0, 2, 5, and 7 of stimulation, cells were collected, and their fluorescence intensity was determined by a flow cytometer (FACSCalibur, San Diego, USA).

Cytokine Assay

To measure the cytokine secretion by T cells after Ag stimulation, the supernatants of T cell culture were collected and analyzed for IFN-γ concentration. The enzyme-linked immunosorbent assay (ELISA) method was used by DuoSet ELISA Kits (R&D Systems, Minneapolis, USA) regarding the manufacturer's instruction.[19]

Assessment of Cell Viability Status

On days 2, 5, and 7 after T cell stimulation, to assess the cell viability using different Ag doses or stimulation methods, cells from different wells were collected and stained with PI (eBioscience, USA), and fluorescent intensity was analyzed by flow cytometry. PI-positive cells were considered dead cells.

Statistical Analysis

Data were analyzed with GraphPad Prism software V8.1 (La Jolla, USA) using non-parametric statistical tests. All data are expressed as mean±standard deviation (SD). Significant differences between analyzed groups were determined using one-way analysis of variance (ANOVA). A statistically significant difference was defined as the p-value$\leq$0.05.

Optimization of a Method for Ag-specific T Cell Clones Enrichment

After analyzing the experiment's results, an eight days course method was optimized for Ag-specific T cell clones enrichment (Figure 1). 21 days before experimental T cell stimulation, mice were infected with live BCG bacterium (priming phase). One day before stimulation, APCs (mitomycin-treated splenocytes) were incubated with the Ag. On day 0, the culture supernatant was removed, and purified T lymphocytes with the fresh culture medium were added. After 4 to 5 days, T cells were collected from wells and transferred to a new plate containing the recently isolated APCs. APCs were treated with mitomycin and incubated with Ag for 24 hours (Ag restimulation) and fresh RPMI including IL-2 cytokine (150 U). Cells were incubated for three more days, and finally, T cell clones were harvested and evaluated for T cells cytokine and CD markers. During the experiment process, depending on the cells' condition, 100 µL of fresh culture medium, including the low dose of IL-2 (50 U), was added to the wells.

RESULT

TB10.4 Production and Purification

In the present study, the target Ag for inducing specific T cell clones was TB10.4. It was a recombinant protein that was successfully cloned, expressed, and purified. TB10.4 is approximately 26.4 kDa exogenous proteins and is observed on the SDS-PAGE gel, as shown in Figure 2.

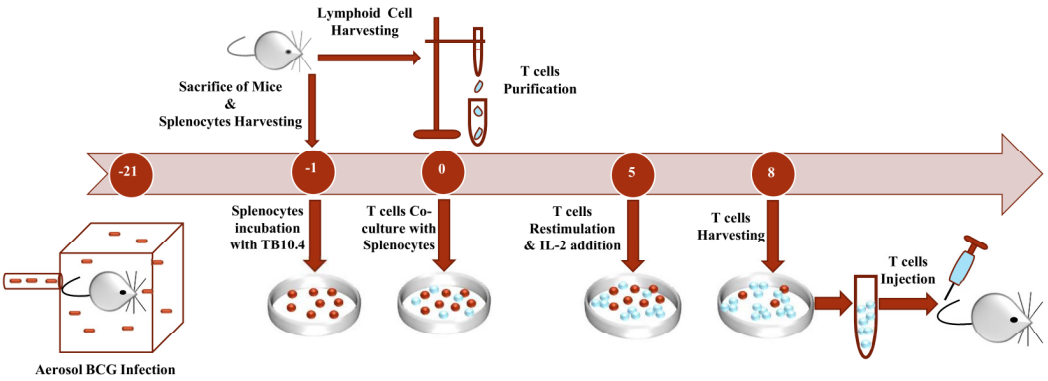

Figure 1. Schematic representation of the experimental schedule of antigen (Ag) stimulation and enrichment of TB10.4 Ag-specific T cell clones. 21 days before experimental T cell stimulation, mice were infected with live BCG bacterium (priming phase). One day before stimulation, antigen-presenting cells (APCs) that were mitomycin treated splenocytes, were incubated with the Ag. On day 0, the culture supernatant was removed, and purified T lymphocytes with the fresh medium were added. After 4 to 5 days, T cells were collected from wells and transferred to a new plate containing the recently isolated APCs. APCs were treated with mitomycin and incubated with Ag for 24 hours (Ag restimulation) and fresh RPMI including IL-2 cytokine (150U). Cells were incubated for three more days, and finally, T cell clones were harvested and evaluated for T cells cytokine and CD markers.

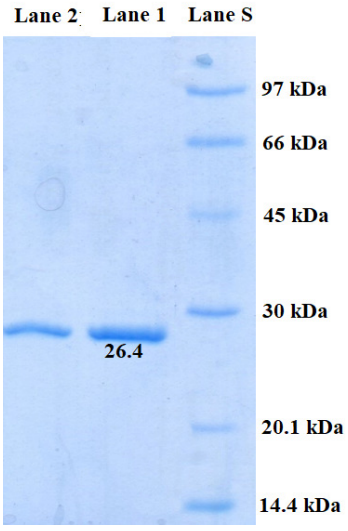

Figure 2. SDS-PAGE gel analysis of recombinant TB10.4 protein after purification by affinity chromatography. Lane S represents protein size standards, and lanes 1 and 2 are related to purified TB10.4 protein.

Assessment of T Cell Purity and Viability after Purification by Nylon Wool

To evaluate the purity of T cells isolated from the lymph node cell population, the cells were assessed for expression of pan T lymphocyte marker (CD3) with a fluorescent conjugated monoclonal antibody. Purification was performed by a negative selection method using the nylon wool column. Before and after nylon wool purification percentage of CD3 positive cells was measured. The results indicate that before purification, the percentage of CD3 positive cells was 64±5%, and after purification, it was 98±2%, as shown in Figure 3. The T cells viability after purification was≥90%.

Assessment of TB10.4 Ag Concentration for Optimal T Cell Stimulation

To adjust the dose of Ag for stimulation of T lymphocytes *in vitro* and induction of specific clones, three different doses of Ag were chosen and used. Then the morphology, proliferation rate, cytokine secretion (IFN-γ), and cell viability were examined in stimulated cells, seven days post Ag stimulation. Figure 4 shows the cell morphology and formation of Ag-specific clones of T lymphocytes on day 7 after Ag stimulation at three Ag concentrations (1, 10, and 100 µg/mL). Proliferating T cells were observed in the presence of different concentrations of Ag. In the 100 µg/mL group, a large number of apoptotic and dead cells is seen.

To compare the functional response of T cells to different concentrations of Ag, cell proliferation and IFN-γ secretion were measured. The proliferation of cells was examined *in vitro* with CFSE dilution. 7 days after Ag stimulation, twenty thousand cells were acquired in a flow cytometer and appropriately analyzed. After initial gating of CFSE positive cells, we calculated division index (DI), proliferation index (Pr.I), and divided cell peaks using Flowjo software.

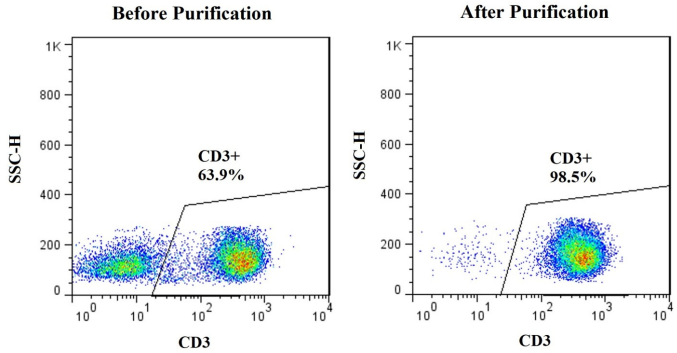

Figure 3. Flow cytometric analysis of the percentage of CD3 positive cells (T cells) in the lymphoid cell population before and after purification. Immune cells from mice lymph nodes were harvested from six lymph nodes of the Bacillus Calmette–Guérin infected mouse model. The T cells were purified by negative selection using the nylon wool column. The figure shows the percentage of $CD3^+$ cells among lymph node cells stained with the anti-mouse CD3 monoclonal antibody.

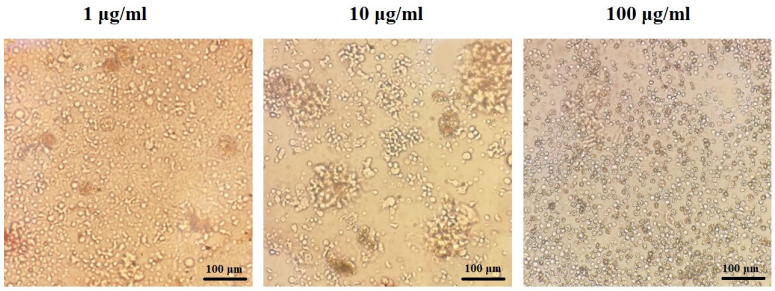

Figure 4. Mice T cells stimulated with different doses of TB10.4 antigen (Ag). Purified T cells isolated from the mice model of Mycobacterium infection were stimulated with three different concentrations of Ag (1, 10, and 100 µg/mL). The morphology of Ag-specific T cells and clone formation, related to 7 days after Ag addition to the cell culture medium, are observed.

As shown in Figure 5, all Ag doses significantly increased Pr.I and DI compared to control wells(Ag:0 µg/mL), and as the amount of Ag in the culture medium increased, the average cell division (Pr.I) in the T cell population increased. The Pr.I and DI means of wells containing 100 µg/mL dose of Ag were significantly (p≤0.05) higher than those of other wells.

Optimization of Ag Stimulation Duration

To optimize the appropriate time course for stimulation of T lymphocytes with Ag and decide on the right time to re-stimulate with the Ag, an experiment was designed. The number of cell divisions means (DI and Pr.I) of T clones in 2, 5, and 7 days after Ag stimulation (Ag: 10 µg/mL) was examined by the

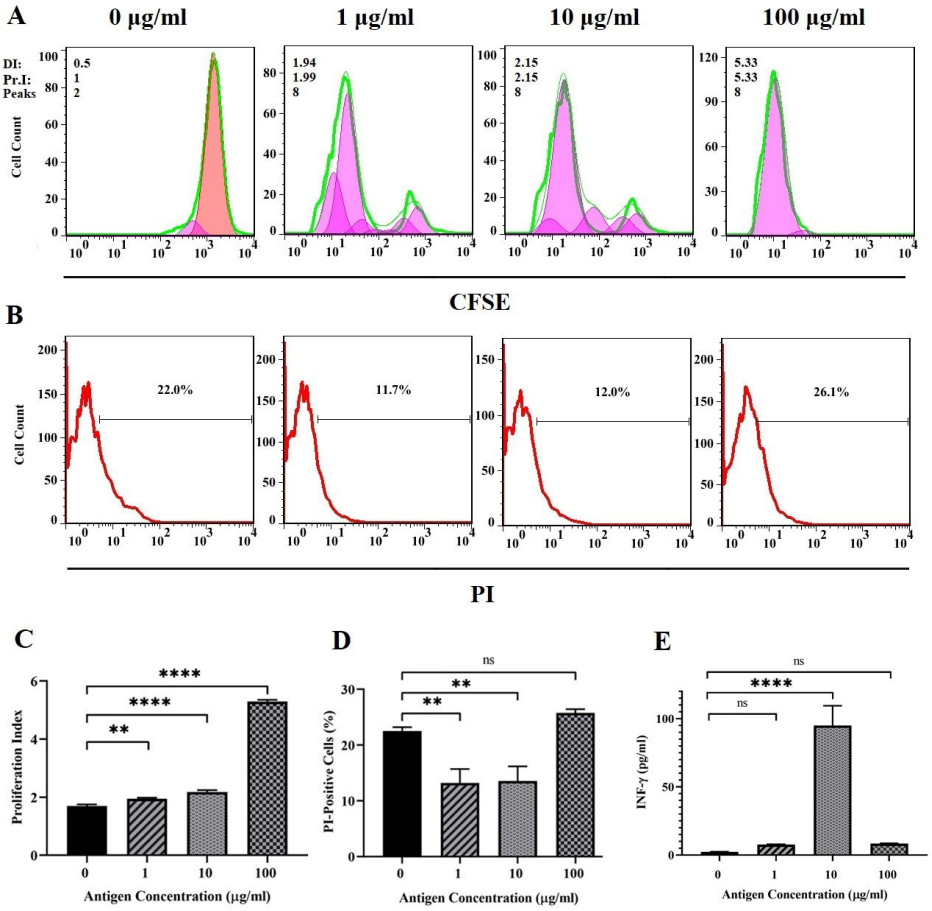

Figure 5. Comparative illustration of proliferation, Interferon-γ secretion, and survival of T cells in response to different TB10.4 antigen (Ag) concentrations (1, 10, and 100 µg/mL) *in vitro*. (A) Carboxyfluorescein diacetate Succinimidyl ester-stained T lymphocytes were cultured with antigen-presenting cells in a ratio of one to four, respectively, and three different concentrations of Ag were added to the wells in triplicate. Seven days after Ag addition, cells were examined by flow cytometry. Division Index (DI), Proliferation Index (Pr.I), and divided cell peaks were calculated and used to compare the experimental groups in terms of the proliferative response to Ag. Pr.I mean ± SD of groups were compared in chart bars (C). Significance of differences (p≤0.05) between Proliferation Index means of stimulated vs. unstimulated control (no Ag) samples are shown. (B) The death rate of T cells in the presence of different Ag doses was assessed by propidium iodide (PI) staining. (D) In bar charts, the percentage of PI^+ cells (dead cells) mean±SD in each group was compared. (E) Comparative Interferon-gamma (IFN-γ) cytokine level in supernatant of T cells culture, 7 days after Ag stimulation between experimental groups is illustrated. Cytokine was determined by the enzyme-linked immunosorbent assay (ELISA) method.

CFSE staining method (Figure 6). The results show that the average number of divisions of T clones (Pr.I and DI) increased over time and the difference between the three groups was significant. The number of positive PI cells also increased over time. There is no significant difference between the mean percentages of dead cells on days 2 and 5, but there is a significant difference ($p \leq 0.05$) between day 7 with others.

Optimization of the Method of Exposure to APCs

To select a preferred method for T-lymphocyte exposure to APCs, the proliferation of T-lymphocytes stimulated using the following two methods in a fixed period (7 days) was examined (Figure 7). In the first method, the Ag was incubated with splenocytes (which were used as stimulus cells) on the day before stimulation to take up the Ag. After 24 hours, the cell supernatant was removed, and T cells were added to the wells with the new culture medium. In the second method, splenocytes and T cells were cultured simultaneously with the Ag-containing medium. The results show that 7 days after Ag stimulation, the proliferation rate of T clones by the first method was significantly higher than the second method. Also, the survival of T cells in the first method is more than in the second method.

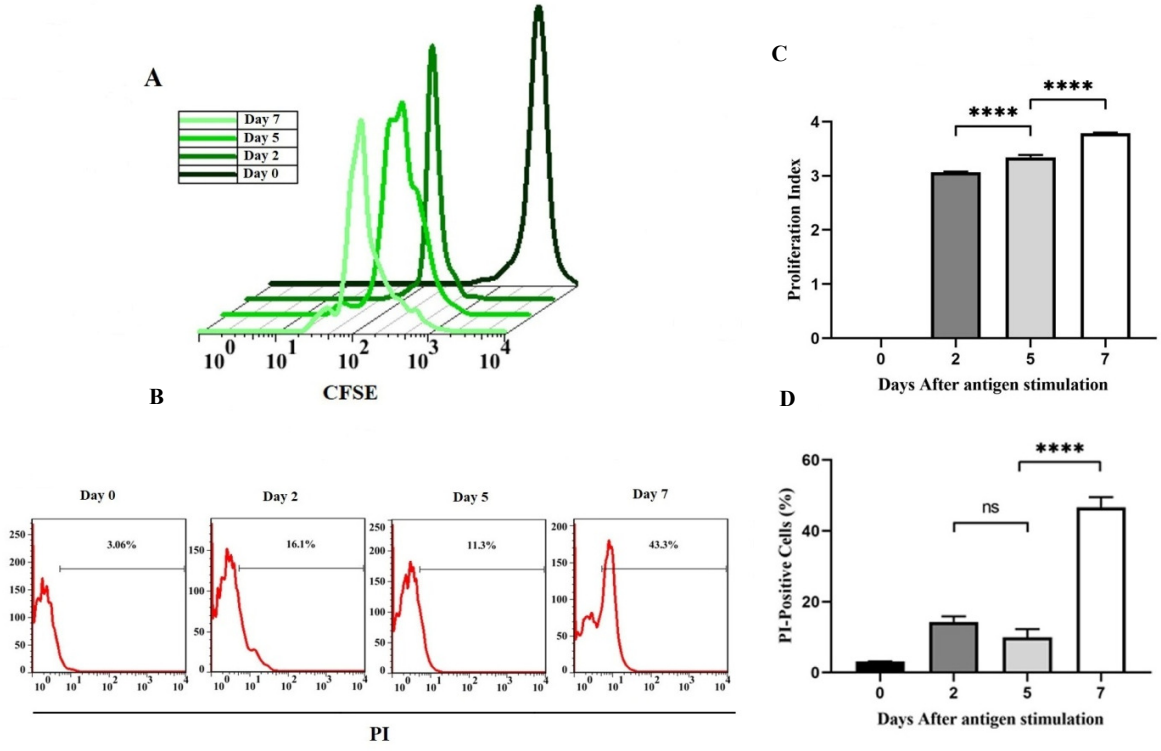

Figure 6. Proliferation rate and survival of T cells, 2-, 5- and 7-days after TB10.4 antigen (Ag) stimulation. (A) Purified mouse T cells were stained with Carboxyfluorescein diacetate Succinimidyl Ester and cultured and stimulated with TB10.4 Ag (Ag: 10 µg/mL). A decrease in cell fluorescence via Flow cytometry was assessed 2, 5, and 7 days after stimulation. (C) The proliferation index of T lymphocytes at all three-time points is compared in the chart. (B) The T cells' survival on days 2, 5, and 7 post Ag stimulation was assessed by propidium iodide (PI) staining. (D) As seen in bar charts, the percentage of PI^+ cells (dead cells) mean±SD in each group was compared. Significance ($p \leq 0.05$) alteration in PI-positive cells and proliferation index means are illustrated.

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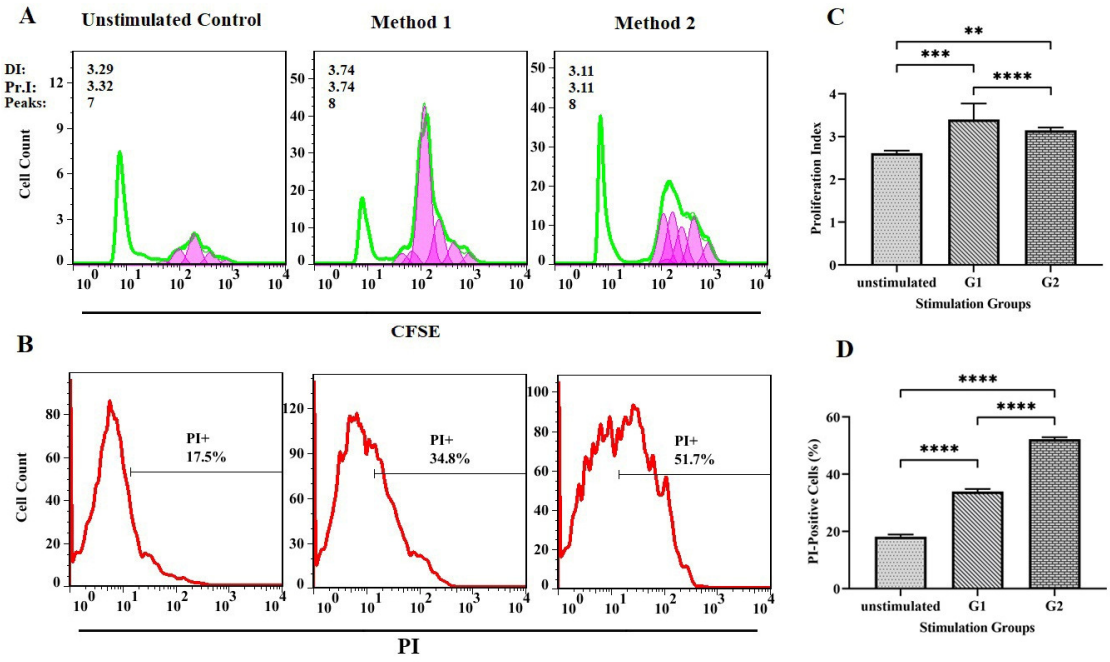

Figure 7. Comparative illustration of Ag induced proliferation and survival of T lymphocytes, using two methods of antigen (Ag) exposure with Ag-presenting cells (APCs). Carboxyfluorescein diacetate Succinimidyl Ester-stained T lymphocytes were co-cultured with APCs (splenocytes), and TB10.4 Ag is added to the culture medium to be picked up and presented to T cells. In the first method, Ags and splenocytes are incubated the day before T cell stimulation. On day 0 of stimulation, the supernatant including suspended cells is removed and T cells are added to the wells. In the second method, T cells and stimulator cells, are cultured simultaneously on day 0 in a culture medium containing Ag. The number of division and survival of T cells induced by Ag stimulation using methods one (A) and two (B) are depicted. As seen in bar charts, the percentage of PI^+ cells (dead cells) and Pr.I mean±SD of each method was compared. Significance ($p \leq 0.05$) alteration in PI-positive cells and proliferation index means are illustrated.

DISCUSSION

Adoptive T-cell therapy is a novel treatment for malignancies and some complications of infectious diseases. An essential challenge in this treatment is to select an appropriate method for stimulating, enriching, and proliferation of specific T cell clones. The ideal method has the least manipulation steps so that T cells do not get exhausted or lose function and can persist for a long time *in vivo*. The procedure should produce polyclonal T cells, all specific for the same Ag, to identify the target cells and allow efficient protection against pathogens.[10,20] Immunotherapy studies usually require a large number of T cells, so we need techniques that eventually produce a large number of cells.[21] MHC Tetramer and IFN-γ secretion assay are known methods to select and enrich the Ag-specific clones with more precision. However, these methods require multiple steps over a long period to expand cells and reach the number needed for immunotherapy, which reduces the viability and function of T cells.[11] This reduces the chance of adaptive transfer success. In this study, we described a one-week process, in which immune cells were isolated from the lymph nodes, T lymphocytes were purified by nylon wool, and stimulated with TB10.4 protein, and expanded by IL2 cytokine. This method prepares a large number of specific T cell clones in a short time. In the present study, we aimed to optimize the method by investigating the effects of factors influencing this process, including Ag concentration, duration of antigenic stimulation, and approach of T cell exposure to APCs and Ag.

Previous studies have shown that the Ag dose has an essential effect on the quality, function, and type of T cells induced *in vitro*.[22,23] Further studies were needed to identify the functions of T cells affected by the Ag dose. Therefore, in this study, we examined three doses of Ag (1, 10, and 100 µg/mL) to stimulate T lymphocytes and investigate the effects of different doses on proliferation, survival, and IFN-γ cytokine secretion of T cells. Based on the results, at all doses of Ag, T cell proliferation was significantly increased compared to the unstimulated control sample and this increase was dose-dependent. At the highest dose (100 µg/mL), proliferation was superior. However, the number of living cells (PI negative cells) or T cell survival in group 100 µg/mL is lower than that in groups 1 and 10 µg/mL. On the other hand, the secretion of IFN-γ cytokine only in group 10 µg/mL is significantly higher than the un-stimulated control. The secretion of the IFN-γ cytokine in the other doses is much lower and the difference in IFN-γ secretion between groups 1 and 100 µg/mL is not significant. IFN-γ cytokine is a principal secretory factor in the Th1 population that plays a crucial role in inducing effective cellular immunity against viral and intracellular bacterial infections and cancer cells.[24] Our findings are consistent with previous studies that show the effect of Ag dose on the type of induced T cell subset (Th1 or Th2) and its cytokine profile. As Hosken et al reported, an increase in the peptide dose increased the development of Th1-like cells, which produced enhanced amounts of IFN-γ. Although at very high and shallow doses of the antigenic peptide, a dramatic switch to the development of Th2-like cells was observed, which produced increasing amounts of interleukin 4 (IL-4) and diminishing level of IFN-γ.[25] Besides, very high Ag doses were shown to induce T cell death as a physiological response to an exceeding strength of signal caused by T cell receptor engagement.[26,27]

The duration of antigenic stimulation is another factor determining the quality of induced specific T cell clones and cell differentiation orientation into functional subsets.[9,27] In this study, the T cells' proliferation at different time points, including 2, 5, and 7 days after Ag stimulation, was measured and compared. Our findings indicated that the average number of cell divisions increases over days and is highest on day 7. Furthermore, on day 7, the rate of cell death (PI-positive cells) is also excessive, while the percentages of dead cells on days 2 and 5 are much lower, and there is no significant difference between them. For this reason, T cells appear to be in a more favorable condition around day 5 in terms of proliferation and survival. In the final step, we compared the two methods of T lymphocyte exposure with stimulator cells (APCs). The results show that the survival and proliferation of T cells with the first method are significantly higher than the second method. This means that 24-hour incubation of Ag and APCs on the day before T cell exposure accelerates the T lymphocytes' proliferation in response to Ags. These results are consistent with the previous studies.[28,29]

In conclusion, in the present study, we investigated the effect of antigen dose, the order of T cells and APCs exposure with Ag, the duration of initial Ag stimulation, and the time point for Ag restimulation of T lymphocytes. The goal was the optimization of the method of enriching and replicating Ag-specific clones. The data demonstrate that a high dose of Ag profoundly increases proliferation in stimulated T lymphocytes. However, this intense stimulation of clones with high doses of antigen eventually induces apoptosis in several clones. This can include high-affinity clones. In addition, high dose Ag can cause T cell exhaustion. As previous studies reveal, T cells generated *in vitro* with a lower antigen dose induced much more functional T cells than cells generated with a high antigen dose. This is most likely due to repertoire selection based on TCR affinity.[22] T clone selection occurs when there is a competition between antigen-specific clones in Ag binding and only those that have a higher affinity for the antigen are amplified, a phenomenon that occurs only in the presence of the optimal dose of antigen.[22] So, we selected the medium Ag dose (10 µg/mL) which induced satisfactory T cell division and caused secretion of a desirable amount of IFN-γ (TH1 cytokine). We also compared the T cells proliferation at 2, 5, and 7 days after Ag stimulation. Despite the higher average number of divisions on day 7, cell viability was significantly reduced on this day. For this reason, T cells are in a more favorable condition around day 5 in terms of proliferation and survival, and it is the desired time for T cell restimulation. Our findings indicate that incubation of antigen and APC cells, the day before exposure to T lymphocytes, increased the method's efficiency. This allows the APC cells to pick up the Ag and be ready to deliver it to the T cells, and both lead to speed up the

Ag stimulation process. To purify T lymphocytes from lymph node immune cells, we used a nylon wool column that provided a sufficient number of T lymphocytes with a purity of more than 90% by negative selection. The cells obtained from this process can be used for T-cell therapy experiments in animal models of infections and malignancies.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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