

ASSESSMENT OF CELL CULTURE MEDIA VARIATION INDUCING CYTOKINE SECRETION IN PBMCs, CD3⁺, AND CD8⁺ IMMUNE CELLS

OLTEANU GHEORGHE-EMILIAN^{1,2}, CRISNIC DANIELA^{1,2,3}, GRIJINCU MANUELA¹, CRISTEA MIRABELA¹, ZAMBORI CSILLA^{1,2}, IVAN ALEXANDRA^{1,2}, BOJIN FLORINA^{1,2}, GAVRILIUC OANA^{1,2}, NEDEA EDUARD^{1,4}, PAUNESCU VIRGIL^{1,2}

¹Clinical Emergency County Hospital "Pius Brinzeu" Timisoara, Center for Gene and Cellular Therapies in the Treatment of Cancer Timisoara – OncoGen; B-dul Liviu Rebreanu No. 156, RO-300723, Timisoara, Romania

²"Victor Babes" University of Medicine and Pharmacy Timisoara, Department of Functional Sciences; Eftimie Murgu Square No. 2A, RO-300041, Timisoara, Romania

³Biodim SRL, Ciprian Porumbescu Street, Timisoara, Romania

⁴Biologos SRL, Curtea de Argeş, Romania

ABSTRACT

In vivo conditions, the innate immune arm signals shape the activation of naive T cells, however, it is unclear how T cells maintain their function in vitro, in experimental settings, where different culture conditions signals influence the effector T-cell function. In this study of two sets of healthy donors designated here group A (n=10) and group B (n=10), we assessed the cytokine profile of PBMCs, cluster of differentiation 3 (CD3⁺) cells and cluster of differentiation 8 (CD8⁺) T cells, which were cultured in different environmental conditions, including Interleukin-2 (IL-2) and/or CD3/CD28 T cell activator, supplemented culture media. The (IL-2), Interleukin-5 (IL-5) and Interferon- γ (IFN- γ) secretion at 24, 48, 72, and 96 hours was determined in a 4.5-hour solid-phase ELISA immunoassay method. The medium containing IL-2 and CD3/CD28 T cell activator was the one that induced increased secretion of IL-2, IL-5, and IFN- γ in all three cell populations, mainly in the CD3⁺ cells, irrespective of the time frame sampled.

Key words: PBMCs, cytokine, CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, ELISA

INTRODUCTION

This study was designed to provide a window in the cytokine profile of peripheral blood mononuclear cells (PBMCs), cluster of differentiation 3 (CD3⁺) cells, and cluster of differentiation 8 (CD8⁺) T cells, grown in different environmental conditions and to ascertain which culture condition provided the best quantifiable secretion of Interleukin-2 (IL-2), Interleukin-5 (IL-5), and Interferon- γ (IFN- γ). All three cytokines of interest in this study are involved to a different degree in various aspects of the immune response, from T cell activation to regulatory induction [1-5]. Overall, the vast majority of evidence found in the literature credits IL-2 with an essential part in the primary immune response, a role in immune memory, induction of regulatory T cells, and finally, IL-2 can also be considered a survival factor for T cells via induction of anti-apoptotic proteins [6-7].

In humans, IL-5 acts mainly on eosinophils and mast cells, and activation of B cells via dependent pathways, therefore IL-5 can be viewed as a chemokine bridge between the innate, and the acquired immune arms of the immune system [8-9]. IFN- γ is a soluble cytokine produced by a variety of immune cells like CD4⁺ helper cells (Th1), CD8⁺ cytotoxic T-lymphocytes (Tc1), non-cytotoxic innate lymphoid cells (ILC), and predominantly by natural killer (NK), and natural killer T (NKT) cells as part of the innate immunological response [10]. Interferon- γ can be viewed as a central node in both the innate and adaptive immune response, with cellular effects like

upregulation of pathogen recognition, inhibition of cellular proliferation, antigen processing and presentation, antiviral, immunomodulation, and leukocyte trafficking [11-12].

Whole blood from two sets of healthy donors designated here group A (n=10) and group B (n=10)

Received 5th of October 2020. Accepted 11th of November 2020. Address for correspondence: Olteanu Gheorghe-Emilian, "Victor Babes" University of Medicine and Pharmacy Timisoara, Department of Functional Sciences, Eftimie Murgu Square No. 2A, RO-300041, Timisoara, Romania; OncoGen-Centre for Gene and Cellular Therapies in the Treatment of Cancer, "Pius Brinzeu" Clinical Emergency County Hospital Timisoara, Romania, Liviu Rebreanu No.156 Blv., Timisoara, RO-300723, Romania; phone/fax: +40356433282; e-mail: olteanugheorgheemilian@gmail.com

was used to provide Ficoll-Paque media density gradient isolation of PBMCs, that were characterized via a flow cytometry analysis. Using negative selection, CD3⁺ and CD8⁺ T lymphocytes were isolated. Afterwards, a flow cytometry analysis was performed to view the distribution of cell populations. The cells of interest, PBMCs, CD3⁺ and CD8⁺ were plated into 24-well plates, and grown using 3 types of environmental conditions, including IL-2 and/or CD3/CD28 T cells activator supplemented culture media.

Secretion of IL-2, IL-5, and IFN- γ was assessed with the 4.5-hour solid-phase enzyme-linked immunosorbent assay (ELISA) immunoassay method on a windowed time frame analysis at 24, 48, 72, and 96 hours [13-19]. All the samples were evaluated in double and when necessary in triple pairs.

MATERIALS AND METHODS

Negative selection of human CD3⁺ T lymphocytes

The selection of human CD3⁺ T lymphocytes was made with the EasySep™ Human T Cell Enrichment Kit (STEMCELLS Technologies, Vancouver, Canada) from frozen human PBMCs (two sets of healthy donors group A (n=10) and group B (n=10)). This kit targets non - T cells for removal with Tetrameric Antibody Complexes and dextran-coated magnetic particles recognizing specific cell surface markers. Unwanted cells are labelled with antibodies and magnetic particles and separated without columns using an EasySep™ magnet. The cells of interest (CD3⁺ T lymphocytes) are simply poured off into a new tube. The cells were resuspended, 1.6×10^7 cells in 320 μ l buffer (EasySep™ Buffer, STEMCELLS Technologies, Vancouver, Canada) into a polystyrene round-bottom tube and incubated with 16 μ l of isolation cocktail (EasySep™ Human T Cell Enrichment Cocktail, STEMCELLS Technologies) for 5 minutes at room temperature. The magnetic particles 16 μ l/sample (EasySep™ D Magnetic Particles, STEMCELLS Technologies) were added to the sample and incubated for 5 minutes at room temperature. The tube was placed into the magnet and incubated for 5 minutes at room temperature, and the enriched cell suspension was poured into a new tube. Following isolation 3×10^6 CD3⁺ T lymphocytes/ml were obtained.

Negative selection of human CD8⁺ T lymphocytes

The selection of human CD8⁺ T lymphocytes was made with the EasySep™ Human CD8⁺ T Cell Enrichment Kit (STEMCELLS Technologies) from frozen human PBMCs (two sets of healthy donors group A (n=10) and group B (n=10)). This kit targets non - CD8⁺ T cells for removal with Tetrameric

Antibody Complexes recognizing specific cell surface markers and glycophorin A, and dextran-coated magnetic particles. Unwanted cells are labelled with antibodies and magnetic particles and separated without columns using an EasySep™ magnet (STEMCELLS Technologies). Following isolation, 3×10^6 CD8⁺ T lymphocytes/ml were obtained. After isolation PBMCs, CD3⁺ and CD8⁺ T cells were plated into 24-well plates. All cell types were plated at 5×10^5 per well (1 ml/well), with three different types of media: 1. RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 5% human serum (HS, Sigma-Aldrich) and 1% Penicillin – Streptomycin (Sigma-Aldrich); 2. RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 5% human serum (HS, Sigma-Aldrich) and 1% Penicillin – Streptomycin (Sigma-Aldrich) and 30 UI/ml IL-2; 3. RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 5% human serum (HS, Sigma-Aldrich) and 1% Penicillin – Streptomycin (Sigma-Aldrich), 30 UI/ml IL-2 (R&D Systems, Minneapolis, USA), and CD3/CD28 Dynabeads (Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation, ThermoFisher Scientific, Waltham, MA USA).

Flow cytometry

After using Ficoll-Paque media for density gradient isolation of PBMCs, the resulting cells were aliquoted and stained with Simultest BD Biosciences (San Jose, California, USA) antibodies CD3 FITC/CD8 PE, afterwards following the instructions provided by the manufacturer a two-colour flow cytometric analysis was performed on the BD FACSVerse™ (San Jose, California, USA) flow cytometer. Subsequently, after the negative selection of human CD3⁺ T lymphocytes from PBMCs, a Multitest BD 4 antibody cocktail (CD3/CD8/CD45/CD4) was used to highlight the distribution of cell populations using the same BD FACSVerse™ platform.

ELISA

The supernatant of all cell types, cultivated in the three different media was collected at distinct time points: 24h, 48h, 72h and 96h. The Quantikine® Human Immunoassay (R&D Systems) was used for assessment of IL-2, IL-5, and IFN- γ secretion of the cells. The procedure followed the instructions provided by the manufacturer. Shortly, the assay employs the quantitative sandwich enzyme immunoassay technique, in which a monoclonal antibody specific for human cytokines is pre-coated onto a 96-well polystyrene microplate; standards and samples are pipetted into the wells in duplicate, and IL-5, IL-2, or

IFN- γ present in the sample is bound by the immobilized antibody. The optical density was determined using Tecan Infinite M200 Pro (Tecan, Männedorf, Switzerland) microplate reader at 450 nm,

Statistical analysis

Statistical analysis was performed using IBM , NY).

with wavelength correction at 570 nm, and each cytokine concentration was calculated based on the standard curve and regression equation.

SPSS Statistics for Windows, Version 20.0. (IBM Corp., Armonk

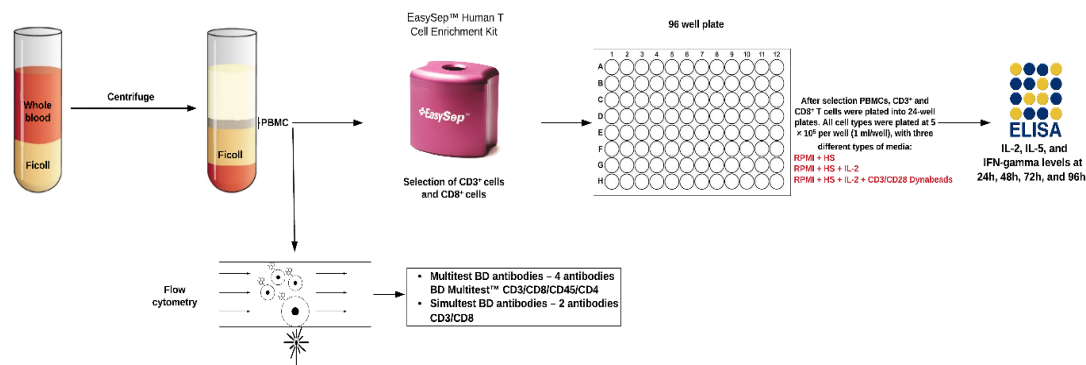


Fig. 1. Graphical abstract of methods used in this study.

RESULTS

Flow cytometry analysis of PBMCs, CD3⁺, and CD8⁺ T lymphocytes

Flow cytometry of PBMCs from both sets of healthy donors, group A (n=10) and group B (n=10)

was analyzed with a gating strategy of CD45 (Ly-5), CD3, CD4 with CD8, and CD8 with CD3, with percentage representation of the cell population, both sets of healthy donors showed similar results on the analysis (Fig. 2).

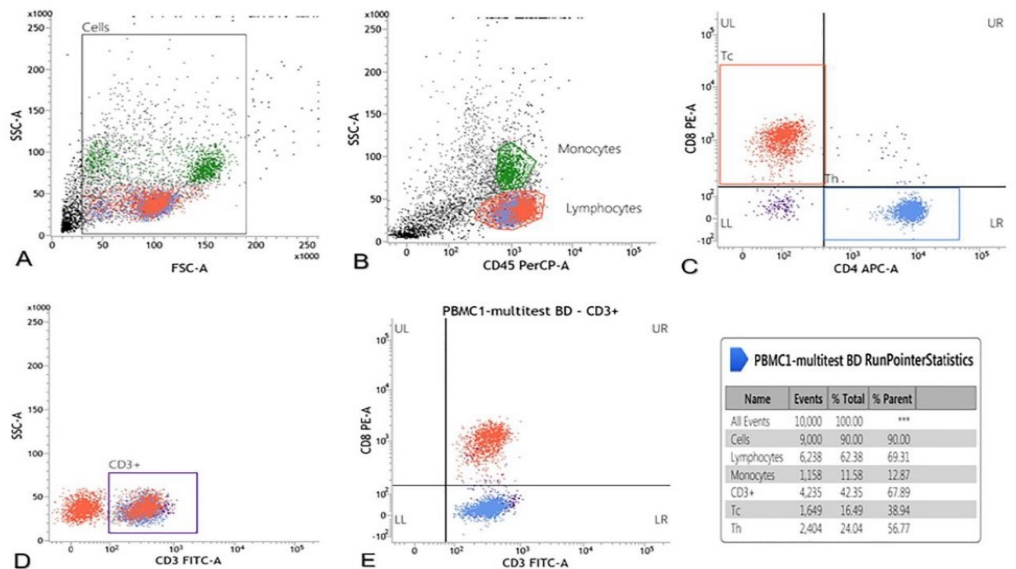


Fig. 2. Gating strategy for lymphocytes population. A. Doted plot representing nucleated cells; B. Monocyte and lymphocyte population based on CD45 expression marker; C. cytotoxic T cells - CTLs (CD8⁺) and T cells (CD4⁺) from total T cells; D. T cells (CD3⁺); E. CD8⁺ cells and percentage distribution of cellular populations

Following the negative selection of CD3⁺ and CD8⁺ T lymphocytes from isolated PBMCs previously quantified and analyzed, a flow cytometry analysis was performed on the isolated CD3⁺ and CD8⁺ T lymphocytes, the cell population was gated and percentages were quantified for both sets of healthy donors used in the study (Fig. 3).

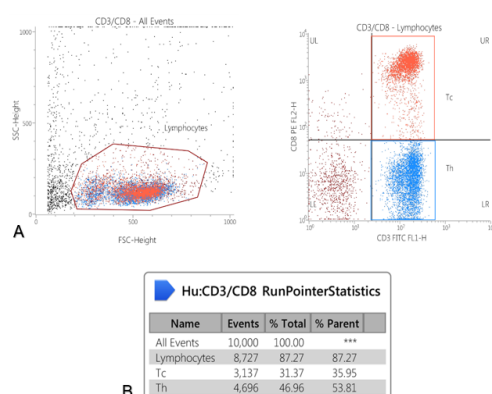


Fig. 3. Flowcytometric analysis of gated selected T cells (CD3⁺). A. Dot plot of total lymphocyte population and CTLs (CD3⁺/CD8⁺); B. Percent value of CTLs and T helper cells from the isolated T cells.

CD8⁺ T cells represented 35.95% of the total CD3⁺ cells, while the CD4⁺ cells were 53.81%. We obtained an enrichment of the total number T cells from 67.89% to 87.27% after cultivation with activator and expansion cocktail.

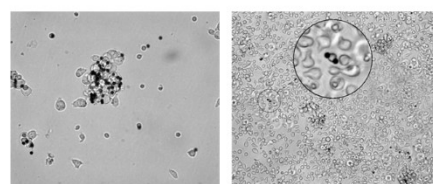


Fig. 4. Cells activated and expanded using CD3/CD28. A. The Dynabeads can be seen coupled with the cells. B. Digital magnification of T-cell coupled with CD3/CD28 Dynabeads.

Solid-phase ELISA analysis of IL-2, IL-5, and IFN- γ IL-2

Although IL-2 was added in the culture media from the beginning, the IL-2 concentration determined by ELISA method shows a time-dependent increase, similar to IL-5. The highest concentration value is reached after 96h by the CD3⁺ cell population, thus confirming synergistic functioning of CD4⁺ and CD8⁺

cells when they are together in cell culture conditions. PBMC whole population has a decreased ability to secrete the mitogenic T cell factor. In Figure 5, the differences between cellular populations concerning IL-2 secretion are well represented with statistical significance bars.

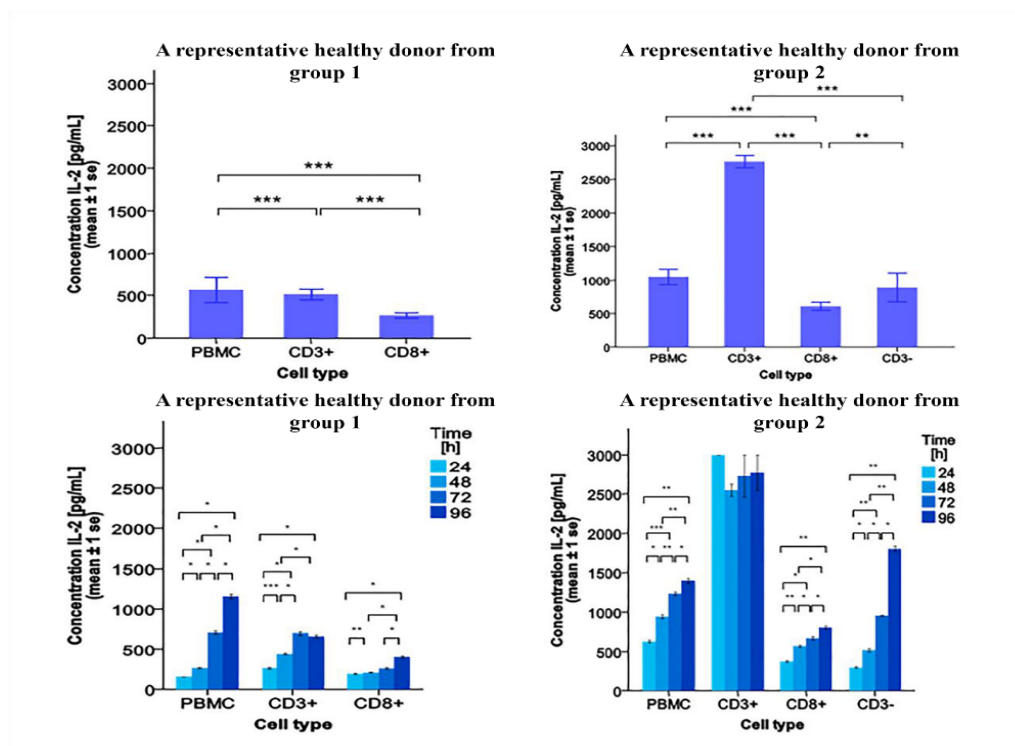


Fig. 5. IL-2 concentration cell types per groups of patients. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Results from posthoc MANOVA GTA Hochberg correction.

IL-5

ELISA assay performed at 24h, 48h, 72h, and 96h revealed that IL-5 concentration increases in a time-dependent manner in all cellular population, for both sets of healthy donors. However, the most important increase was observed for CD3⁺ T cells, with

a significant statistical difference compared with PBMC, CD8⁺ or CD3⁻ cellular populations. Interestingly, the CD3⁻ cellular populations secreted large amounts of IL-5, probably due to monocytes and granulocytes present in the cell culture. ($p \leq 0.001$ between PBMC and CD3⁻; Fig. 6)

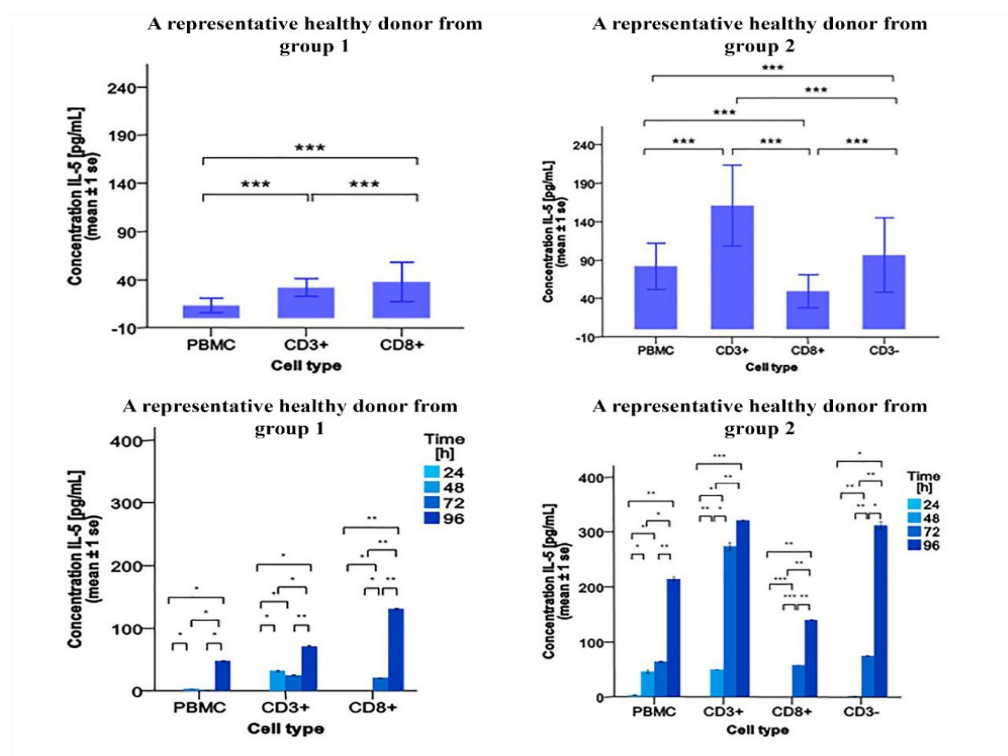


Fig. 6. IL-5 concentration cell types per patient. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Results from posthoc MANOVA GTA Hochberg correction.

INF- γ

INF- γ , although normally secreted also by Tc1 CD8⁺ cells, it has the maximum concentration in the supernatant of the CD3⁺ cells, where the contribution is due to Th1 CD4⁺ cells (Fig. 6). Even though we used the same culture media for all cellular types, supplemented with CD3/CD28 activator and IL-2, it

seems that the PBMC population is not able to contribute with higher amounts of functional cytokines compared to CD3⁺ population of cells. CD3⁺ cells have the optimum cytokine secretion; further usage of this cell population in further in vitro and in vivo studies of interleukin production is recommended.

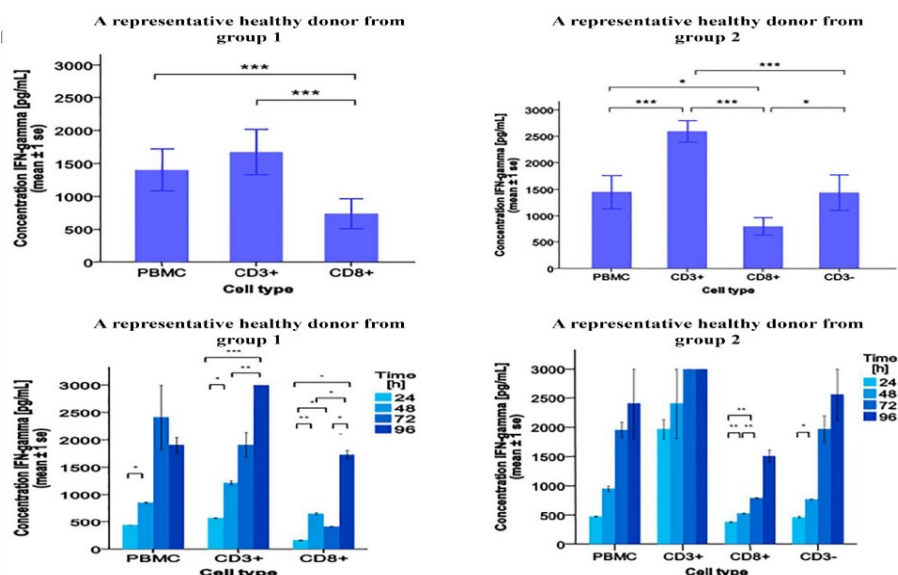


Fig. 7. IFN-gamma concentration cell types per both sets of healthy donors. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Results from posthoc MANOVA GTA Hochberg correction.

DISCUSSION

Scientific data credits IL-2 with a central role in T regulatory (Treg) cell production and homeostasis, with present studies attributing IL-2 a part in T memory recall responses [6]. IL-5's vital role in eosinophil activation and generation is well established, thus, IL-5 expands the innate immune response, and it accentuates the acquired immune response linking the two main branches of the immune system [8]. IFN- γ harmonizes many regulatory cellular programs via its effects on transcriptionally relevant genes in the immune response, specifically those that pertain to macrophage activity [11]. To revenue the best output of cytokines concerning stimulation of production and secretion of cytokines, a well-established stimulatory cocktail is used. In this study, the best outcome, or revenue (quantifiable for cytokines secreted), is in the culture medium supplemented with CD3/CD28 Dynabeads, a stable, well-used activator and expansion kit [20-22].

CONCLUSION

In our present study, there was no observable statistical difference in cytotoxicity in the cell populations in the first and second growth environment, notable IL-2, IL-5, and IFN- γ secretions were registered in the third growth environment supplemented with IL-2, and CD3/CD28 T cells activator. The cell population with the most efficient effector function was CD3+ irrespective of the time frame sampled. Therefore, considering the findings in our study and the evidence in the literature, the best growth environment for the quantitative secretion of IL-2, IL-5, and IFN- γ can be viewed as a cocktail between normal growth media, IL-2, and CD3/CD28 T cells activator supplementation.

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EVALUAREA VARIAȚIEI MEDIULUI DE CULTURĂ ASUPRA SECREȚIEI DE CITOKINE A PBMCS, LIMFOCITE T CD4+ ȘI CD8+

REZUMAT

În condiții *in vivo*, sistemul imun înăscut modulează activarea limfocitelor T naîve, dar este încă neclară modalitatea prin care limfocitele T reușesc să își mențină funcția *in vitro*, în condiții experimentale, care influențează funcția limfocitelor T efectoare. În acest studiu, pe două seturi de donatori sănătoși, denumiți ca grup A (n=10) și grup B (n=10), am testat profilul citokinic al PBMCS, limfocitelor T CD3+ și al limfocitelor T CD8+, care au fost cultivate în medii de cultură diferite, care au inclus suplimentarea cu IL-2 și/sau activator al limfocitelor T, CD3/CD28. A fost determinată secreția citokinelor de tip IL-2, IL-5 și - γ (IFN- γ) la 24, 48, 72 și 96 de ore, prin testări assay-uri imune de tip ELISA. Mediul de cultură conținând IL-2 și activator limfocitar CD3/CD28 a fost cel care a dus la secreția crescută de IL-2, IL-5 și IFN- γ în toate cele 3 populații celulare investigate, mai ales la nivelul limfocitelor T CD3+, indiferent de intervalul de timp la care a fost investigată.

Cuvinte cheie: PBMCS, citokine, limfocite T CD4+, limfocite T CD8+, ELISA