EJMO

DOI: 10.14744/ejmo.2024.64067
EJMO 2024;8(1):40–48

Research Article

Immunomodulatory Effects of MDA-MB-231-derived Exosome Mimetic Nanovesicles on CD4+ T Cell Line

Mustafa Oztatlici,[1] Alper Tunga Ozdemir,[2] Hulya Oztatlici,[3] Serife Kucukhuyuk,[1] Rabia Bilge Ozgul Ozdemir,[4] Hilmi Orhan,[5] Kemal Ozbilgin[6]

[1]Department of Histology and Embryology, Faculty of Medicine, Gaziantep Islam Science and Technology University, Gaziantep, Türkiye
[2]Department of Medical Biochemistry, Merkez Efendi State Hospital, Manisa, Türkiye
[3]Department of Histology and Embryology, Faculty of Medicine, Gaziantep University, Gaziantep, Türkiye
[4]Department of Immunology and Allergy, Manisa City Hospital, Manisa, Türkiye
[5]Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Ege University, Izmir, Türkiye
[6]Department of Histology and Embryology, Faculty of Medicine, Manisa Celal Bayar University, Manisa, Türkiye

Abstract

Objectives: The aim of this study is to investigate the immunomodulatory effects of MDA-MB-231 cells or MDA-MB-231-derived exosome-mimetic nanovesicles (NVs) on CD4+ Jurkat cells.

Methods: NVs were produced by the breakdown of MDA-MB-231 cells and the characterization of generated NVs were performed by using direct-ELISA and Flow cytometry methods. We co-cultured CD4+ Jurkat cells with MDA-MB-231 cells or MDA-MB-231-derived NVs for 48 h. Subsequently, expressions of pro-inflammatory and anti-inflammatory cytokines, and related transcription factors of CD4+ Jurkat cells were evaluated by qPCR method.

Results: Clustering, which is the indicator of activation, was not seen in the CD4+ Jurkat cells co-cultured with MDA-MB-231 cells. However, CD4+ Jurkat cell clusters were observed in the co-culture experiments with all NV concentrations. In addition, it was determined that the expressions of pro-inflammatory cytokines significantly increased while the expressions of anti-inflammatory cytokines was dramatically decreased in the NV-treated groups. On the contrary, opposite results were obtained in CD4+ Jurkat cells co-cultured with MDA-MB-231 cells. Moreover, TNF-α and Gata3 expressions were decreased in all groups.

Conclusion: These preliminary findings from in-vitro experiments suggested that NVs could be a potential tool in cancer immunotherapy, but our data need to be supported by more comprehensive studies.

Keywords: Breast cancer, extracellular vesicles, exosome-mimetics, exosomes, immunomodulation

Cite This Article: *Oztatlici M, Ozdemir AT, Oztatlici H, Kucukhuyuk S, Ozgul Ozdemir RB, Orhan H, et al. Immunomodulatory Effects of MDA-MB-231-derived Exosome Mimetic Nanovesicles on CD4+ T Cell Line. EJMO 2024;8(1):40–48.*

Breast cancer is one of the most common types of cancer in women, and one out of every eight women suffers from this disease. Today, the life expectancy of patients can be extended with the use of biological agents, but tumors that do not express estrogen receptors, progesterone receptors, and HER2, classified as "triple-negative", are resistant to current treatments and have a fatal course.[1, 2] Promising results are obtained with cancer immunotherapy, a new and effective approach to treating these resistant patients.[3] However, there are several challenges, tumor cells interact intensely with the surrounding tissue, reducing the success rate of both conventional

Address for correspondence: Mustafa Oztatlici, MD. Department of Histology and Embryology, Faculty of Medicine, Gaziantep Islam Science and Technology University, Gaziantep, Türkiye
Phone: +90 554 927 68 27 **E-mail:** mustafaoztatlici@gmail.com / mustafa.oztatlici@gibtu.edu.tr

Submitted Date: August 17, 2023 **Revision Date:** February 03, 2024 **Accepted Date:** February 11, 2024 **Available Online Date:** March 06, 2024

treatments and immunotherapy approaches. In tumor-immune interface, there are different cells defined like as, tumor-associated macrophage (TAM), cancer-associated fibroblast (CAF), mesenchymal stem cells (MSC), and tumor-infiltrating lymphocytes (TIL) are included.[4] These cells play an important role, especially in the escape of tumor cells from the immune system. Strong suppressors such as IL-10, TGF-b, IDO, and PGE2 expressed by tumor microenvironment components cause the inactivation of anti-tumor cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.[5-7] Tumor microenvironment components not only contribute to the escape of tumor cells from the immune system but also negatively affect the success of immunotherapy applications.[8] Intensive studies are also carried out on the roles of exosomes secreted from tumor cells in the development of the tumor microenvironment or apoptotic cell remnants formed due to the death of cells during rapid proliferation.[9]

It has been shown that exosomes play important roles in the transfer of intracellular molecular components to other cells.[10] The effects of molecular components transferred via exosomes on immune cells is one of the popular research topics of nowadays.[11] These structures, are vesicles of 30-100 nm in size and released from the cells, enable the cytoplasmic content of the cell from which they originate to be transferred paracrine or endocrine. However, the exosome production capacity of each cell differs, and the amounts of exosomes released from some beneficial cells may be insufficient to achieve a clinical effect. At this point, exosome-mimetic nanovesicles (NV), a structure similar to exosomes and obtained by mechanical disintegration of cells, come into play. Serial extrusion of cells of different origins through filters with reduced pore sizes produces high amounts of exosome-mimetic NV. With the serial extrusion method, much larger amounts of NV can be produced from the same number of cells compared to secreted exosomes. NVs have the same size, morphology, and protein markers as exosomes.[12, 13]

CD4 T lymphocytes (T helper cells), which play the most important role in the formation of adaptive immune response in both the eradication of pathogens and tumor immunity, differentiate into subgroups that manage different specific immune responses with cytokine stimulus and antigen presentation from their environment. Th1 cells play a role in the eradication of intracellular pathogens and provide pro-inflammatory activation of the immune system with its basic cytokine interferon gamma (IFN-γ). The lineage-specific transcription factor (TF) of these cells is known as T-bet and plays a critical role in the differentiation of naive T lymphocytes into Th1 cells after antigen presentation and cytokine stimulation.[14] Another important lymphocyte subgroup is Th2 cells. These cells manage the immune response to the eradication of extracellular pathogens and parasites. The main cytokines of Th2 cells are IL-4, IL-5, and IL-13 moreover Gata3 TF is activated by the differentiation of naive T cells into Th2 cells.[15]

Another subset of T lymphocytes responsible for directing the extracellular pathogens and neutrophil response is Th17 cells. The specific cytokines of these cells are IL-17a, IL-21, and IL-22. RoRC2 TF plays a critical role in the activation of these cells after antigen presentation.[16] Regulatory T cells (Treg) play a critical role in the termination of the existing immune response and the formation of tolerance to self-antigens after the eradication of pathogens. Almost all immune cells are suppressed by the specific cytokines of Treg cells, IL-10 and TGF-β1. Lineage-specific TFs of Treg cells were defined as FoxP3.[17] Treg cells also play a role in inhibiting the immune elimination of cancer cells. In this regard, many studies show that Treg cells' presence in tumor tissue is associated with a poor prognosis.[18]

In this study, we aimed to investigate the immunomodulatory effects of the MDA-MB-231 cells, an invasive breast tumor cell line, and MDA-MB-231-derived NVs on the Jurkat cells which is a CD4+ T cell line. In this way, we had the opportunity to comparatively evaluate the effects of cellular composition transferred by MDA-MB-231-derived NVs, and the effects of MDA-MB-231 cells on pro-inflammatory and anti-inflammatory cytokines, and related transcription factors in the Jurkat cells, where they establish direct cell-cell contact.

Methods

Cell Culture

MDA-MB-231 (Metastatic breast cancer, ATCC® HTB-26™) cells were cultured in DMEM-F12 medium while CD4+ Jurkat (T lymphocyte, ATCC® TIB-152 ™) cells were cultured in RPMI 1640 medium. 10% heat-inactivated fetal bovine serum (FBS) was added to the media. All media were supplemented with 1% antibiotic-antimycotic and also 1% L-glutamine was added to RPMI 1640 medium. The morphology and proliferation of the cells were examined daily under an inverted microscope and the medium was changed every 3 days. In addition, cells were regularly monitored for mycoplasma contamination. All cells were cultured in a humidified incubator at 37°C and 5% CO_2.[19]

Preparation of Exosome-Mimetic Nanovesicles (NV)

MDA-MB-231 cells were cultured in 75 cm^2 flasks and when the confluency reached 80-90%, the medium in the flask was discarded and the cells were washed two times with sterile and filtered phosphate-buffered saline

(PBS). Adherent MDA-MB-231 cells were detached by scraping and the cells were resuspended at a concentration of 3x106 cells/mL in PBS. Using a small-spray syringe system (mini-extruder, Avanti Polar Lipids) sterilized under UV, the cell suspensions were passed 5 times through 10 µm polycarbonate membrane filters (Whatman), and the cells were separated into small pieces. After the filtration process, the filter in the small-spray syringe system was replaced with 5 and 1 µm polycarbonate membrane filters, respectively, and the filtration process was repeated (Fig. 1).

Iodixanol (Axis-Shield PoC AS) density gradient ultracentrifugation (UCF) was performed to separate and purify exosome-sized NVs from cellular particles and other vesicles. To form a step gradient, 50% iodixanol was placed at the bottom of an ultracentrifuge tube, overlaid with 10% iodixanol and the extruded sample, respectively, and sample loaded ultracentrifuge tubes were placed in the SW40 Ti ultracentrifuge rotor. Ultracentrifugation was performed at 100.000g for 2 hours 10 minute at +4ºC. NVs were collected with a micropipette from the interface of 50%-10% Iodixanol layers and obtained NVs were aliquoted and stored at -80ºC until needed.[12]

Protein Amounts of NVs

The total protein contents of NVs obtained from MDA-MB-231 cells were determined with the Qubit 4.0 device using the Qubit Protein Assay (Q33211, Thermo Scientific) kit and the amount of NVs in µL was determined.

Characterization of NVs with Flow Cytometry

Exo-FACS kit (HBM-FACS-C, HansaBioMed, Estonia) was used to determine the expression of CD9 and CD63 proteins, which are exosome and NV-specific surface markers. Latex- beads in the kit were incubated overnight at +4°C with NVs. After washing step, NV-loaded latex beads were incubated with CD9 FITC (1F-208-T100, Exbio, Czech Republic) and CD63 Alexa 488 (HBM-CD63-20, HansaBioMed, Estonia) antibodies for 2 hours at +4°C in the dark for labelling. At the end of the incubation, 4 mL of washing solution was added to the tubes to remove non-adherent antibodies, and each tube was centrifuged for 5 minutes at 4500 g. The supernatant was removed and 500 µL of washing solution was added to the pellet and the tubes were gently mixed. NVs were analyzed with the Accurie C5 Flow cytometry device (BD Bioscience, USA). Histogram analyzes were performed with FlowJo v10 software (BD Company, USA).[20]

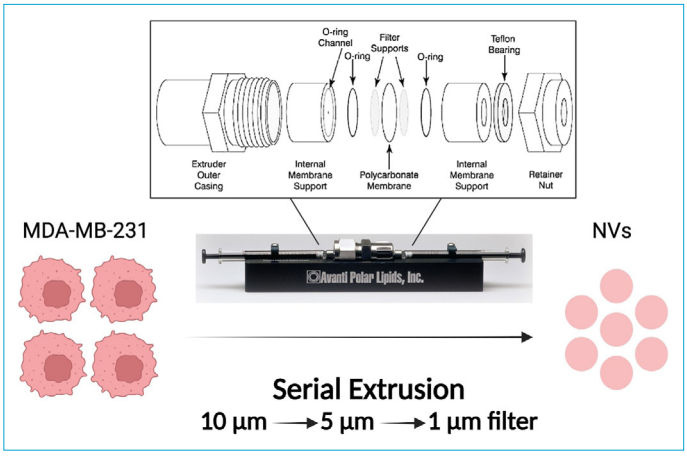

Figure 1. Schematic illustration of the procedure for the generation of exosome-mimetic nanovesicles (NVs) from MDA-MB-231 cells.

Characterization of NVs with ELISA

Direct ELISA method was used to detect the expression of CD9 and CD63 marker proteins. Briefly, 500 ng of NVs in 100 µl of PBS were added to a 96-well plate and incubated overnight at 4°C. At the end of the incubation, plates were blocked with 1% BSA/PBS for 1 h. After that, the anti-CD9 (sc-13118) and anti-CD63 (sc-5275) antibodies were added and incubated for 2 h (all antibodies were in a 1:300 dilution and purchased from Santa Cruz Biotechnology). After washing with PBS, HRP-conjugated secondary antibody (1:2000 dilution, GE Healthcare) was added and samples were incubated for 1 h. After washing with PBS, the luminescent signal was measured with the BM Chemiluminescence ELISA Substrate (BD Biosciences, San Jose, CA) by using Varioskan Flash (Thermo) device.[21]

Co-culture

Co-culture experiments were performed in 6-well culture plates to observe the immunomodulatory interactions between CD4+ Jurkat cells and MDA-MB-231 cells or MDA-MB-231-derived NVs. For this purpose, 1x106 CD4+ Jurkat cells were cultured in the 6 well culture dish for 48 hours within the media containing 5 µg/mL or 50 µg/mL NVs, respectively. 1x105 MDA-MB-231 cells were seeded into the another 6 well culture dish and cultured for 24 hours to adhere. Then, 5x105 or 1x106 CD4+ Jurkat cells were added into wells and co-cultured for 48 hours (Fig. 2). At the end of the culture, the suspended CD4+ Jurkat cells were collected with a micropipette and transferred to 1.5 mL eppendorf tubes. Eppendorf tubes were centrifuged at 1000 rpm for 10 minutes at +4ºC. The supernatant was discarded, and 1 mL of sterile PBS was added to the pellet and mixed well with a micropipette. Again, it was centrifuged at 1000 rpm, for 10 minutes at +4ºC. This process was repeated 2 times. The supernatant was completely removed with a micropipette and the pellet was stored at -80ºC for quantitative polymerase chain reaction (qPCR).

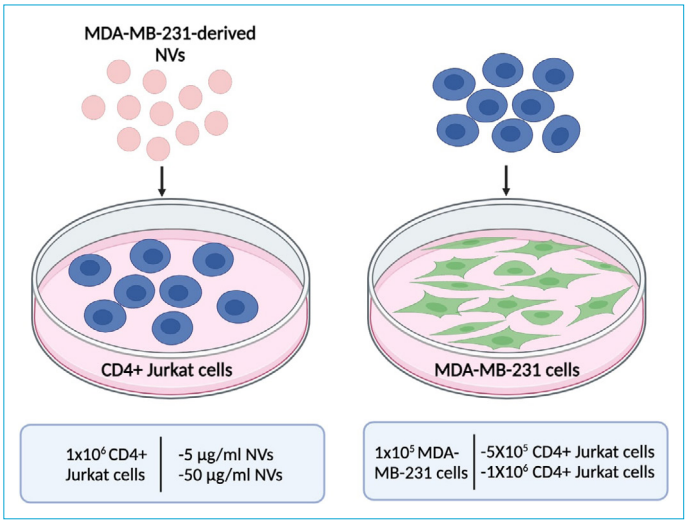

Figure 2. Schematic summary of co-culture experiments (NVs: Exosome-mimetic nanovesicles from MDA-MB-231 cells).

Quantitative PCR (qPCR)

To observe the immunomodulatory effects of MDA-MB-231 cells or MDA-MB-231-derived NVs on CD4+ Jurkat cells, *IFN-γ, TNF-α, IL-4, IL-17a, Tbet, Gata3, RoRC2, IL-10, TGF-β1, FoxP3* and *GAPDH* gene expressions of CD4+ Jurkat cells were evaluated. For this, the total RNA was isolated from the collected CD4+ Jurkat cells using PureLink RNA Mini Kit (Cat# 12183018A, ThermoFisher, USA) according to the kit protocol. Subsequently, cDNAs were synthesized from the isolated RNAs by using the high-capacity cDNA Reverse Transcription Kit (Cat# 4368814, Invitrogen, USA). Forward and reverse primer sets of target genes were purchased from Sentegen Biotech, Ankara, Turkey. Changes in mRNA expressions were detected with StepOnePlus™ Real-Time PCR System (Cat# 4376600 Applied Biosystems, USA) by using SYBR® Green PCR Master Mix (Cat# 4344463, ThermoFisher, USA). GAPDH was used as reference gene, and the relative gene expression differences were calculated by ΔΔCT (cycle threshold) method. The primers of target genes are shown in Table 1.[22]

Statistical Analysis

All experiments were performed in triplicate and repeated three times. The data obtained from the experimental groups were statistically compared by PRISM v7 (GraphPad, California, USA) software. All the data were presented as mean±standard deviation (SD). First, it was determined whether the data were in the normal distribution or not by Column analysis. Normally distributed data were analyzed using the One-Way ANOVA test. $p<0.05$ values were considered statistically significant.

Table 1. Sequences of primer pairs for qPCR.

Target genes	Primer pairs	Base counts
IFN-γ	F 5′-ACTGACTTGAATGTCCAACGCA-3′	
	R 5′-ATCTGACTCCTTTTTCGCTTCC-3′	44
TNF-α	F 5′-ATGAGCACTGAAAGCATGATCC-3′	
	R 5′-GAGGGCTGATTAGAGAGAGGTC-3′	44
IL-4	F 5′-CCAACTGCTTCCCCCTCTG-3′	
	R 5′-TCTGTTACGGTCAACTCGGTG-3′	40
IL-17a	F 5′-CAATCCCACGAAATCCAGGATG-3′	
	R 5′-GGTGGAGATTCCAAGGTGAGG-3′	43
Tbet	F 5′-CCAGTTCATTGCCGTGAC-3′	
	R 5′-AGGATACTGGTTGGGTAGGA-3′	38
Gata3	F 5′-TCAGACCACCACAACCACAC-3′	
	R 5′-CACTTTTTGGATTTGCTAGA-3′	40
RoRC2	F 5′-GTGGGGACAAGTCGTCTGG-3′	
	R 5′-AGTGCTGGCATCGGTTTCG-3′	38
IL-10	F 5′-TCAAGGCGCATGTGAACTCC-3′	
	R 5′-GATGTCAAACTCACTCATGGCT-3′	42
TGF-β1	F 5′-GGTTGAGCCGTGGAGGGGAAAT-3′	
	R 5′-TGCCATGAATGGTGGCCAGGT-3′	43
FoxP3	F 5′-GAAACAGCACATTCCCAGAGTTC-3′	
	R 5′-ATGGCCCAGCGGATGAG-3′	40
GAPDH	F 5′-GAGTCAACGGATTTGGTCGT-3′	
	R 5′-GACAAGCTTCCCGTTCTCAG-3′	40

F: Forward; R: Reverse.

Results

MDA-MB-231 and CD4+ Jurkat Cell Culture

MDA-MB-231 breast cancer cells were grown in 75 cm^2 culture flasks in DMEM-F12 medium. In the microscopic evaluation, MDA-MB-231 cells were observed as adherent and had spindle morphology. Cultivation of MDA-MB-231 cells in co-culture experiments was done with RPMI 1640 medium. Microscopically, it was determined that using the RPMI 1640 medium did not affect the cell morphology and confluency time of MDA-MB-231 cells. CD4+ Jurkat cells were observed as suspension and had spheroid morphology. The light microscopy images of the cells are shown in Figure 3.

Production and Quantification of NVs

To obtain cell-derived NVs, MDA-MB-231 cells were harvested and serially extruded through a series of polycarbonate membranes with pore sizes of 10, 5, and finally 1 µm. Iodixanol density gradient ultracentrifugation was performed to separate and purify exosome-sized NVs (Fig. 4). Protein amounts of isolated NVs were determined using the Qubit protein assay kit. Total protein concentration of NVs was found as 2064 µg/mL.

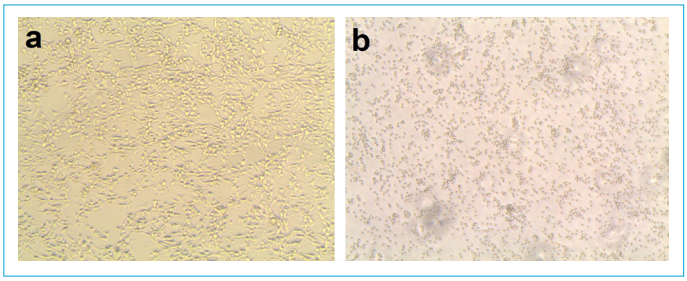

Figure 3. Microscopic images of MDA-MB-231 **(a)** and CD4+ Jurkat cells **(b)**. Magnification 100X.

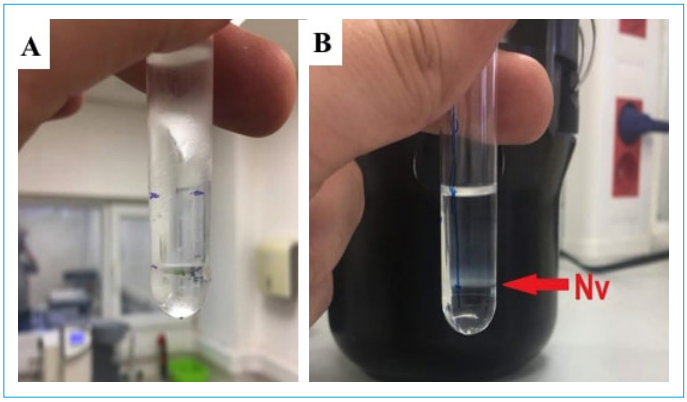

Figure 4. Serially extruded cell lysate in ultracentrifuge tube **(a)** and purified exosome-sized NVs **(b)**.

Characterization of Exosome-Mimetic NVs

CD9 and CD63, exosome and NV-specific marker proteins, were found positive in the flow cytometry analysis of the isolated and purified NVs (Fig. 5). Additionally, the direct ELISA assay also confirmed that NVs were positive for the CD9 and CD63 marker proteins.

Co-culture Experiments

Clustering, which is the indicator of activation, was not seen in the CD4+ Jurkat cells co-cultured with MDA-MB-231 cells at 1:5 and 1:10 ratios. Additionally, there were no significant morphological differences in the CD4+ Jurkat cells. However, CD4+ Jurkat cell clusters were observed in the co-culture experiments with all NV concentrations. Clustering was more prominent in the CD4+ Jurkat cells applied to 50 µg/mL NVs. Light microscopy images of the co-culture experiments are shown in the Figure 6.

qPCR Results

The changes in gene expressions of inflammatory cytokines and transcription factors in Jurkat cells co-cultured with MDA-MB-231 cells or MDA-MB-231-derived NVs were analyzed by qPCR method. We observed that the expression of pro-inflammatory cytokines (IFN-γ, IL-4 and IL-17a) increased significantly with 5 µg/mL NV application ($p<0,0001$, $p<0,0001$, and $p<0,0001$, respectively), but decreased significantly

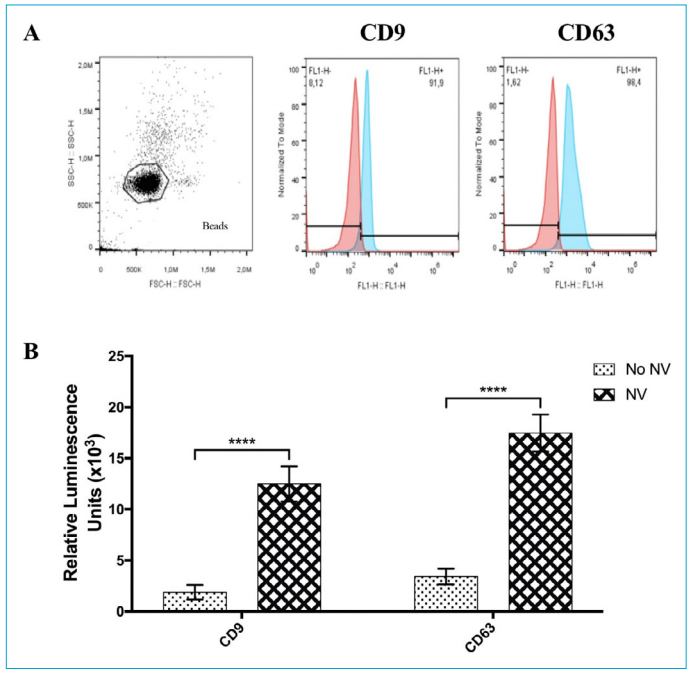

Figure 5. Flow cytometry histogram graphics **(a)** and direct ELISA results **(b)** showing the expression of CD9 and CD63 on the NVs. The results are presented as the mean ± SD. ****p-Value < 0.0001.

in CD4+ Jurkat cells, which interacted directly with MDA-MB-231 cells at a ratio of 1/5, except for IL-17a ($p<0,0001$, $p<0,0001$, and $p=0,1088$, respectively). Expressions of IFN-γ, IL-4 and IL-17a increased significantly with 50 µg/mL NV administration ($p<0.0001$, $p<0.0001$ and $p<0.0001$, respectively), whereas it was significantly decreased in 1/10 co-cultures ($p<0.0001$, $p<0.0001$ and $p<0.0001$, respectively). On the other hand, TNF-α expressions decreased significantly with

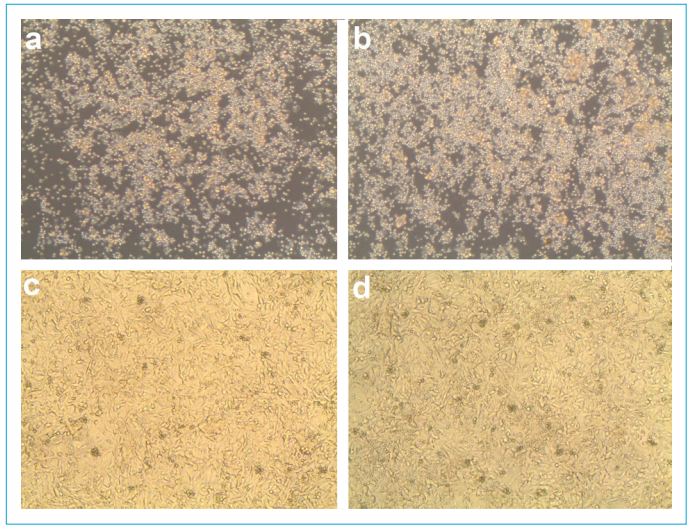

Figure 6. Representative microscopy images of co-cultured experiments: 1x106 CD4+ Jurkat cells co-cultured with 5 µg/mL **(a)** and 50 µg/mL NVs **(b)**, MDA-MB-231 cells and CD4+ Jurkat cells co-culturred at 1:5 **(c)** ve 1:10 cell ratios **(d)**. Magnification 100X.

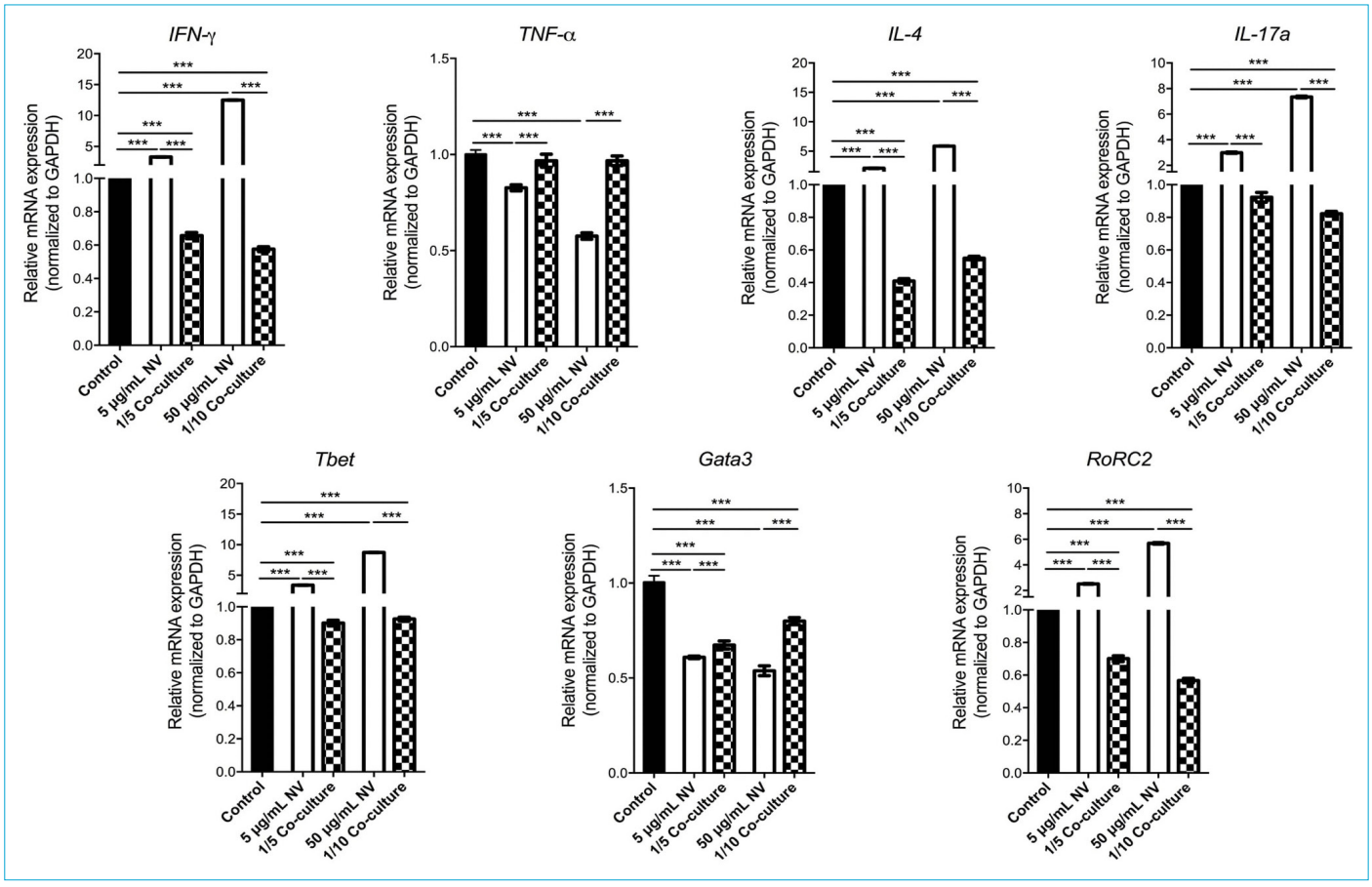

Figure 7. The comparison graphs of fold changes in gene expression of pro-inflammatory molecules. The results are presented as the mean ± SD. ****p-Value < 0.0001.

5 and 50 µg/mL NV treatments (p<0.0001 and p<0.0001, respectively) but did not change in 1:5 and 1:10 co-cultures (p=0,2299 and p=0.2183, respectively). Transcription factors associated with pro-inflammatory cytokines were evaluated separately. Expressions of Tbet (p<0.0001 and p<0.0001, respectively) and RoRC2 (p<0.0001 and p<0.0001 respectively) increased with 5 and 50 µg/mL NV treatments, but it was significantly decreased in the 1/5 and 1/10 co-cultures (p<0.0001 and p=0.0003, respectively for Tbet, p<0.0001 and p<0.0001 for RoRC2, respectively). Gata3 expressions were found both with 5 and 50 µg/mL NV treatments (p<0.0001 and p<0.0001, respectively) and in 1:5 and 1:10 co-cultures (p<0.0001 and p<0,0001 respectively) decreased significantly. Comparison graphs of the changes in gene expressions of pro-inflammatory cytokines are presented in Figure 7 and the summary table of the obtained results in Table 2.

The anti-inflammatory cytokines IL-10 and TGF-β1 and the transcription factor FoxP3, which is associated with these cytokines, were also investigated. IL-10 and TGF-β1 expressions were significantly decreased by 5 µg/mL (p<0.0001 and p<0.0001 respectively) and 50 µg/mL (p<0.0001 and p<0.0001 respectively) NV treatments. However, in 1:5 and 1:10 direct co-culture experiments, CD4+ Jurkat cells' IL-10 (p<0.0001 and p<0.0001, respectively) and TGF-β1 (p<0.0001 and p<0.0001, respectively) expressions increased significantly. FoxP3 expressions were decreased significantly both with 5 and 50 µg/mL NV treatments (p<0.0001 and p<0.0001, respectively) and in 1:5 and 1:10 co-cultures (p<0.0001 and p<0.0001 respectively). Comparison graphs of the changes in gene expressions of anti-inflammatory cytokines are presented in Figure 8 and the summary table of the obtained results in Table 2.

Discussion

T helper (Th) cells are divided into two main classes CD4 and CD8 also CD4 T cells are divided into various subgroups such as Th1, Th2, Th9, Th17, Th22, and Treg to perform different functions.[23] Although the main task of Th1 cells is the eradication of intracellular pathogens, they also have important roles in cancer and organ-specific autoimmune diseases. IFN-γ is intensely secreted from Th1 cells, and T-bet stands out as the responsible transcription factor in the regulation of these cytokines.[24] We observed significant differences between the direct interaction of

Table 2. Fold changes in gene expressions of pro-inflammatory and anti-inflammatory cytokines, and related transcription factors

	5 µg/mL NV	50 µg/mL NV	1/5 Co-culture	1/10 Co-culture
Pro-inflammatory				
IFN-γ	3.29±0.03	12.52±0.025	0.657±0.018	0.575±0.014
TNF-α	0.826±0.015	0.574±0.016	0.966±0.033	0.966±0.024
IL-4	2.09±0.034	5.67±0.009	0.409±0.013	0.550±0.011
IL-17a	2.99±0.052	7.34±0.067	0.923±0.029	0.822±0.014
Tbet	3.35±0.026	8.69±0.023	0.900±0.016	0.925±0.010
Gata3	0.609±0.006	0.583±0.025	0.673±0.021	0.799±0.018
RoRC2	2.51±0.026	5.67±0.055	0.700±0.017	0.566±0.013
Anti-inflammatory				
IL-10	0.476±0.009	0.440±0.013	2.34±0.031	3.967±0.088
TGF-β1	0.793±0.019	0.621±0.017	1.71±0.054	2.04±0.062
FoxP3	0.356±0.004	0.228±0.004	0.861±0.029	0.853±0.034

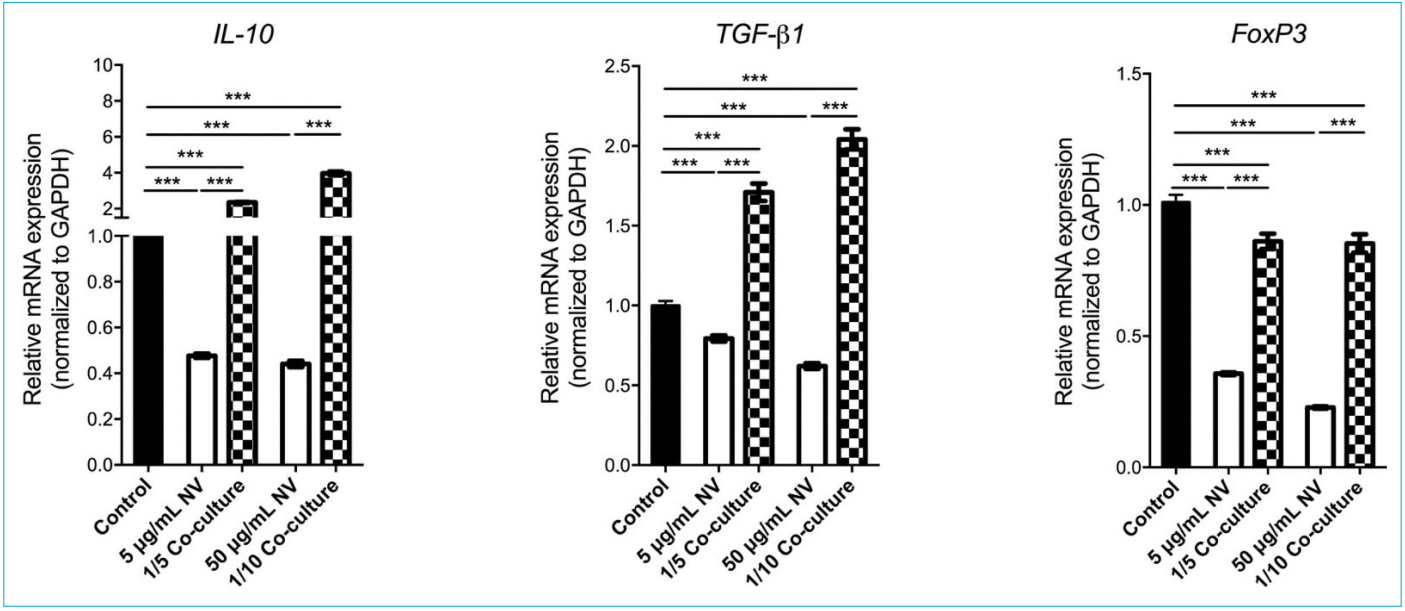

Figure 8. The comparison graphs of fold changes in gene expression of anti-inflammatory molecules. The results are presented as the mean ± SD. ****p-Value < 0.0001.

CD4+ Jurkat cells with MDA-MB-231 tumor cells or their interaction with MDA-MB-231-derived NVs. IFN-γ and T-bet expressions of CD4+ Jurkat cells cultured with NV were significantly increased and these increases were dose-dependent. In contrast, there was a significant reduction in co-culture experiments where cell-cell interaction occurred. NVs are the packaged form of the cytoplasmic contents of MDA-MB-231 cells. In this state, they contain many tumor-associated mutated DNA, RNA and proteins, as well as specific molecules expressed in the tumor cells. Under normal conditions, antigen presentation via MHC class II molecules is required for the activation of CD4 T cells, and MHC class II molecules are expressed mainly by professional antigen-presenting cells.[25] Unlike the direct co-culture where cell integrity is preserved, NVs that may integrate with the CD4+ Jurkat cell membrane transfer all the contents of the MDA-MB-231 cells to the CD4+ Jurkat cell cytoplasm. In this way, Toll-Like Receptors (TLR3, TLR7, TLR8, and TLR9) may activated by specific DNA and RNA sequences in the cell can be activated by tumor cell-derived genomic materials.[26] Even IFN-γ and T-bet expressions in CD4+ Jurkat decreased in the direct co-culture experiments, their increase with NV treatments supports this hypothesis.

Th2 cells are responsible for the eradication of parasitic infections, but these cells are also involved in pathologies such as allergy and asthma.[23] Recent studies reveal that Th2 cells may also have important contributions to the anti-tumor immune response.[27] IL-4, IL-5, and IL-13 are the main cytokines secreted from Th2 cells, and Gata3 is the transcription factor

associated with these cytokines.[28] In our analysis, we found that IL-4 expression in the CD4+ Jurkat cells increased significantly with NV applications but decreased significantly in direct co-culture experiments. However, Gata3 expressions were significantly reduced in both approaches. It has been reported that MDA-MB-231 cells intensely express IL-4 and can suppress the formation of anti-tumor effects in this way.[29] Th1 and Th2 responses are mutually suppressive processes, that is, when the Th1 response is triggered, a decrease in the Th2 response occurs.[30] In this respect, while the decrease in Gata3 accompanying the increase in T-bet expressions is expected, the source of the increase we detected in IL-4 with NV applications most probably comes from the MDA-MB-231-derived NVs because MDA-MB-231 cells also normally express a higher amount of IL-4.[29]

Th17 cells are responsible for the elimination of extracellular bacteria and yeasts. However, it is closely related to the pathogenesis of many autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis. The main cytokines of Th17 cells are IL-17a, IL-17b, IL-21, and IL-22 molecules, and the basic transcription factors have been defined as RoRC2 and STAT3.[16] Since Th17 cells are cells that show extreme plasticity and can transform into pro-inflammatory Th1 and anti-inflammatory Treg cells, different results have been reported regarding their association with cancer.[31] In our study, we found that both IL-17a and RoRC2 expressions increased significantly with NV applications, whereas they decreased significantly in direct co-culture experiments. As mentioned in Th1 cells, genomic/cytoplasmic tumor material transferred by NVs may have caused pro-inflammatory activation of CD4+ Jurkat cells by activating different defense receptors, especially TLRs. Our findings support this approach.

Treg cells are responsible for maintaining the self-tolerance mechanism with the potent inhibitory cytokines IL-10 and TGF-β1 they secrete. FoxP3 is a characteristic transcription factor for these cells. Loss of function in Treg cells causes allergies, asthma, and autoimmune diseases,[32] while their unwarranted activation helps tumor cells to escape immune elimination.[33] In our study, we observed that anti-inflammatory IL-10, TGF-β1, and FoxP3 expressions were significantly decreased in contrast to pro-inflammatory cytokines, which we found to increase dramatically with NV applications. This situation was vice versa in direct co-culture settings; direct interaction decreased pro-inflammatory cytokines while significantly increased anti-inflammatory cytokines. Many studies are showing that tumor cells affect both CTLA-4 in activating Treg formation and PD-L1 in increasing Treg activation and suppressing activated pro-inflammatory Th1, Th2, and Th17 cells. It has been reported that a significant immune suppression occurs in proportion to PD-L1 expression in MDA-MB-231 cells.[34-37] In this respect, cell-cell interaction may have led to the suppression of pro-inflammatory molecules and an increase of anti-inflammatory molecules through the activation of these potent inhibitors (CTLA-4 and PD-L1) on the surface of the membranes. Unlike direct cell interaction, this may not have occurred in NVs, and our findings support this approach.

In conclusion, our study revealed that dramatic contrasts between the tumor cell itself and the effects of NVs isolated from them on CD4+ Jurkat cells. It was determined that MDA-MB-231 cell content (genomic/cytoplasmic material) transferred to CD4+ Jurkat cells by NVs triggered an anti-tumor effective pro-inflammatory response through different mechanisms while suppressing the anti-inflammatory response leading to tumor immune evasion. On the contrary, direct cell interaction produces the opposite effect. Our study provided preliminary data that NVs could be a potential tool in cancer immunotherapy, but our data need to be supported by more comprehensive studies. Further techniques and analyzes are needed to understand the exact reason for these results.

Disclosures

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Financial Disclosure: The Scientific Research Projects Commission of Manisa Celal Bayar University provided financial support for the project, reference number 2019/035.

Authorship Contributions: Concept – M.O., A.T.O.; Design – M.O., A.T.O., H.O.; Supervision – M.O., K.O., H.O.; Funding – K.O., M.O.; Materials – M.O., A.T.O., H.O.; Data collection &/or processing – M.O., A.T.O., H.O.; Analysis and/or interpretation – M.O., A.T.O., H.O., R.B.O.O.; Literature search – M.O., A.T.O., H.O., S.K.; Writing – M.O., A.T.O., H.O., S.K., R.B.O.O., K.O., H.O.; Critical review – M.O., A.T.O., H.O., R.B.O.O.

References

1. Corona SP, Sobhani N, Ianza A, Roviello G, Mustacchi G, Bortul M, et al. Advances in systemic therapy for metastatic breast cancer: Future perspectives. Med Oncol 2017;34:119.
2. Sönmez PK, Öztatlıcı M, Özbilgin K. Effects of verteporfin-mediated photodynamic therapy in breast cancer cells. Indian J Biochem Biophys 2020;57:560–6.
3. Masoud V, Pagès G. Targeted therapies in breast cancer: New challenges to fight against resistance. World J Clin Oncol 2017;8:120–34.
4. Dai X, Zhu K. Cold atmospheric plasma: Novel opportunities for tumor microenvironment targeting. Cancer Med 2023;12:7189–206.
5. Goswami KK, Ghosh T, Ghosh S, Sarkar M, Bose A, Baral R. Tu-

mor promoting role of anti-tumor macrophages in tumor microenvironment. Cell Immunol 2017;316:1–10.
6. Munn DH, Sharma MD, Johnson TS, Rodriguez P. IDO, PTEN-expressing Tregs and control of antigen-presentation in the murine tumor microenvironment. Cancer Immunol Immunother 2017;66:1049–58.
7. Özkara G, Öztürk O, Yılmaz AH. Cancer and metastasis: Importance of cell adhesion molecules and cell junctions. Experimed 2020;10:38–48.
8. Liedtke C, Kolberg HC. Systemic therapy of advanced/metastatic breast cancer - current evidence and future concepts. Breast Care Basel 2016;11:275–81.
9. Maia J, Caja S, Strano MMC, Couto N, Costa-Silva B. Exosome-based cell-cell communication in the tumor microenvironment. Front Cell Dev Biol 2018;6:18.
10. Batagov AO, Kurochkin IV. Exosomes secreted by human cells transport largely mRNA fragments that are enriched in the 3'-untranslated regions. Biol Direct 2013;8:12.
11. Greening DW, Gopal SK, Xu R, Simpson RJ, Chen W. Exosomes and their roles in immune regulation and cancer. Semin Cell Dev Biol 2015;40:72–81.
12. Jang SC, Kim OY, Yoon CM, Choi DS, Roh TY, Park J, et al. Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. ACS Nano 2013;7:7698–710.
13. Jo W, Jeong D, Kim J, Park J. Self-renewal of bone marrow stem cells by nanovesicles engineered from embryonic stem cells. Adv Healthc Mater 2016;5:3148–56.
14. Zhao X, Liu J, Ge S, Chen C, Li S, Wu X, et al. Saikosaponin A inhibits breast cancer by regulating Th1/Th2 balance. Front Pharmacol 2019;10:624.
15. Kanhere A, Hertweck A, Bhatia U, Gökmen MR, Perucha E, Jackson I, et al. T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. Nat Commun 2012;3:1268.
16. Unutmaz D. RORC2: The master of human Th17 cell programming. Eur J Immunol 2009;39:1452–5.
17. Rudensky AY. Regulatory T cells and Foxp3. Immunol Rev 2011;241:260–8.
18. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. Adv Cancer Res 2010;107:57–117.
19. Ozdemir AT, Kirmaz C, Ozdemir RBO, Degirmenci P, Oztatlici M, Degirmenci M. In-vitro evaluation of effects of mesenchymal stem cells on TLR3, TLR7/8 and TLR9-activated natural killer cells. Eurasian J Med Oncol 2021;5:71.
20. Ozdemir AT, Kirmaz C, Ozgul ORB, Oztatlici M, Kilicarslan SP, Tuglu MI. In-vitro evaluation of immunomodulation effects of mesenchymal stem cell-derived exosomes in refractory chronic spontaneous urticaria. Asthma Allergy Immunol 2023;21:45–54.
21. Crescitelli R, Lässer C, Jang SC, Cvjetkovic A, Malmhäll C, Karimi N, et al. Subpopulations of extracellular vesicles from human metastatic melanoma tissue identified by quantitative proteomics after optimized isolation. J Extracell Vesicles 2020;9:1722433.
22. Ozdemir AT, Oztatlici M, Ozgul OR, Cakir B, Ozbilgin K, Dariverenli E. The effects of preconditioning with IFN-γ, IL-4, and IL-10 on costimulatory ligand expressions of mesenchymal stem cells. Int J Med Biochem 2021;4:121–30.
23. Eagar TN, Miller SD. Helper T-Cell subsets and control of the inflammatory response. In Rich RR, Fleisher TA, Shearer WT, Schroeder HW, Frew AJ, Weyand CM, editors. Clinical Immunology 5th ed. London: Elsevier; 2019. p. 235–45.e1.
24. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P, et al. The adaptive immune system. In Molecular Biology of the Cell. 4th ed. New York: Garland Science; 2002.
25. Kotsias F, Cebrian I, Alloatti A. Antigen processing and presentation. Int Rev Cell Mol Biol. 2019;348:69–121.
26. Cen X, Liu S, Cheng K. The role of toll-like receptor in inflammation and tumor immunity. Front Pharmacol 2018;9:878.
27. Lorvik KB, Hammarström C, Fauskanger M, Haabeth OAW, Zangani M, Haraldsen G, et al. Adoptive transfer of tumor-specific Th2 cells eradicates tumors by triggering an in situ inflammatory immune response. Cancer Res 2016;76:6864–76.
28. Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: Induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res 2006;16:3–10.
29. Chen K, Satlof L, Stoffels G, Kothapalli U, Ziluck N, Lema M, et al. Cytokine secretion in breast cancer cells – MILLIPLEX assay data. Data Brief 2020;28:104798.
30. Li J, Zeng Z, Wu Q, Chen J, Liu X, Zhang J, et al. Immunological modulation of the Th1/Th2 shift by ionizing radiation in tumors (Review). Int J Oncol 2021;59:50.
31. Asadzadeh Z, Mohammadi H, Safarzadeh E, Hemmatzadeh M, Mahdian-Shakib A, Jadidi-Niaragh F, et al. The paradox of Th17 cell functions in tumor immunity. Cell Immunol 2017;322:15–25.
32. Dominguez-Villar M, Hafler DA. Regulatory T cells in autoimmune disease. Nat Immunol 2018;19:665–73.
33. Ohue Y, Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? Cancer Sci 2019;110:2080–9.
34. Mitsuiki N, Schwab C, Grimbacher B. What did we learn from CTLA-4 insufficiency on the human immune system? Immunol Rev 2019;287:33–49.
35. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. Cell Res 2017;27:109–18.
36. Cai J, Wang D, Zhang G, Guo X. The role Of PD-1/PD-L1 Axis in Treg development and function: Implications for cancer immunotherapy. OncoTargets Ther 2019;12:8437–45.
37. Zheng Y, Fang YC, Li J. PD-L1 expression levels on tumor cells affect their immunosuppressive activity. Oncol Lett 2019;18:5399–407.