The golden doses of anti CD3/CD28 microbeads plus IL-15 promote expansion of specific T cells in human cytomegalovirus adaptive immunotherapy

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ABSTRACT

In this study, the use of anti-CD3/CD28 magnetic microbeads and IL-15 in the culture of T lymphocytes *in vitro* had a significant role in the proliferation and cytotoxicity of these cells. Peripheral blood mononuclear cells of 6 (Cytomegalovirus) HCMV positive volunteers were harvested, then the pure population of lymphocytes were isolated. HCMV-Specific T Cells were expanded in 4 groups consisting of IL-2, IL-15 and anti-CD3/CD28 microbeads. A significant effect of IL-15 along with anti-CD3/CD28 magnetic microbeads on the proliferation rate of HCMV-specific T lymphocytes was evaluated.

*Keywords***:** Cytomegalovirus, T cell, adaptive immunotherapy, expansion

INTRODUCTION

Immunosuppression prevents the development of Graft-versus-Host Disease (GvHD) in a wide range of transplants and subsequent use of high-intensity chemotherapy in the treatment of cancers can potentially prolong immune

reconstitution. As a result, the patient's sensitivity to opportunistic infections increases [1]. Among these infections, viral infection is the main cause of infectious deaths in 30 % of patients after transplantation. There are some viruses that are responsible for one-third of deaths attributed to infection such as Cytomegalovirus (CMV), Epstein-Barr Virus (EBV), and Human adenovirus (AdV). Meanwhile, human CMV is the most common infection that occurs in patients receiving HSCT [2].

CMV is a beta human herpes virus which involves 60 % to 90 % of the world's population, and its seropositivity increases in older ages. CMV infection is usually asymptomatic, and the initial infection causes a lifelong latency in the myeloid progenitor cells of the host's bone marrow [3]. This cytopathogenic virus can be reactivated in patients who have received Solid Organ Transplantation (SOT) and chemotherapy related to leukemia, and multiply widely as a result of immunosuppression. A high viral load that occurs due to HCMV replication can increase the risk of post-transplant mortality [4,5]. Today, in order to fight against this infection and reduce its death rate, various HCMV antiviral treatment

methods such as antiviral drugs, vaccination, passive immunotherapy and adaptive immunotherapy with HCMV-specific T cells are undergoing clinical trials to confirm the current treatments [6].

The defense mechanisms of the innate immune system using natural killer cells and antigen-presenting cells, as well as adaptive immunity with the help of cellular immunity (CD4$^+$/CD8$^+$ T cells) and humoral immunity (antibodies) lead to inhibition of HCMV multiplication [7]. Effective HCMV-specific T-cell immunity plays a significant role in inducing long-term immunity against HCMV infection and reactivation [8]. In an HCMV seropositive individual, a significant proportion (average 10 %) of the total population of CD4$^+$/CD8$^+$ memory T cells are HCMV specific [9]. In HSCT, immunity mediated by T cells following immunosuppression is disrupted, and reducing the protective effect of these cells leads to prolonged viral infection and in some cases fulminant infection because the recovery of T cells, especially HCMV-specific CTLs, takes several months [10]. On the other hand, the HCMV virus interferes with the function of dendritic cells as antigen-presenting cells to T cells and leads to disturbances in the initiation of

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adaptive immune responses and suppression of the immune system [11].

Since T-cell immunity helps control many viral pathogens, adaptive immunotherapy with Virus-Specific T cells (VSTs) is an effective approach against severe viral diseases in immunosuppressed patients [12]. For the successful generation and expansion of HCMV-specific T cells, it is important to determine the most immunogenic epitopes presented by Antigen-Presenting Cells (APC) to increase the activation and proliferation of viral peptide-specific T cells [13]. A large number of antigens expressed during different stages of viral replication are involved in the activation of HCMV-specific $CD8^+$ and $CD4^+$ T cells, which are known as mediators of the immune response against the virus [14]. In HCMV, immunodominant epitopes include pp50, Immediate Early protein-1 (IE-1), IE-2, phosphoprotein 65 (pp65), along with subdominant epitopes such as glycoprotein-H and pp28, which are responsible for most antiviral immune responses [15].

In adaptive immunotherapy with HCMV-specific T cells, various strategies have been used to generate virus-specific T cells.

In the *ex vivo* expansion method, the presentation of virus-specific peptides or proteins using antigen-presenting cells to T cells *in vitro*, leads to the generation of virus-specific T cells [16]. The advantages of this method include the lack of HLA type restriction, [17] the small amount of blood required and the production of polyclonal T cells [18]. Also, the disadvantages of this method are the long-time culturing and the need for seropositive volunteers [19].

In the direct selection method using specific peptide-MHC (pMHC), the use of pMHC multimers enables the isolation of T cells based on the ability of the TCR receptor of these cells to bind to a complex mixture of recombinant HLA class-I molecules loaded with a virus-specific peptide [20]. The advantage of this method is the possibility of reducing time and improving the quality of the final product and minimizing alloreactivity [16]. However, the main disadvantages of this method are related to the limitation in the isolation method of $TCD4^+$ or $CD8^+$ cells and the irreversibility of the connection of these cells, which can cause changes in the phenotype of T cells and then changes in the function of these cells [21,22]. In direct selection using Cytokine Capture System (CCS), HCMV-specific T cells can be

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selected using IFN-γ Cytokine Capture System (CCS). This method is a rapid assay that allows the selection and enrichment of IFN-γ secreting T cells that have been previously stimulated using viral antigens [23,24]. This strategy provides the possibility of choosing a T cell that, unlike pMHC, has no restrictions on HLA, and one of the benefits of this method is to stimulate and obtain a polyclonal population of TCD4$^+$ and/or TCD8$^+$ cells. However, the need for seropositive volunteers and the large volume of blood required are negative points of this method [25,26]. Another direct selection method is the isolation and enrichment of activated virus-specific T cells after antigenic stimulation based on the identification of specific surface markers that are selectively expressed or up-regulated after T cell activation, such as CD25. CD69, CD137 and CD154 (CD40 ligand). This approach allows simultaneous targeting of antiviral T helper cells and effector cells. Having no need for antigen presenting cells and having no restrictions on HLA are also benefits of this method. Also, being time-consuming and difficult to isolate and expand functional cells are negative points of this method [27-29].

Other interesting strategy in the treatment of cancers are Chimeric Antigen Receptors (CAR) or gene modification of patient lymphocytes with tumor-specific T cell Receptors (TCRs) [30,31]. The generation of autologous CAR-T cells, as a candidate immunotherapy against HCMV, enable antigen recognition in an MHC-independent manner and can specifically target conserved and essential epitopes of the targeted antigen [32]. The positive points of TCR-T cells include high affinity for specific antigens, strong activation of T cells when faced with a small amount of antigen, and the use of natural T cell signaling mechanisms. In contrast, only targeting surface antigens and restriction to epitope for CAR-T cells and targets recognition limitation through MHC molecules lead to activation of T lymphocytes for TCR-T cells. In addition, this technique is extremely expensive and thus, it is one of the disadvantages of this method [33,34].

As it is known, the stimulation and subsequent activation of T lymphocytes requires two signal transductions. The first is generated by antigen recognition through the TCR complex, in which CD3 and zeta proteins are responsible for creating a signal that leads to T cell activation. On the

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other hand, binding of CD28 co-stimulatory molecule to B7-1/B7-2 and CD4 and CD8 molecules to MHC, at the APC, provides the second signal transduction for complete activation of T lymphocytes [35]. In adaptive immunotherapy with HCMV-specific T cells through *ex vivo* expansion, expansion of T lymphocytes *in vitro* takes place following appropriate stimulation of these cells. Traditionally, mitogenic lectins such as Phytohemagglutinin (PHA) and Concanavalin (Con A) have been used to stimulate polyclonal T cells. More related methods have shown that the use of microbeads coated with anti-CD3 and anti-CD28 stimulates T lymphocytes in a manner almost mimicking antigen-presenting cells [36]. Signal transduction of cytokines through their specific receptors on the surface of cells regulates cell proliferation, differentiation and survival, and as a result, affects cell function and fate [37]. Interleukin-2 is widely used to enhance immune cells, especially T cells, and has been called a T-cell growth factor because of its potential capacity to enhance T-cell proliferation and differentiation *in vitro*. This cytokine has been molecularly cloned and is used as a selective cytokine for the proliferation of T cells in cell cultures [38]. Due to the stimulatory functions of this cytokine in T cell growth *in vitro*, IL-2 has been used for many years to culture and expand various T cell products, including tumor infiltrating lymphocytes (TILs), Virus-Specific T cells (VSTs), TCR-T cells and CAR-T cells [39, 40]. In addition, the role of interleukin-15 in the development and immunobiology of NK and T CD8$^+$ cells have been reported. Viral infection induced IL-15 has been shown to influence the migration and function of NK and CD8$^+$ T cells and thus, has great potential for modulating antiviral immune responses and vaccine development. The results of *in vitro* studies had shown that IL-15 promotes the expansion and maintenance of T CD8$^+$ and NK cells. For this reason, it is being evaluated as a main cytokine for adaptive immunotherapy and vaccine strategies [41]. So based on previous *in vitro* researchers and another comprehensive of our team's study [42], the aim of this research is to achieve the optimal dose of anti CD3/CD28 microbeads along with IL-15, in order to expand HCMV-specific T cells with a high rate of proliferation and cytotoxicity.

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MATERIALS AND METHODS

Volunteers and Ethic Statement

In this study, 6 CMV seropositive healthy donors were selected. The personal consent form of the donors was signed and approved by the ethics committee of Taleghani Hospital (code: IR.SBMU.RETECH.REC.1400.094), and Royan Institute (code: IR.ACECR.ROYAN.REC.1401.030). Leukapheresis sample contains many cells such as Pan T cells. Leukapheresis samples were prepared with Spectra Optia Apheresis System in the bone marrow transplant ward of Taleghani Hospital in Tehran.

Isolation of Mononuclear Cells

Three to five cc of leukapheresis samples was prepared from each donor and placed at room temperature for 15 min. The sample was diluted to a volume equal to RPMI 1640 (Gibco) and gently poured onto one-third of the total volume of the ficoll (Sigma-Aldrich). The sample was centrifuged at 349 g (eppendorf 5810 R) for 30 min. The buffy coat was separated and mixed with equal volume of PBS solution (Gibco-BRL, Uxbridge, UK). After

mixing, the sample was centrifuged for 12 min at 349 g and the washing step with PBS and centrifugation was repeated two more times. The supernatant was discarded and the resulting cell pellet was reduced to 1 cc. Cell count was performed with Neubauer Chamber, survival rate was evaluated with trypan blue solution (Gibco) and was reported more than 98 % for all samples. In order to increase the purity of T cells in this study, T lymphocytes were isolated from the MC cells population of each volunteer by MACS (magnetic-activated cell sorting system) technique.

Isolation of T Lymphocytes by MACS KIT

T cells were isolated using a cell isolation negative selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the respective protocol. Briefly, first, a hapten-conjugated primary monoclonal antibody cocktail (anti-CD11b, CD16, CD19, CD36 and CD56) to eliminate dendritic cells, monocytes, granulocytes, B cells, platelets, primary erythroid progenitor cells and NK cells) at a ratio of 1:5 was added to the cells (20 μl of primary monoclonal antibody per 1×10^7 cells resuspended in 80 μl of wash buffer) and

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the cells were washed twice with wash buffer.

Monoclonal secondary antibody (MACS paramagnetic microbeads coupled with monoclonal anti-hapten antibody) was added to the cells at a ratio of 1:5, similar to the first step, and incubation was performed for 30 min. Then the cells were washed with washing buffer and resuspended at an optimum amount of 1×10^8 per 500 microliters of washing buffer. Finally, the cells were passed through a 30 μm filter (Miltenyi Biotec, München, Germany) to remove any cell masses. In the next step, the preparation of the AS column was done before passing the cells and it was sterilized with 70 % ethanol and finally it was placed inside the magnet. To prevent non-specific binding, the column was filled from bottom to top by injecting cold PBS/2 % FCS for 1 hour. Then the cell suspension was added to the column and the non-attached cells passed through it. Cold PBS/2 % FCS with a tenfold the volume of the column was injected into the column. And finally, eluted T cells were collected.

Assessment of Purity of the Negatively Selected T Lymphocytes Subset by Flowcytometry

The cells were spun at 300 g at room temperature for 10 min and resuspended in 1 mL of culture medium. Then, in order to check the viability of the cells, 90 μL of the cell suspension was mixed with 1 μL of trypan blue and their viability was reported to be above 98 %. Finally, the concentration of cells was set to 1×10^6 cells/mL in the culture medium. In order to check the efficiency of T cells, three-color flow cytometry was performed using monoclonal antibodies conjugated with PerCP/FITC/PE (Becton Dickinson, Mountain View, CA). For this purpose, 50 μL of cell suspension was incubated with specific monoclonal antibodies CD3-PerCP, CD4-FITC, CD8-PE and isotype control monoclonal antibody at 4 °C for 30 min. Then the cells were washed with cold PBS/2 % FCS and analyzes were performed by flow cytometry (Becton Dickinson, San Jose.) the percentage of CD3$^+$ T cells in the live cell gate was checked and then it was evaluated what proportion of these cells are CD4$^+$ T and CD8$^+$ T cells.

In vitro Expansion of HCMV

In this study, the expansion of HCMV-specific T cells includes 4 groups of these cells with the IL-2 (200 U/ml) and IL-15 (20 ng/ml), as well as anti-CD3/CD28 magnetic microbeads (Invitrogen, Carlsbad, CA) at a 1:1 ratio of cells to magnetic microbeads. For this purpose, 2×10^6 cells were cultured in a 24-well plate (Costar, Cambridge, MA) with RPMI 1640 (Gibco). Study culture groups included IL-2 (control group), IL-2+IL-15, IL-2+Beads, and IL-2+IL-15+Beads. For the activation of T lymphocytes against HCMV, pp65 (Miltenyi Biotec PepTivator®) peptide was prepared and added to each culture group according to the relevant protocol. Cytokines and anti-CD3/CD28 microbeads were applied every 2 and 4 days. On the fourteenth day of cell culture, after counting the cells by Neubauer slide and determining the percentage of living cells with trypan blue solution (Survival rate > 98 %), the proliferation rate and cytotoxicity of these cells were evaluated in each study group.

HCMV Proliferation Assay

For Carboxyfluorescein Succinimidyl Ester (CFSE), Molecular Probes, labeling assay,

T cells in each group were stained with CFSE. For this purpose, T cells were suspended in the amount of 5×10^6 cells/mL in PBS. Then, CFSE with a concentration of 1 μM was added to the cell suspension and incubated for 10 min at 37 °C. After washing, the cells were counted again and then cultured in RPMI-1640 culture medium containing 5 % FBS and the proliferation ratio of CFSE-low T cells was measured by flow cytometry. To control the intensity of CFSE staining of non-proliferating cells, 5×10^6 CFSE-labeled T cells were cultured alone according to the above conditions.

In vitro HCMV-Specific TCD8⁺ Cells Cytotoxicity assay on EBV-LCLs

EBV transformed lymphoblastoid cell lines as target cells and HCMV-Specific TCD8⁺ cells as effector cells were co-cultured with the 1:1 and 2:1 ratio of effector cells to target cells in a 96-well plate for 4 h. The cytotoxic activity of the TCD8⁺ cells was measured by flow cytometry-based cytotoxicity assay with CFSE-labeled EBV-LCLs and Propidium Iodide (PI). 2 μM of fluorescence labeling were used to stain target EBV-LCLs, as described in manufacturer's instruction. 50 μl of CFSE-labeled EBV-LCLs (1×10^5cell/ml) were

placed in 5 ml round-bottomed tubes, and expanded TCD8^{+} cells were added into the tubes as effector cells. Effector or target cells alone were used as negative controls. After 4 h incubation at 37 °C, 50 µl of 20 µl g/ml PI was added and cells were incubated for an additional 15 min. CFSE and PI were measured by LSR II flow cytometer (Becton Dickinson, San Jose, CA). Cytotoxic activity was calculated as follows:

Specific lysis (%) = ((TC-TE) / TC) × 100

TC is the percentage of live CFSE^{+} target cells in control tubes (target cells alone) and TE is the percentage of live CFSE^{+} target cells in test tubes (target cells + effector cells).

Assessment of Produced IFN-γ in Co-culture Supernatant of Effector Cells with Target Cells by ELISA Technique

T CD8^{+} cells which secreted IFN-γ were measured by an Enzyme-Linked Immunosorbent Assay (ELISA) technique, using the protocol of Human IFN-gamma Quantikine ELISA kit (R&D System, Bio-Techne, MN, USA). Briefly, this assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for IFN-γ has been

precoated onto a microplate. Standards and samples are pipetted into the wells and any IFN-γ present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IFN-γ is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IFN-γ bound in the initial step. The color development is stopped and the intensity of the color is measured.

RESULTS

Cytokine activated T cells proliferation was augmented in presence of magnetic microbeads

To evaluate the proliferation of T CD4^{+} and T CD8^{+} cells individually, these cells were purified separately and treated with cytokine(s) and/or microbeads (beads). The treatment groups were: IL-2, IL-2+IL-15, IL-2+Beads and IL-2+IL-15+Beads. The beads to cells ratio for both types of T cells were 1:1. Treatment with IL-2+IL-15 did not improve CD4^{+} cells proliferation in comparison with IL-2 treatment individually. Likewise, the difference of

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proliferation between IL-2 and IL-2+IL-15 activation for T CD8$^+$ groups was not statistically significant. In contrast, adding beads to both cytokine regimes, IL-2+Beads (Median: 42.5, rang: 35.6 to 47.4) (p<0.01), IL-2+IL-15 +Beads (Median: 62.95, rang: 53.3 to 70.1) (p<0.001), drove T CD4$^+$ cells significantly comparing to their counterpart groups without beads including IL-2 (Median: 29.55, rang: 25.21 to 35.13) and IL-2+IL-15 (Median: 31.1, rang: 26.3 to 35.6) (Figure 1). The same pattern of proliferation increments in T CD8$^+$ proliferation in IL-2+Beads (Median: 49.2, rang: 44.2 to 55.8) (p<0.01) and IL-2+IL-15+Beads (Median: 67.33, rang: 61.11 to 75.6) (p<0.001) were acquired by combining with IL-2 (Median: 34.2, rang: 29.6 to 39.4) or IL-2+IL-15 (Median: 36.45, rang: 33.70 to 39.5) respectively (Figure 1).
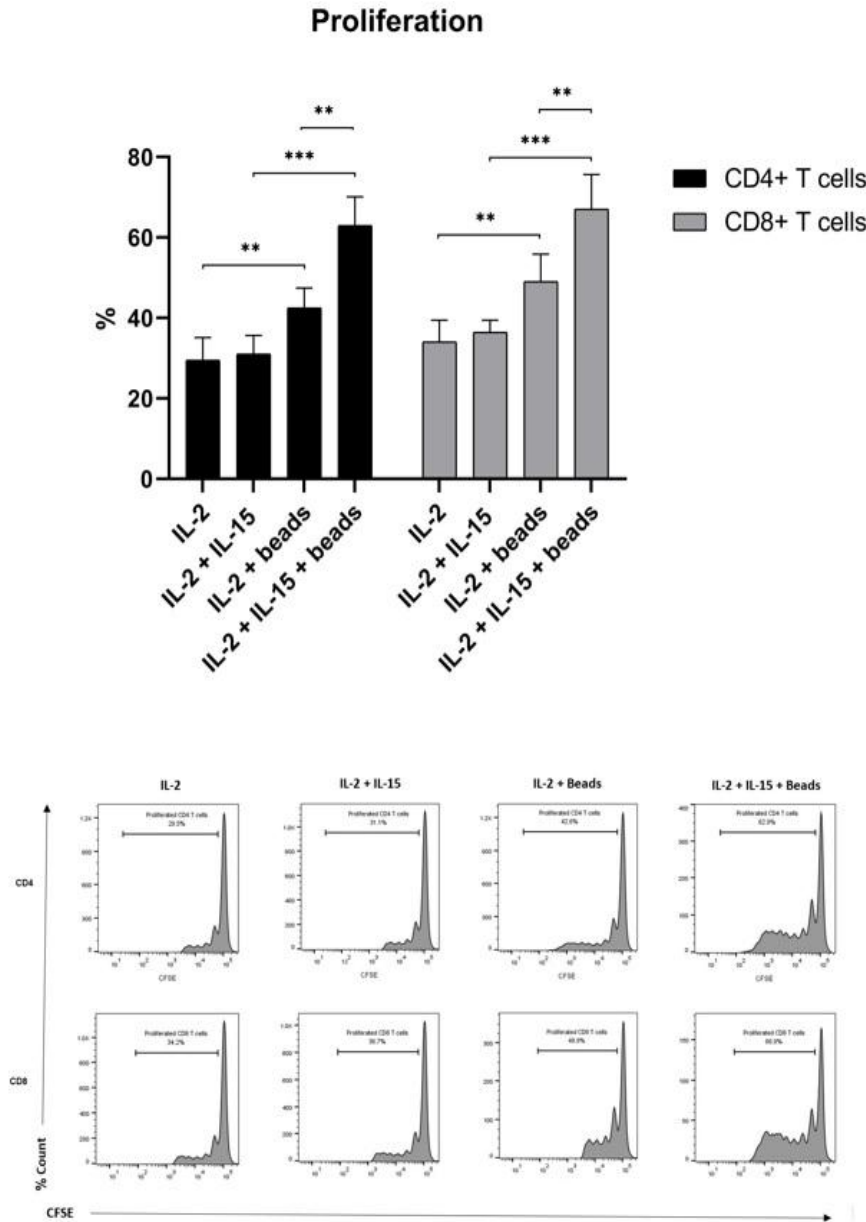
Figure 1. Assessment of CD4^{+} and CD8^{+} T cells proliferation. The CD4^{+} and CD8^{+} T cells were purified individually and their proliferation was evaluated after being activated in four different treatment groups including IL-2, IL-2 + IL-15, IL-2 + Beads and IL-2 + IL-15 + Beads. Evaluation of T CD4^{+} cells' proliferation in above mentioned treatment groups. Proliferation assessment of T CD8^{+} cells in above mentioned treatment groups. Statistical comparison of CD4^{+} CD8^{+} T cells in all treatment groups was performed using Mann–Whitney test. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ and ****: $p<0.0001$:) (n=3).

Microbeads along with activation cytokine regime potentiated cytotoxicity of T CD8$^+$ cells

Next, to explore the effects of exploiting beads and IL-2 and IL-15 on cytotoxicity of T CD8$^+$ cells, these cells were activated in all for mentioned groups at beads to cells ratio of 1:1. To assess the cytotoxicity of T CD8$^+$cells, EBV-LCLs were used as the target cell at the effector to target ratios: 1:1 and 2:1. As it shown in the Figure 2, at effector to target ration: 1:1, cytotoxicity of T CD8$^+$ cells in to IL-2 + IL-15 + Beads (Median: 18.1, rang: 21.4 to 16.5) (p<0.05) group is higher than IL-2 + IL-15 (Median: 14.5, rang: 11.8 to 17.3) group significantly. Moreover, cytotoxicity of IL-2 + Beads (Median: 7.01, rang: 5.6 to 8.8) group at 1:1 effector to target ratio was significantly lower than IL-2 + IL-15 + Beads (Median: 18.1, rang: 21.4 to 16.5) (p<0.001) group. Surprisingly, presence of beads did not apply significant change in T CD8$^+$ cells cytotoxicity while IL-2 and IL-15 were used simultaneously at effector to target ratio 2:1. The significantly higher cytotoxicity of CD8$^+$ T cells in the group of IL-2 + IL-15 + Beads (Median: 22.3, rang: 19.1 to 25.9) (p<0.01) compared to IL-2 + Beads (Median: 13.9, rang: 10.3 to 17.9) group was also observed at effector

to target ratio of 2:1, which was similar to another studied ratio.

IL-15 and microbeads elevated IFN-γ production by both CD4$^+$ and CD8$^+$ T cells

In the following step, the impact of different activation regimes on IFN-γ production was assessed. The IFN-γ production in the group treated with IL-2 + IL-15 (Median: 40.7, rang: 38.6 to 42.6) (p<0.0001) was higher than IL-2 treatment group (Median: 21.1, rang: 15.4 to 28.4). IFN-γ production raised significantly in IL-2 + IL-15 + Beads (Median: 56.5, rang: 52.2 to 59.3) (p<0.01) group comparing to IL-2 + IL-15 treatment group (Median: 40.7, rang: 38.6 to 42.6). A similar significant increment was observed in the counterpart group at 2:1 effector to target ratio. The production of IFN-γ increased in IL-2 + IL-15 (Median: 61.76, rang: 51.9 to 71.3) (p<0.001) and IL-2 + Beads (Median: 37.7, rang: 31.2 to 44.6) (p<0.05) group was higher than IL-2 (Median: 21.1, rang: 15.4 to 28.4) treatment group. Likewise, the production of IFN-γ was upregulated in IL-2 and IL-15 + Beads (Median: 68.4, rang: 57.9 to 85.6) (p<0.01) comparing to the group treated with IL-2 + Beads (Figure 3).
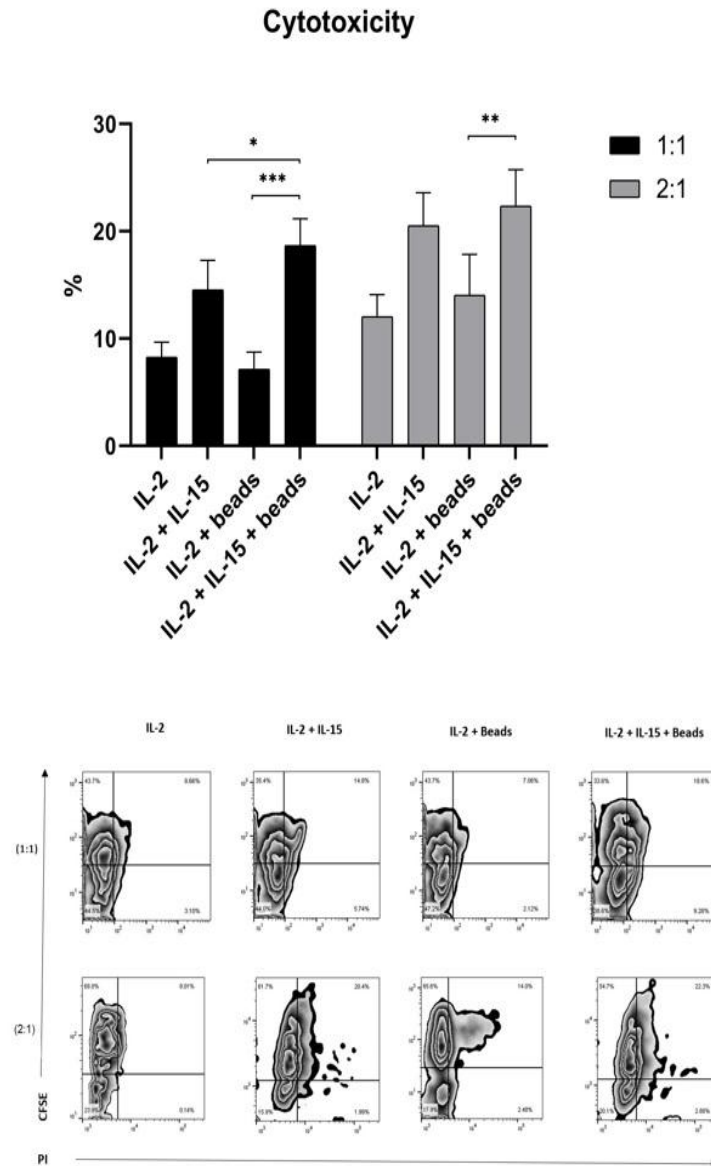
Figure 2. Assessment of T CD8^{+} cells Cytotoxicity. Purified T CD8^{+}cells were evaluated for their cytotoxicity capacity on EBV-LCLs with being activated in four different treatment groups including IL-2, IL-2 + IL-15, IL-2 + Beads and IL-2 + IL-15 + Beads at same time. Evaluation of T CD8^{+} cells' cytotoxicity at 1:1 effector to target ratio. Evaluation of T CD8^{+} cells' cytotoxicity at 2:1 effector to target ratio. Statistical comparison of cytotoxicity activity in all treatment groups carried out using Mann–Whitney test was used to compare difference between groups of interest. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$:) (n=3).
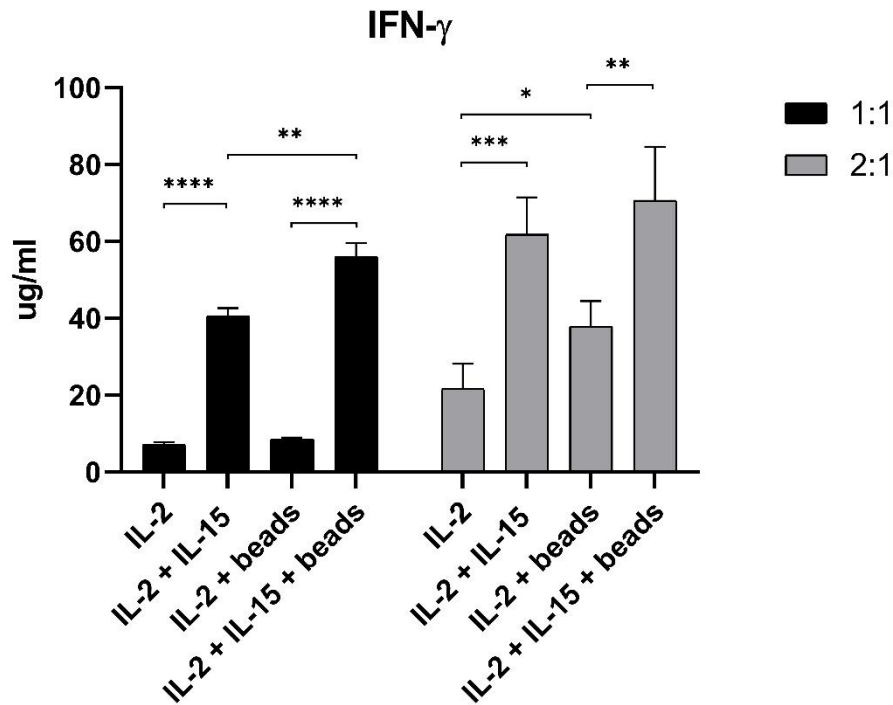
Figure 3. Measurement of IFN-γ production in T CD8$^+$ cells and EBV-LCLs coculture supernatant. Purified T CD8$^+$ cells were cocultured with EBV-LCLs and activated in four different treatment groups including IL-2, IL-2 + IL-15, IL-2 + Beads and IL-2 + IL-15 + Beads. The IFN-γ production was quantified at 1:1 and 2:1 effector to target ratio coculture supernatants. Statistical comparison of cytotoxicity activity in all treatment groups. Mann–Whitney test was used to compare difference between groups of interest. (*: $p < 0.05$, **: $p < 0.01$, $p < 0.001$ and ****: $p < 0.0001$:) (n=3).

DISCUSSION

Cytomegalovirus (CMV) is a threat to human health, especially for patients with weakened immune systems, in the world. This virus causes opportunistic infections, congenital infections and central nervous system infections. Treatment of infections CMV is difficult due to the special life cycle, mutation and latent characteristics of this virus. CMV infection is associated with high prevalence and mortality in immunocompromised patients, especially in recipients of HSCT and SOT transplants

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[43]. The protective function of CMV-specific T cells in HSCT patients has shown that the insufficient response of these specific cells in patients with late CMV infection after transplantation is important as a risk for CMV infection and late death [44]. The relation between T cell deficiency and dysfunction, especially CD4^{+} deficiency and CMV reactivation, has been shown in patients with HIV infection and subsequent solid organ transplantation. In HSCT patients, decreased lymphocytes number and CD4^{+} T cell counts less than 50 per microliter at 3 months post-transplant are risk factors for developing late-onset CMV infection. Hakki and colleagues have shown that a decrease in the number of CD4^{+} T cells (less than 100 per microliter) and a decrease in the number of CD8^{+} T cells (less than 50 per microliter) are associated with inefficient CMV-specific immunity [45].

Various treatment approaches are being implemented in order to reduce the adverse effects of CMV infection following HSCT and SOT in recipients. The first line of anti-CMV drugs is ganciclovir (and valganciclovir), which inhibits viral DNA polymerase. However, foscarnet and cidofovir are also used to manage resistant infections. Letermovir (terminase inhibitor) is a new drug approved for prophylaxis after allogeneic HSCT in recipients [46]. However, the side effects of these drugs, such as neutropenia and the emergence of drug-resistant infections, lead to reconstitute the recipient's immune that, it is be important in treatment approaches. Adoptive CMV-specific T-cell therapy is an emerging clinical tool with the potential to guide antiviral prophylaxis, a preventive therapy and treatment of CMV disease after transplantation [47,48].

In order to activate and expand T lymphocytes in adaptive immunotherapy, many studies are being conducted to generate optimal conditions for different laboratory protocol. In this new treatment approach, T cells are harvested directly from blood or tissue. Due to the decreased number of cells and poor response in immunocompromised patients, they must be activated and expanded *in vitro* before being transferred to patients [49].

Previous studies have shown several T cell expansion techniques, including [50] soluble anti-CD3, soluble anti-CD3 antibody in the presence of Fc receptor-bearing peripheral cells [51], immobilized anti-CD3 and immobilized anti-CD3 plus anti-CD28 [52]. Recently, the use of

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expensive paramagnetic beads coated with anti-CD3 and anti-CD28 have been used for T cell expansion [53]. The use of beads to cells with a ratio of about 0.1-5 has a positive effect on the expansion of T cells. However, values greater than 5 are not effective in increasing the rate of cell proliferation [54]. In adaptive immunotherapy by T cells, an equal ratio of anti-CD3/CD28 microbeads to cells creates optimal conditions for maintaining the balance between increased cell proliferation and stability of cell vitality, and its combination with homeostatic cytokines such as IL-2, IL-7, and IL-15 further improves cell yield, while a high microbead-to-cell ratio (3:1) helps generate bioactive cells [55]. A recent study showed that the combination of IL-2, IL-15, and IL-21 can effectively induce the expansion of tumor-infiltrating lymphocytes (TIL) with anticancer properties [56]. In another study of this group, with the effect of these cytokines on lymphocytes, it was reported that the expansion of HCMV T cells from healthy volunteers or cancer patients in the presence of IL-2, IL-15, 1L-21 and CMV-pp65 led to an increase in proliferation and generation of high-affinity CMV-specific T cells that express CCR6 and CXCR3 and caused them to migrate to virally infected

tissues, effectively reducing CMV load and possibly clearing the infection [57].

In our study, in order to optimize the conditions for increasing proliferation and output of HCMV-specific T cells, we used 1:1 the ratio of microbeads to cells along with IL-2 and IL-15. This study has shown that in the adaptive immunotherapy of HCMV-specific T cells, the combination of IL-15 along with anti CD3/CD28 microbeads promotes *in vitro* cells proliferation. Thus, in both T CD4$^+$ cells and T CD8$^+$ cells in the group where both cytokines were used together with beads, we observed the highest significant proliferation rate. Also, in the significant comparison of culture groups that contained or did, we reported the main role of B:C (1:1) ratio in the proliferation of both subsets of T lymphocytes for the expansion of these cells in HCMV adaptive immunotherapy.

On the other hand, the generation and expansion of specific CD8$^+$ cytotoxic T cells *in vitro* is also a key element in the induction of anti-tumor and anti-viral responses. Teschner and colleagues have shown that the activation and expansion of T cells from peripheral blood mononuclear cells using anti-CD3/CD28 microbeads

along with IL-2 during two weeks of culture increases the rate of cell expansion compared to the use of only IL-2. Also, the use of anti-CD137 microbeads plus anti-CD3/CD28 for a period of more than two weeks compared to the use of anti-CD3/CD28 microbeads alone causes a significant increase in the expansion of tumor-specific CTLs [58]. In another study, in order to *in vitro* produce type 1 and type 2 cytotoxic T cells (Tc1 and Tc2), different culture conditions were used. For both types of CTL cells, the cell culture medium contained IL-2. In this study, it was revealed that the cultured cells containing anti-CD3 and anti-CD28 showed the highest level of cytotoxic activity. Also, Tc1 cells obtained from cultures containing anti-CD3 and anti-CD28 have anti-tumor activity *in vivo* [59]. Montes and colleagues have also shown that a significant expansion of antigen-specific T cells, up to 50 % of the TCD8$^+$ cell population, can be achieved with the presence of IL-2 and IL-15. These cells have the ability to secrete cytolytic IFN-γ [60]. Interestingly, in a study conducted by Alves, the effect of IL-15 on non-antigen-dependent of differentiation and expansion of human TCD8$^+$ cells have been shown. They reported that naïve TCD8$^+$ cells

successfully downregulated CD45RA and CD28, but retained CD27 expression. Simultaneously, with these phenotypic changes, naïve T cells acquired the ability to produce IFN-γ and TNF-α, express perforin and granzyme B, and acquire cytotoxicity properties [61]. In another study, it has been shown that the migration of activated TCD8$^+$ cells is mediated by IL-15-induced CCR5, and CCL5/CCR5 interaction increases the proliferation and cytotoxic capacity of IL-15-activated TCD8$^+$ cells [62].

We have also shown that the use of cytokines with beads *in vitro* leads to an increase in the cytotoxicity of TCD8$^+$ cells and the secretion of IFN-γ. Thus, in both ratios of effector to target cells of this study, the presence of IL-15 along with beads and IL-2 caused a significant increase in the cytotoxicity of CTLs. Also, in a ratio of 1:1, we have reported the promoting role of beads in combination with IL-2 and IL-15. In addition, the secretion of IFN-γ in both reported ratios indicates that IL-15 in the presence and absence of beads has a significant ability to increase the secretion of this cytokine, and subsequently plays a significant role in the cytotoxicity of CTLs. However, in the IL-2+IL-15+Beads group, we had the highest level of IFN-γ secretion,

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which indicates the important role of beads along with IL-15.

CONCLUSION

According to the results obtained from our study, in adoptive HCMV-specific T-cell therapy, the use of cytokines such as IL-15 along with anti-CD3/CD28 magnetic microbeads in TCD4$^+$ and TCD8$^+$ cell cultures could significantly increase cell proliferation. Also, these expanded cells show increased cytotoxicity toward target cells and produce more IFN-γ *in vitro*. Therefore, the new therapeutic approach of using virus specific T cells in patient with an inefficient immune system could play an improving role in controlling and eliminating viral infections.

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