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Phenotypic and genetic evaluation of human monocyte-derived dendritic cells generated from whole blood for immunotherapy

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Abstract

Background: Dendritic cells (DCs) recognize different pathogens and cancer cells and activate the adaptive immune response. The generation of effective DC-based cancer vaccines depends on the appropriate differentiation of monocytes in vitro. This study aimed to standardize a protocol for the in vitro differentiation of human peripheral blood monocytes into immature DCs upon treatment with growth factors and generate monocyte-derived DCs (MoDCs). Peripheral blood mononuclear cells were separated from peripheral blood. After monocyte enrichment by plastic adhesion, monocytes were cultured for 6 days in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4 to generate immature DCs. The cells were examined by microscopy. Using flow cytometry, DCs were evaluated for the expression of the CD83 and HLA-DR surface antigens, for the uptake of fluorescein isothiocyanate conjugated dextran, and also for the expression of CD80 and CD86 mRNA.

Results: CD80 and CD86 genes expression was upregulated at day six and exhibited a significant difference ($P < 0.05$). DCs showed positive expression of the CD83 and HLA-DR surface antigens by flow cytometry and FITC-conjugated dextran uptake.

Conclusion: This study represents a preliminary trial to generate immature MoDCs in vitro from blood monocytes collected by the flask adherence method. It offers a panel of surface markers for DCs characterization and provides Immature DCs for experimental procedures after 6 incubation days.

Keywords: Adherence, Monocyte-derived, Cell separation, Dendritic cell, Phenotype

Background

Dendritic cells (DCs) recognize and uptake various antigens, including tumors and viruses in peripheral tissue. Once activated, they migrate to lymph nodes, where they initiate an adaptive immune response. To achieve this, DCs process the engulfed antigens into peptides, which are presented on major histocompatibility complex (MHC) molecules. Peptides loaded onto MHC II molecules are recognized by antigen-specific CD4+ T helper cells. Similarly, peptides loaded onto MHC I molecules are recognized by antigen-specific CD8+ T cells, leading to their proliferation and activation of cytotoxic activity [1].

DCs may be categorized as immature and mature according to their morphology and function. Once foreign antigens are presented to the pattern recognition receptors (e.g., toll-like receptors, C-type lectins, or complement receptors) expressed on the cell membrane, iDCs undergo changes and maturation.

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During this maturation process, antigen uptake receptors are downregulated, whereas antigen presentation molecules are upregulated. Mature DCs express the major histocompatibility complexes I and II in addition to the co-stimulatory molecules CD80 and CD86, which mediate antigen presentation and T lymphocyte activation [2].

DCs stimulate adaptive immunity and are widely used for cancer immunotherapy, particularly in DC vaccination. With DC-based vaccines, cancer patients are injected with mature tumor antigen-loaded DCs, which are prepared ex vivo. These specialized cells stimulate cytotoxic tumor-specific T lymphocytes that eliminate cancer cells and induce memory to prevent further cancer growth and recurrence [3].

This study aims to provide an effective strategy to generate DCs from human blood monocytes collected using the immunotherapy flask adherence method.

Methods

This study was conducted in the Medical Biochemistry Department, and written informed consent was obtained from all participants.

Peripheral blood mononuclear cell (PBMC) isolation

Fresh peripheral blood (100 ml) was collected from 20 healthy donors under complete aseptic conditions (laminar flow work area). Heparinized blood was diluted 1:1 with phosphate buffer saline (PBS) (Sigma, USA) in 15 ml Falcon tubes. Using a sterile plastic Pasteur pipette, the diluted blood was layered entirely onto an identical volume of a density gradient containing 5.6% Ficoll reagent and 9.6% diatrizoate with a density of 1.077 g/ml and an osmolarity of 300 mOsm. We situated falcon tubes at a 45° angle, allow diluted blood to run down the side of the tube without allowing the two solutions to mix [4].

Samples were centrifuged for 30 min at 400×*g* at room temperature. PBMCs were collected and washed twice with PBS, and 2% heat-inactivated FCS solution (Invitrogen, Grand Island, NY, USA) (PBS/2% FCS) centrifuged for 10 min at 400×*g*, and the PBMC pellet was collected at the bottom.

PBMC viability, counting, and culture

The pellet was resuspended in 2 ml of RPMI (Sigma, Catalog. no. R8758, USA) and counted by hemocytometer. The cells were tested for viability by trypan blue exclusion (Merck Darmstadt, Germany). A total of 1×10^6 cells per ml were cultured in RPMI-1640 containing 10% fetal calf serum and 2% penicillin-streptomycin (Gibco, Catalog. no. 15140-122, USA), then placed in a 6-well plate, containing 50 ng/ml of GM-CSF (R&D Systems, catalog no 215-GM, Minneapolis, USA) and 10 ng/ml of IL-4 (R&D Systems, catalog no 204-IL, Minneapolis, USA). The 6-well plate was incubated for two hours at 37 °C in a humidified, 5% CO_2 atmosphere. After 2 h, the non-adherent cells were removed.

Preparation of monocyte-derived DCs

A fresh medium enriched with GM-CSF and IL-4 was added to the culture. We used an inverted microscope at × 400 magnification power to examine the adherent cells in order to detect monocytes. Every 2 days, complete fresh media was added to the monocytes to generate the immature monocyte-derived DCs (MoDCs). After culturing for 6 days, iDCs were developed.

Characterization of monocyte-derived DCs (MoDCs)

Phenotypic analysis of DC by flow cytometry

DCs were stained with specific monoclonal antibodies CD83-PE and HLA-DR-FITC surface markers (eBiosciences, USA). One hundred microliters of cells at 75×10^3/ml were incubated in the dark for 30–45 min with 20 μl of FITC-conjugated antibodies at 4 °C. The cells were washed with cold phosphate buffer saline (PBS), fixed in 1% formaldehyde, and resuspended in 200 μl PBS (Applied Biosystems) [5].

FITC-dextran assay

Cells were harvested in a fresh complete culture medium containing 0.5 mg/mL FITC-dextran (Sigma, St. Louis, MO, USA) and incubated for 30 min at 5% CO_2 and 37 °C (negative control was incubated on ice) to evaluate the ability of DCs regarding the uptaking of soluble antigens from the culture medium. The uptake was stopped by adding cold PBS with 1% FCS. Then, the cells were washed four times using a refrigerated centrifuge and then analyzed by flow cytometry [6].

Quantitative real-time PCR for measuring CD80 and CD86 gene expression

Total RNA was extracted from cultured cells using a kit obtained from (iNtRON Biotechnology, South Korea). RNA was reverse-transcribed to cDNA using the TIAG EN Fast Quant RT Kit (with gDNase). The primers (Biolegio, Nijmegen, Netherlands) sequences used were as follows: CD80 [7] forward 5′- CATCCAAGTGTCCATA CCTC -3′ and reverse 5-CTCTCATTCCTCCTTCTC TC-3′, for CD86 [8] forward 5′- TGGTGCTGCTCCTC TGAAGATTC-3′ and reverse 5′-ATCATTCCTG TGGGCTTTTTGTG-3′, and β-actin [9], forward 5′-GGT TCC GCT GCC CTG AGG-3′ and reverse 5′-GTC CAC GTC ACA CTT CATG-3′. The amplification was done using qPCR (Stratagene Mx3005P) with the following protocol: initial denaturation and polymerase activation at 95 °C for 30 s. For CD86, 40 cycles of denaturation at 95 °C for 15 s were done, followed by

annealing and elongation at 67 °C for 1 min. For CD80, 40 cycles of denaturation at 95 °C for 15 s were done, followed by annealing and elongation at 63 °C for 1 min. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method, as described by Livak and Schmittgen [10].

Statistical analysis

The collected data were statistically analyzed using the SPSS program (Statistical Package for Social Science) version 20. Quantitative data were expressed as the mean ± SD (standard deviation). The paired sample *t*-test was considered statistically significant at *P*-values < 0.05, highly statistically significant at *P*-values < 0.001, and non-significant at *P*-values > 0.05.

Results

Morphological characterization of cultured monocytes and DCs by inverted microscope

Microscopic examination of the mononuclear cells obtained by Ficoll-Hypaque gradient centrifugation from fresh blood samples was tested on day 0 of the experiment. The cell counting and viability of PMNC and monocytes were evaluated in the Table 1. Monocytes were spherical and rounded. They were large in size and contained a large nucleus (Fig. 1a). Morphological examination of DCs was done on the seventh day.

Biochemical results

Immunophenotypic analysis of DCs by flow cytometry

DCs (gated cells) showed positive CD83 and HLA-DR expression. The measured mean fluorescent intensity (MFI) ± SD for three experiments was 105 ± 4.3 for CD83 and 95 ± 3.4 for HLA-DR. Each value is the mean of 3 performed experiments ± SD (Fig. 2a–c).

Antigen uptake assay

DCs express mannose receptors which facilitate dextran endocytosis and enable their active uptake to reflect the endocytic antigen capacity. The data revealed that DCs exhibited a high ability to incorporate FITC-dextran; the mean value of uptake capacity ± SD was 64 ± 2.3% (Fig. 3a–d).

Quantitative real-time PCR analysis of CD80 and CD68 genes

The expression of CD80 and CD86 mRNA was assessed by qRT-PCR relative to β-actin expression. The result demonstrated a significant upregulation in the expression of both CD80 and CD86 in DCs compared with untreated monocytes. The mean value ± SD of CD80 and CD86 in DCs relative to monocytes was 2.5 ± 0.532 and 4 ± 0.42, respectively (Fig. 4).

Discussion

DCs exhibit the highest potency for antigen uptake and presentation among the immune cells [11, 12]. They show many immune regulation features that balance the complex system of inflammatory and inhibitory immune reactions in the tumor tissue microenvironment [13]. DCs are involved in innate and adaptive immunity. They can modulate immune function, reverse immunosuppression, and decrease cancer immunotolerance [14].

Human monocytes isolated from PBMCs differentiate to produce iDCs so that DCs are usually generated from blood monocytes in vitro. iDC maturation to mature DCs occurs through an antigen-loading step [15].

Human DCs are loaded with recombinant proteins, lysates of tumor cells, or peptides, the latter representing the most common process [16].

DC vaccines aim to stimulate cancer-specific effector T cells to eradicate tumor cells and to stimulate immunological memory to control cancer recurrence [16]. Peptide-loaded DCs can present Ags to naïve T lymphocytes [17]. The proliferative and cytolytic function of tumor-specific cytotoxic T-lymphocytes requires Ag identification by the T cell receptor (TCR). These antigens are linked to MHC class I molecules on APCs [16].

This study evaluated the DC generation from monocytes isolated from whole peripheral blood for immunotherapy in an in vitro model. These DCs were isolated using Ficoll-Hypaque gradient centrifugation.

There are multiple methods for collecting monocytes from whole peripheral blood, including flask adherence [18], density gradient centrifugation [19], and separations using a specific marker such as magnetic-activated cell sorting [2] or monocytes separation by bipolar tetrameric antibody [20]. In our study, we used the flask adhesion method for monocyte separation. Delirezh and Shojaeefar [21] demonstrated that magnetic-activated cell sorted monocytes' viability was slightly higher than Flask-DCs. They express higher levels of $CD14^{-}$ compared with Flask-DCs but a higher IFN-γ to IL-4 ratio, IL-12, and an IL-12 to IL-10 ratio in the flask group. Also, Flask-DCs polarized the immune response toward a Th1 cytokine profile and cell-mediated immunity, a desired feature in cancer immunotherapy.

Table 1 The cell count and viability of peripheral blood mononuclear cells and monocytes at day 0

	Cell count (cells/ml)	Mean ± SD	Viability %
Mononuclear cells	5 to 5.0×10^7	5.0×10^7 ± 0.52	95–98
Monocytes	5 to 15×10^5	10×10^5 ± 3.251	70–75

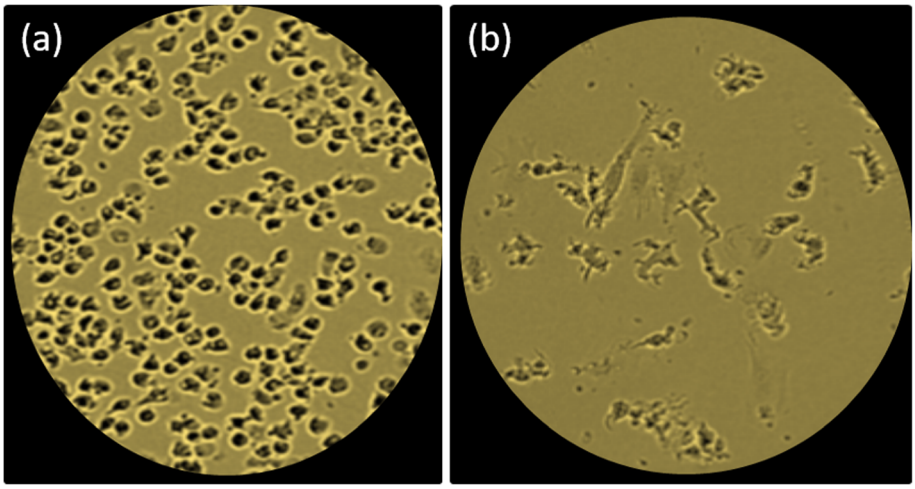

Fig. 1 Microscopic image showing morphological changes during in vitro culture of monocytes and the generation of dendritic cells (× 40). **a** Morphologic appearance of cultured adherent monocytes on day 0; the cells were small and rounded with a large nucleus. **b** Generated immature DCs on the day; cells were semi-adherent with multiple projections

For MoDC generation through human monocytes isolation, Posch et al. [2] used the anti-human CD14 magnetic nanoparticle for the positive selection of CD14 leukocytes. The advantages of this technique compared with other protocols are the high purity and speed. MoDCs are the most popular model used for DC generation because the direct isolation of DCs from biopsies or cord blood $CD34^+$ stem cells is a more complex technique. Also, it leads to inefficient cell numbers.

Elkord et al. [22] showed that the positive selection of monocytes by anti-CD14 coated microbeads inhibits lipopolysaccharide (LPS)-induced production of IL-12, IL-10, and TNF-α from DCs. However, for Flask-DCs, LPS induced much higher levels of IL-12, IL-10, and TNF-α cytokines and CTLs.

DCs were phenotypically and genetically confirmed. HLA-DR, CD 83, and dextran uptake were detected by flow cytometry. Also, the expression of CD80 and CD86 genes was measured by quantitative real-time PCR.

Our results demonstrated DC-positive expression of CD83 and HLA-DR by flow cytometry, DCs showed high positive uptake of dextran and significant upregulation of CD80 and CD86 genes expression in DCs compared to monocytes.

Pan et al. [23] and Elkord et al. [24] reported similar findings.

Pan et al. [24] observed positive CD83 and HLA-DR and positive expression of CD80 and CD86. In their model, PBMCs were isolated from healthy donors' peripheral blood using Ficoll-Hypaque gradient centrifugation, and the monocytes were separated by the flask

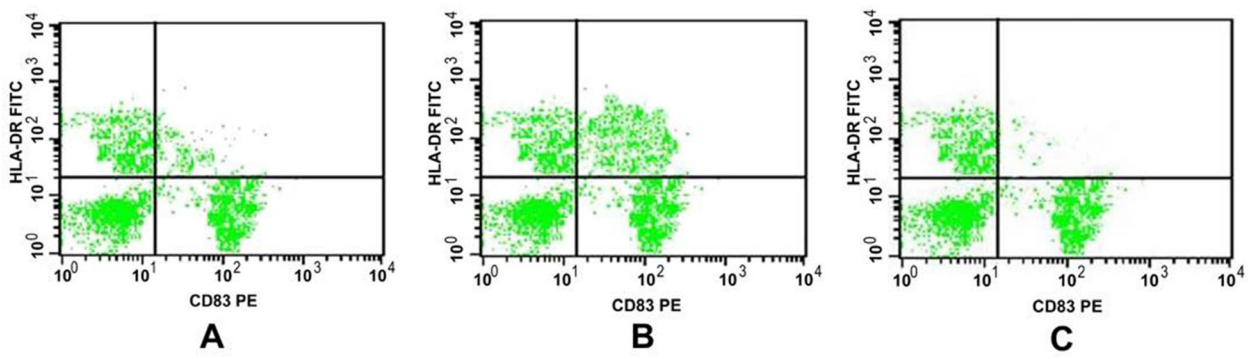

Fig. 2 The expression of CD83 PE (lower right) and HLA-DR FITC (upper left) on dendritic cells by flow cytometry; dendritic cells (gated cells) showed positive surface expression of CD83 and HLA-DR

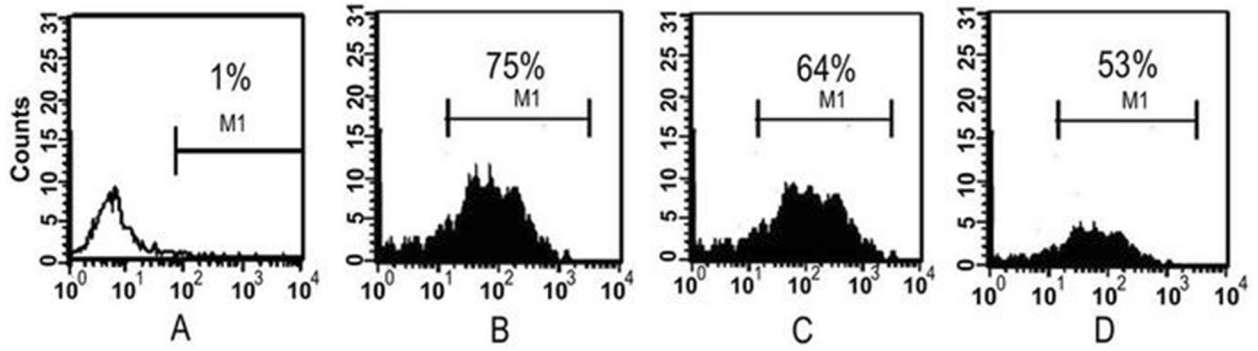

Fig. 3 Active uptake FITC-dextran by dendritic cells; **a** Dextran uptake by monocytes (control). **b**–**d** First, second, and third experiments of evaluation of dextran uptake by dendritic cells, respectively

adherence method. Non-adherent cells (T cells) were removed. Monocytes were harvested with media enriched with IL-4 (400 U/ml) and GM-CSF (1000 U/ml) to produce DCs under conditions identical to our study.

Elkord et al. [22] demonstrated that monocytes exhibit lower levels of CD14 expression and higher levels of HLA-DR and CD86 expression. iDCs express CD1a and low levels of CD80 on their cell surface but do not express CD83.

Our study used IL-4 and GM-CSF at concentrations of 10 ng/mL and 50 ng/mL, respectively, and the cells were cultivated for 6 days, consistent with El-Sahrigy et al. [23]. They demonstrated that iDCs were generated by culturing monocytes selected by the flask adhesion method and examining their viability. The gated cells (iDCs) showed positive CD1a, HLA-DR, CD11c, and CD83 expression. The monocyte culture media was enriched with 50 ng/ml GM-CSF and 20 ng/ml IL-4.

However, Colić et al. [25] provided evidence that GM-CSF (100 ng/ml) with IL-4 (5 ng/ml) was effective in iDC generation from monocytes at the same concentration of GM-CSF and ten times higher concentration of IL-4 (50 ng/ml). iDCs were characterized by HLA-DR, CD80, CD86, and CD1a positive expression and down-regulation of CD14 and an absence of CD83. At lower concentrations of IL-4, a high number of cells were adherent, and DC generated at low concentrations of IL-4 (5 ng/ml) showed more robust anti-tumor capacity against the Jurkat cell line than DC generated at higher IL-4 concentrations. Adherent cells cultured with only

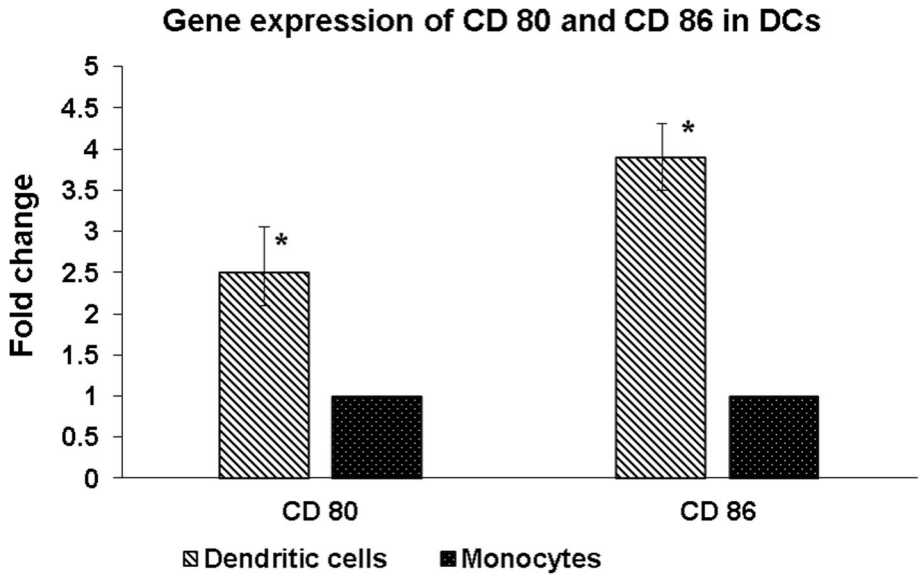

Fig. 4 CD80 and CD86 gene expression in dendritic cells showing significant upregulation for both markers in comparison with monocytes at day 0 (*P*-value < 0.05). Each value is the mean of 3 experiments ± SD

GM-CSF were primarily macrophages, as confirmed by CD14-positive expression.

El Ashmawy et al. [26] reported that cultured cells were supplemented with GM-CSF (20 ng/mL) and IL-4 (20 ng/mL). Cells were characterized by morphological change and appeared to be semi-adherent with branched projections, and they showed positive expression of CD83 and CD86 on the cell surface.

Other researchers used variable growth factor concentrations, and their trials were successful regarding DC generation. The concentrations were as follows: GM-CSF 50 ng/ml and IL-4 50 ng/ml [27], GM-CSF 100 ng/ml and IL-4 20 ng/ml [28], GM-CSF 100 ng/ml and IL-4 20 ng/ml [29], and GM-CSF 10 ng/ml and IL-4 20 ng/ml [30].

In our study, we cultured the cells in a 6-well culture plate. Each well contained 4 ml of culture media (RPMI-1640 containing 10% FCS, 2% penicillin-streptomycin with GM-CSF (50 ng/mL) and IL-4 (10 ng/mL)). Tkachenko et al. [31] used different media for the generation of iDCs. They used RPMI 1640 with 2% human serum albumin, RPMI 1640 with 2% TCH serum replacement, Panserin 501, and X-VIVO 15. Flow cytometry showed that in all previous media, the iDCs were CD45+ CD83+ and lost CD14.

Our results were also in line with Osugi et al. [32], who reported that MoDCs derived in in vitro culture did not express the CD1a surface antigen but expressed high levels of the HLA-DR CD86, CD83, and CD40 surface antigens. Kolli et al. [29] also characterized the phenotype of DCs by flow cytometry for other surface markers, including MHC class II, CD11b, CD11c, CD86, and CD80.

DCs have mannose receptors on their cell membrane that facilitates dextran uptake and phagocytosis [33]. In a functional study of DCs using FITC-dextran, we demonstrated that DCs showed a high capacity for FITC-dextran uptake, which is in line with Encabo et al. [6], who confirmed that fresh monocytes and immature MODCs showed an increased ability for accumulating FITC-dextran. After exposure to TNF-α for 2 days, mature Mo-derived DCs reduced their ability to internalize FITC-dextran.

Conclusion

In conclusion, we revealed that the flask adherence method is a successful procedure for mediating monocytes differentiation into immature MoDCs, which are efficient for experimental studies after 6 incubation days. Further studies are recommended for the study of monocyte differentiation kinetics into DCs. Also, further maturation of iDCs is recommended for the use of mature DCs in DC tumor vaccines.

Abbreviations

APCs: Antigen-presenting cells; DCs: Dendritic cells; MHC: Major histocompatibility complexes; iDCs: Immature dendritic cells; PBS: Phosphate-buffered saline; FCS: Fetal calf serum; RPMI: Roswell Park Memorial Institute; GM-CSF: Granulocyte monocyte colony-stimulating factor; IL: Interleukin; PBMCs: Peripheral blood mononuclear cells; MoDCs: Monocyte-derived DCs; FITC: Fluorescein isothiocyanate; MFI: Mean fluorescent intensity; RT-qPCR: Quantitative real-time PCR; CTLs: Cytotoxic T lymphocytes; TCR: T cell receptor; LPS: Lipopolysaccharide; TNF-α: Tumor necrosis factor-alpha

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Authors' contributions

YMH and NR prepared the idea and designed the study. ANA did the data statistical analysis. DMH and NR performed all the laboratory investigations. All authors wrote, read, and approved the final manuscript.

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Availability of data and materials

Not applicable

Declarations

Ethics approval and consent to participate

This study has been approved by the Faculty of Medicine, Zagazig University, Institutional Review Board (IRB) (reference number is 4464/13-3-2018). Written informed consents were obtained from all participants.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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