

Research Article

Blockade of PD-1/PD-L1 Axis May Improve NK-92 Cell Inhibition Caused by Mesenchymal Stem Cells

 Alper Tunga Ozdemir,¹  Cengiz Kirmaz,²  Rabia Bilge Ozgul Ozdemir,³  Mustafa Oztatlici,⁴
 Mehmet Ibrahim Tuglu,⁴  Pinar Kilicarslan Sonmez,⁴  Kamil Vural,⁵  Afif Berdeli⁶

¹Medical Biochemistry Laboratory, Merkezefendi State Hospital, Manisa, Turkey

²Department of Allergy and Clinical Immunology, Manisa Celal Bayar University, Faculty of Medicine, Manisa, Turkey

³Department of Allergy and Clinical Immunology, Manisa City Hospital, Manisa, Turkey

⁴Department of Histology and Embryology, Manisa Celal Bayar University, Faculty of Medicine, Manisa, Turkey

⁵Department of Pharmacology, Manisa Celal Bayar University, Faculty of Medicine, Manisa, Turkey

⁶Department of Molecular Genetics, Ege University, Faculty of Medicine, Izmir, Turkey

Abstract

Objectives: In this study, it was aimed to investigate how the effects of Mesenchymal stem cells (MSCs) on the anti-tumor properties of NK-92 cells change with programmed death-ligand-1 (PD-L1) blocking antibodies.

Methods: NK-92 cells were co-cultured with MDA-MB-231 breast tumor cells and MSCs. To evaluate the effect of anti-PD-L1 antibodies, cells were cultured for 48 hours with and without the addition of 1, 5, and 10 µg/ml anti PD-L1 antibody. IFN-γ, TNF-α, IL-10 and IDO levels of medium supernatants were determined by ELISA. CCK-8 kit was used to evaluate cytotoxic activity.

Results: IFN-γ and TNF-α expressions of NK-92 cells co-cultured with MDA-MB-231 increased significantly, but this increase was significantly decreased in culture groups with MSCs. IDO expressions increased significantly in co-culture groups with MSCs only. Cytotoxic effects of NK-92 cells were significantly reduced in culture groups with MSCs. However, the suppression effects caused by MSCs improved in the presence of anti-PD-L1 antibodies and in a dose dependent manner.

Conclusion: In our findings, we found that MSCs are a highly effective inhibitors, and the IDO enzyme they secrete may play a major role in this. However, the suppressive effects caused by MSCs may be significantly improved by blocking the PD-1/PD-L1 axis.

Keywords: Mesenchymal stem cells, NK-92 cells, tumor microenvironment, PD-L1, IDO

Cite This Article: Ozdemir AT, Kirmaz C, Ozgul Ozdemir RB, Oztatlici M, Tuglu MI, Kilicarslan Sonmez P, et al. Blockade of PD-1/PD-L1 Axis May Improve NK-92 Cell Inhibition Caused by Mesenchymal Stem Cells. EJMI 2021;5(3):334–340.

Breast cancer is the most common type of cancer in women. In addition to conventional approaches such as surgery, chemotherapy, and radiotherapy to treat breast cancer, new alternatives are emerging every day and the most

prominent of these is immunotherapy. Mutations that occur in tumor cells cause them to be identified and eliminated by immune cells. However, the immune evasive adaptations acquired in addition to these mutations dramatically reduce

Address for correspondence: Alper Tunga Ozdemir, MD. Tibbi Biyokimya Laboratuvarı, Merkezefendi Devlet Hastanesi, Manisa, Turkey

Phone: +90 505 297 32 69 **E-mail:** alpertungaozdemir@outlook.com / alpertunga.ozdemir@saglik.gov.tr

Submitted Date: March 31, 2021 **Accepted Date:** August 24, 2021 **Available Online Date:** September 22, 2021

©Copyright 2021 by Eurasian Journal of Medicine and Investigation - Available online at www.ejmi.org

OPEN ACCESS This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License.



the effectiveness of immune elimination.^[1] Self-tolerance mechanisms are triggered by bioactive molecules secreted on tumor cell surfaces or in the environment, and the effects of anti-tumor cells such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are suppressed. Inhibitory cell surface molecules such as programmed death-ligand 1 (PD-L1), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), human leukocyte antigen G (HLA-G) and TIM3 (T-cell immunoglobulin domain and mucin domain 3) effectively suppress immune cells.^[2,3] Apart from direct cell contact, there are molecules that affect immune cells by paracrine mechanisms. In addition to suppressing cytokines such as interleukin (IL)-10 and transforming growth factor beta (TGF- β), enzymes such as indoleamine 2,3-dioxygenase (IDO) that metabolize tryptophan, which is critical for T cells, dramatically reduce immune cell activation. The kynurenine, forming from the interaction of tryptophan with IDO, is a potent activator of aryl hydrocarbon receptors (AHRs) in regulatory T (Treg) lymphocytes, thereby enhancing the effectiveness of immune-inhibition.^[4,5] Thanks to antibodies that block immune checkpoint molecules such as PD-1, PD-L1 and CTLA-4, therapeutic alternatives that provide immune elimination of tumor cells are gaining increasing popularity.^[6] However, despite these superior effects, the rate of patients benefiting from the treatment remains at 20-30%.^[7,8]

Tumor tissue is a microenvironment made up of cells of different origins in addition to tumor cells. Cells such as tumor-associated macrophages (TAMs), Treg and myeloid derived suppressor cells (MDSCs) are of immune origin, cancer-associated fibroblasts (CAFs) and mesenchymal stem/stromal cells (MSCs) are of somatic origin. Although these cells have different contributions in tumor progression, the common effects of all are inhibition of immune cells.^[9,10] Among these cells, MSCs stand out with a different feature. MSCs are cells that suppress T and B lymphocytes, macrophages, and NK cells non-selectively and non-specifically thanks to their strong immunomodulation properties.^[11-14] In addition to the molecules they secrete such as IL-10, TGF- β , IDO and PGE2, inhibitory checkpoint molecules such as PD-L1 and HLA-G play a critical role in the emergence of these effects.^[15] For this reason, MSCs are used in the treatment of many autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, and Crohn's disease, especially acute graft-versus-host disease resistant to steroids, which can be fatal.^[16] Having such strong immunosuppressive properties, the effects of MSCs on the tumor microenvironment stand out as an important target that needs to be investigated. In this study, we aimed to demonstrate how PD-L1 blocking affects the anti-tumor effects of NK cells on breast tumor cell lines and how these changes in the presence of MSCs.

Methods

Cell culture

Human adipose tissue MSCs, human breast tumor cells MDA-MB-231 and NK-92 cells were purchased from the American Type Culture Collection. MSCs and MDA-MB-231 cells were cultured by using Dulbecco's Modified Eagle's Medium F12 (Biosera, USA) medium that including 10% fetal bovine serum (FBS) (Biosera, USA), 100 U/ml penicillin, 100 μ g/ml streptomycin (Biosera, USA) and 1% 2 mM L-glutamate (Biosera, USA) at 37°C and 5% CO₂ incubator. NK-92 cells were cultured by using Minimum Essential Medium Eagle- Alpha Modification (Biosera, USA) that including 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1% 2 mM L-glutamate and 500 IU/ml IL-2 (Proleukin[®], Prometheus Therapeutics, USA) at 37°C and 5% CO₂ incubator.

NK-92 cells were activated by culturing in medium containing cytokines 500 ng/ml IFN- α (Reprokine, Israel), 500 IU/ml IL-2 (Proleukin[®], Prometheus Therapeutics, USA) and 50 ng/ml IL-15 (Reprokine, Israel) for 24 hours. 5x10⁴ MDA-MB-231 cells were seeded in appropriate wells of 24 well culture dishes. Shortly after MDA-MB-231 and NK-92 cells were co-cultured at a ratio of 1:10, and MSCs, MDA-MB-231 and NK-92 cells were co-cultured at a ratio of 1: 1: 10. To evaluate the effect of anti-PD-L1 antibodies, cells were cultured for 48 hours with and without the addition of 1 μ g/ml, 5 μ g/ml and 10 μ g / ml polyclonal goat PD-L1 IgG antibody (R&D Systems, USA).

Flow Cytometry

Flow cytometry analyzes were performed using FITC fluorescently labeled CD69 (Clone: FN50; Biolegend, USA) and CD107a (Clone:H4A3; Biolegend, USA) antibodies to observe the activation status of NK-92 cells. Phycoerythrin (PE) labeled anti human CD274/PD-L1 antibody (Clone: 29E.2A3, Exbio, Czech Republic) was used to evaluate PD-L1 expression of MSCs and MDA-MB-231 cells. Fluorescence changes were detected with NovoCyte 2060R (Aligent, USA) device and analyzes were performed with FlowJo V.10 software (BD, USA).

ELISA

Medium supernatants were collected after cell culture incubations were completed. Supernatants were centrifuged at 4000 rpm for 10 min to remove the cells. Alterations in IFN- γ (Cat# 950.000.192 Diaclone, France), TNF- α (Cat# 950.090.096 Diaclone, France), IL-10 (Cat# 950.060.192 Diaclone, France) and IDO (Cat# KTE62917 Scientific, China) levels were determined by ELISA method. Absorbance changes were detected with the Multiskan [™] FC Microplate Photometer (Thermo Fisher Scientific, USA).

Cytotoxicity

Cell proliferation assays were performed with Cell Counting Kit-8 (Cat # KTC011001 Abbkine Scientific, China) to observe activation of NK-92 cells and their killing activity against tumor cells. For this, 5×10^3 cells of MDA-MB-231 and MSCs were seeded separately into the appropriate wells of the 96-well culture dish. MDA-MB-231 and MSCs were seeded at $5 \times 10^3 + 5 \times 10^3$ cells for co-culture and cultured for 24 hours to attach. Meanwhile, NK-92 cells were activated with cytokines of 500 ng/ml IFN- α , 500 IU/ml IL-2, 50 ng/ml IL-15. At the end of the culture, 5×10^4 activated NK-92 cells were added onto the adherent cells, which were cultured single and together. To evaluate the effect of anti-PD-L1 antibodies, cells were cultured for 24 hours with and without the addition of 1 μ g/ml, 5 μ g/ml, and 10 μ g/ml polyclonal goat PD-L1 IgG antibody (R&D Systems, USA). After 24 hours of co-culture, the NK-92 cells were removed and 10 μ l of Cell Counting Kit-8 was added to each well with fresh medium and cultured for 4 hours. Color changes at the end of the culture were detected at 450 nm using an ELX800 microplate reader.

Statistic

Each experiment was repeated three times. Prism v7 (GraphPad, CA, USA) program was used for statistical analysis of the data obtained from the experiments. First, whether the data had a normal distribution or not was determined by Shapiro-Wilk test. Groups with normal distribution were analyzed using the parametric method and the One Way Anova statistical method. Values of $p < 0.05$ were considered statistically significant.

Results

Flow Cytometry

In flow cytometry analysis, we found that the PD-L1 positive cell frequency of AD-MSCs was 54.16 ± 1.05 % and MDA-MB-231 was 19.66 ± 2.99 %. After cytokine stimulation of NK-92 cells, we found that CD69 ($p < 0.005$) and CD107a ($p < 0.01$) median florescent intensity values, which are activation markers, increased significantly. Histogram graphs obtained from flow cytometry analyzes are presented in Figure 1.

ELISA

IFN- γ expression of NK-92 cells co-cultured with MDA-MB-231 cells increased significantly independent of antibody administration (without anti PD-L1 $p < 0.001$, for 1 μ g/ml $p < 0.001$, for 5 μ g/ml $p < 0.001$, for 10 μ g/ml $p < 0.001$ respectively). However, in the presence of MSCs, IFN- γ expressions were significantly decreased in all groups (with-

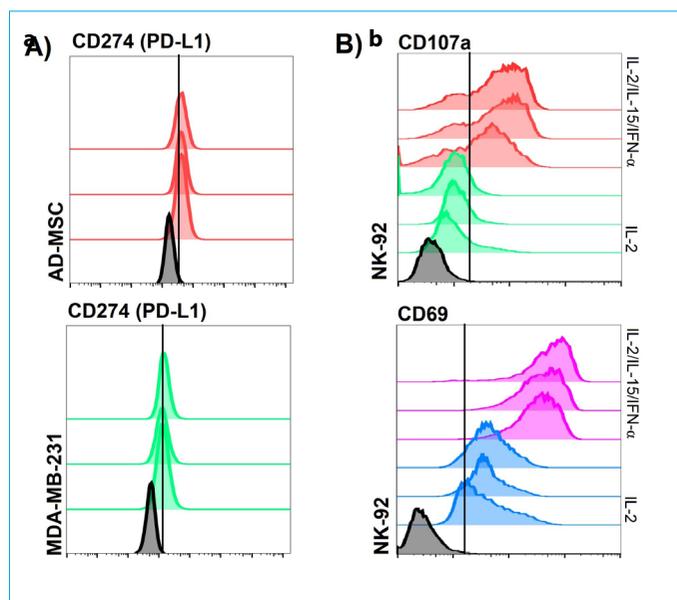


Figure 1. Histogram graphs obtained from flow cytometry analysis. Comparison histograms of adipose derived mesenchymal stem cells (AD-MSCs) and MDA-MB-231 tumor cells analyzes made from three different cell cultures and positive for CD274/PD-L1 (a). Histograms of CD69 and CD107a expression of NK-92 cells (b).

out anti PD-L1 $p < 0.001$, for 1 μ g/ml $p < 0.001$, for 5 μ g/ml $p < 0.001$ respectively) except for the 10 μ g/ml antibody dose ($p > 0.99$). Similarly, TNF- α expression of NK-92 cells co-cultured with MDA-MB-231 cells increased significantly independent of antibody administration (without anti PD-L1 $p = 0.002$, for 1 μ g/ml $p = 0.03$, for 5 μ g/ml $p < 0.001$, for 10 μ g/ml $p < 0.001$ respectively). However, in the presence of MSCs, TNF- α expressions were significantly decreased in without PD-L1 ($p < 0.001$), and 1 μ g/ml dose ($p < 0.001$). There was no significant difference at the 5 μ g/ml dose ($p > 0.99$), but a significant increase at the 10 μ g/ml dose ($p < 0.001$). IL-10 expression of NK-92 cells cultured with MDA-MB-231 was significantly decreased at all doses (for all groups $p < 0.001$). IL-10 expressions were significantly decreased in all groups also in the presence of MSCs (for all groups $p < 0.001$). Interestingly, IL-10 expression of NK-92 cells was significantly suppressed at all doses in the presence of MSCs compared to NK-92 cells cultured with MDA-MB-231 cells alone (for all groups $p < 0.001$). IDO expressions in NK-92 cells cultured with MDA-MB-231 cells without PD-L1 ($p > 0.99$), and 1 μ g/ml ($p = 0.70$) antibody doses did not show a significant change, however, there was a significant increase at the 5 μ g/ml ($p = 0.03$) and 10 μ g/ml ($p = 0.003$) doses. There was a significant increase in IDO expressions at all doses in the presence of MSCs (for all groups $p < 0.001$).

We observed that 1 μ g/ml, 5 μ g/ml, and 10 μ g/ml PD-L1 antibody applications did not significantly change the IFN- γ (for 1 μ g/ml $p > 0.99$, for 5 μ g/ml $p > 0.99$, for 10 μ g/ml

$p > 0.99$ respectively) TNF- α (for 1 $\mu\text{g/ml}$ $p = 0.43$, for 5 $\mu\text{g/ml}$ $p > 0.99$, for 10 $\mu\text{g/ml}$ $p = 0.52$ respectively), IL-10 (for 1 $\mu\text{g/ml}$ $p > 0.99$, for 5 $\mu\text{g/ml}$ $p > 0.99$, for 10 $\mu\text{g/ml}$ $p > 0.99$ respectively) and IDO (for 1 $\mu\text{g/ml}$ $p > 0.99$, for 5 $\mu\text{g/ml}$ $p > 0.99$, for 10 $\mu\text{g/ml}$ $p > 0.99$ respectively) expressions of NK-92 cells. IFN- γ expression of NK-92 cells cultured with MDA-MB-231 cells did not change significantly with 1 $\mu\text{g/ml}$ ($p > 0.99$) and 5 $\mu\text{g/ml}$ ($p = 0.07$) PD-L1 antibody, but a significant increase occurred with 10 $\mu\text{g/ml}$ ($p < 0.001$). TNF- α expression of NK-92 cells cultured with MDA-MB-231 cells did not change significantly with 1 $\mu\text{g/ml}$ PD-L1 antibody ($p = 0.97$), but a significant increase occurred with 5 ($p < 0.001$) and 10 $\mu\text{g/ml}$ ($p < 0.001$). In contrast, IL-10 expressions were not significantly altered with 1 $\mu\text{g/ml}$ PD-L1 antibody ($p > 0.99$), but a significant reduction occurred with 5 ($p = 0.04$) and 10 $\mu\text{g/ml}$ ($p < 0.001$). PD-L1 antibodies did not have a significant effect on IDO expression of NK-92 cells co-cultured with MDA-MB-231 (for 1 $\mu\text{g/ml}$ $p > 0.99$, for 5 $\mu\text{g/ml}$ $p = 0.23$, for 10 $\mu\text{g/ml}$ $p = 0.06$ respectively). IFN- γ , TNF- α and IDO expressions of NK cells co-cultured with MSC and MDA cells did not change with 1 $\mu\text{g/ml}$ PD-L1 antibody ($p > 0.99$, $p > 0.99$ and $p = 0.75$ respectively), but administration of 5 $\mu\text{g/ml}$ ($p < 0.001$, $p = 0.003$ and $p < 0.001$ respectively) and 10 $\mu\text{g/ml}$ ($p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively) antibody led to a significant increase. In contrast, IL-10 expressions were not significantly altered with 1 $\mu\text{g/ml}$ PD-L1 antibody ($p > 0.99$), but a significant reduction occurred with 5 ($p = 0.003$) and 10 $\mu\text{g/ml}$ ($p < 0.001$). The measurements of ELISA analysis of the groups are presented in $\gamma\epsilon$ -1 and comparison graphics are presented in Figure 2.

Cytotoxicity

In our CCK-8 assays, we found that the administration of different doses of anti PD-L1 antibody did not have a sig-

nificant effect on the proliferation of MDA-MB-231 cells (for 1 $\mu\text{g/ml}$ $p = 0.38$, for 5 $\mu\text{g/ml}$ $p = 0.87$, for 10 $\mu\text{g/ml}$ $p > 0.99$ respectively). There was a significant decrease in the proliferation of MDA-MB-231 cells co-cultured with NK cells at all doses (for all groups $p < 0.001$). Increasing the anti PD-L1 antibody dose did not have a significant effect on these effects (for 1 $\mu\text{g/ml}$ $p > 0.99$, for 5 $\mu\text{g/ml}$ $p > 0.99$, for 10 $\mu\text{g/ml}$ $p = 0.97$ respectively). There was no significant change in the proliferation of MDA-MB-231 cells in the presence of MSCs in co-culture groups without PD-L1 antibody ($p = 0.34$) and with 1 $\mu\text{g/ml}$ antibody ($p > 0.99$), however, there was a significant decrease at the 5 $\mu\text{g/ml}$ ($p = 0.01$) and 10 $\mu\text{g/ml}$ doses ($p < 0.001$). The cytotoxic effects of NK cells against MDA-MB-231 cells co-cultured with MSCs did not change significantly at the 1 $\mu\text{g/ml}$ ($p > 0.99$) and 5 $\mu\text{g/ml}$ ($p = 0.10$) dose, but a significant increase occurred at the 10 $\mu\text{g/ml}$ dose ($p < 0.001$). Optical density measurements of the groups obtained from CCK-8 assays are presented in Table 1 and comparison graphics are presented in Figure 2.

Discussion

In this study, we observed that anti PD-L1 antibodies significantly increase the anti-tumor activity of NK-92 cells on MDA-MB-231 cells, and this has a dose-dependent pattern. There was an increase in proinflammatory cytokines such as IFN- γ and TNF- α consistent with the dose of anti PD-L1 antibody administered, but a significant decrease in anti-inflammatory IL-10 levels. The immunosuppressive properties of MSCs have been well elucidated in the literature. It has been reported that MSCs express PD-L1 and in this way their immune suppression abilities are increased.^[15] As expected, in our findings the antitumor activity of NK-92 cells was significantly reduced in the presence of MSCs. However, this suppressive effect produced by MSCs was

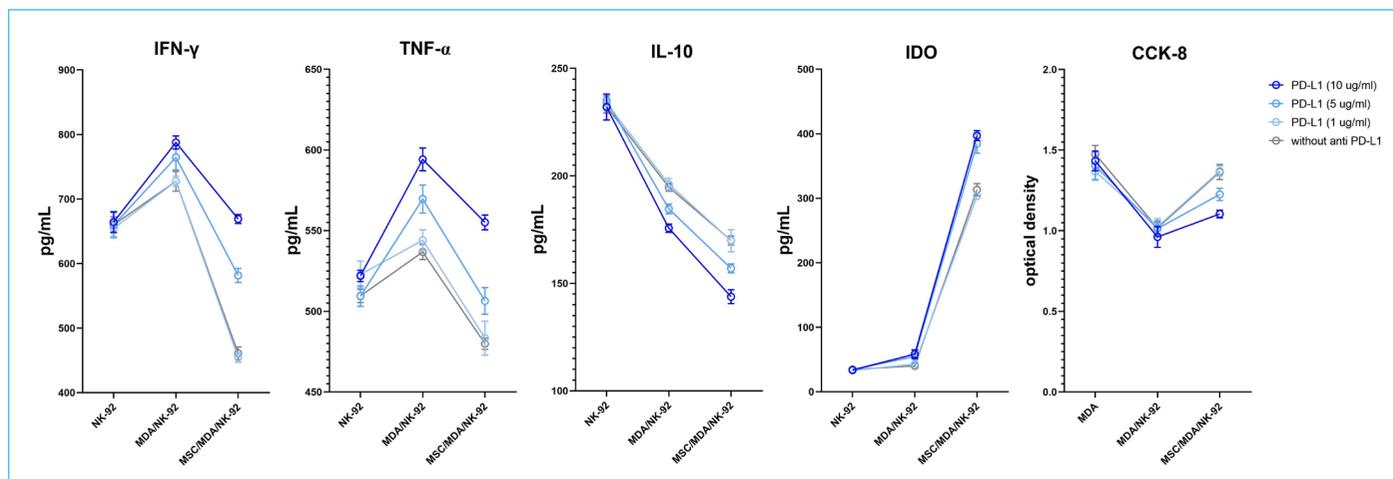


Figure 2. Comparison graphics of IFN- γ , TNF- α , IL-10 and IDO ELISA measurements and CCK-8 optical densities of experiments with different doses of anti PD-L1 antibody (without, 1 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$) added. Data are presented as mean and standard deviation.

Table 1. Values obtained from ELISA and CCK-8 measurements

| | Without anti PD-L1 | 1 µg/ml PD-L1 | 5 µg/ml PD-L1 | 10 µg/ml PD-L1 |
|---|--------------------|---------------|---------------|----------------|
| NK-92 | | | | |
| IFN-g (pg/ml) | 660±12.4 | 655±12.5 | 659±19.5 | 664±16.1 |
| TNF-a (pg/ml) | 510±4.07 | 523±8.41 | 509±6.29 | 522±3.58 |
| IL-10 (pg/ml) | 232±3.16 | 234±4.04 | 235±2.09 | 232±6.03 |
| IDO (pg/ml) | 34.6±1.08 | 32.8±1.38 | 34.5±1.83 | 33.8±2.55 |
| NK-92 and MDA-MB-231 Co-culture | | | | |
| IFN-g (pg/ml) | 728±15.4 | 728±7.66 | 765±19.5 | 788±10.1 |
| TNF-a (pg/ml) | 537±4.69 | 544±6.49 | 570±8.68 | 5974±7.06 |
| IL-10 (pg/ml) | 195±1.91 | 196±2.73 | 185±2.25 | 176±1.99 |
| IDO (pg/ml) | 40.1±3.32 | 43.0±3.24 | 54.6±2.04 | 58.3±6.75 |
| CCK-8 (OD) | 1.02±0.03 | 1.03±0.05 | 1.01±0.05 | 0.96±0.06 |
| NK-92, MDA-MB-231 and MSC Co-culture | | | | |
| IFN-g (pg/ml) | 461±9.8 | 455±7.26 | 582±10.9 | 669±6.98 |
| TNF-a (pg/ml) | 480±3.47 | 483±10.5 | 506±8.28 | 555±4.59 |
| IL-10 (pg/ml) | 170±2.13 | 170±5.22 | 157±2.07 | 144±3.26 |
| IDO (pg/ml) | 313±9.66 | 304±3.37 | 385±15.0 | 397±7.89 |
| CCK-8 (OD) | 1.36±0.04 | 1.37±0.03 | 1.23±0.03 | 1.10±0.02 |
| MDA-MB-231 cells | | | | |
| CCK-8 (OD) | 1.48±0.05 | 1.37±0.05 | 1.40±0.08 | 1.43±0.06 |

significantly decreased especially at the dose of 10 µg/ml anti PD-L1 antibody.

The PD-1 / PD-L1 interaction is a pathway that is particularly involved in the suppression of CD4 and CD8 T lymphocytes, and its blocking results in the recovery of the anti-tumor immune response.^[17] However, there are studies in the literature showing that NK cells also express PD-1 and that the increase in PD-1 positive NK cell frequencies in cancer patients is associated with poor prognosis.^[18] There are different dimensions of anti-tumor activity created by anti PD-L1 antibodies in terms of NK cells. Many anti PD-L1 antibodies used in treatment have the Fc domain. CD16, which is intensely expressed in NK cells, is an Fc receptor and in this way antibody dependent cellular cytotoxicity (ADCC) is triggered. It has been shown that PD-L1 positive tumor cells are effectively eliminated by NK cells and by ADCC in the presence of anti PD-L1 antibodies.^[19] Another effect of blocking the PD-L1 axis on NK cells is that it increases the expression of proinflammatory cytokines. Oyer et al. reported that anti PD-L1 antibodies increased IFN-γ and TNF-α expressions of NK cells significantly.^[20] In our findings, we detected that the IFN-γ and TNF-α expressions of NK-92 cells co-cultured with MDA-MB-231 cells were significantly increased, and this was potentiated by high anti PD-L1 antibodies (Fig. 2). However, we observed that adding MSCs as a third variable to the equation significantly suppressed the increase in IFN-γ and TNF-α levels.

Although this suppression effect caused by MSCs could not be completely eliminated, we found that high doses of anti-PD-L1 antibodies could significantly reduce this effect. IL-10, one of the other molecules we evaluated in our study, is a strong immunosuppressive cytokine. IL-10 activates the STAT3 signaling pathway by interacting with its receptor, IL-10R1. This interaction causes an increase in IL-10 expression while decreasing the expression of the proinflammatory cytokines IFN-γ and TNF-α.^[21] It has been reported that excessively activated CD8 T lymphocytes increase IL-10 expression and thus limit their own activity.^[22] In addition, it has been shown that the cytokines IFN-α, IL-2 and IL-15, which we use to activate NK-92 cells, can activate the same signal pathway as IL-10, namely STAT3.^[23] In our findings, we found that the IL-10 expressions of NK-92 cells that we activated with the cytokine cocktail were significantly higher than the group co-cultured with MDA cells, and in contrast, there were significant increases in IFN-γ and TNF-α expressions (Fig. 2). The reason for this increase in IL-10 expression may be that the cytokine cocktail we applied to NK-92 cells simultaneously caused IL-10 secretion. Direct interaction with MDA-MB-231 cells may trigger activator receptors such as NKG2D, NCRs and DNAM-1 in NK-92 cells, leading to an increase in TNF-α and IFN-γ.^[24]

Nevertheless, what was more interesting in our findings was that IL-10 expression was significantly decreased in addition to IFN-γ and TNF-α in the presence of MSCs. MSCs

are known to be powerful immunosuppressive cells. In addition to molecules such as IDO, PGE2, IL-10 and TGF- β , suppressive surface ligands such as PD-L1 also play a role in the emergence of these effects.^[13,25] Interestingly, MDA-MB-231 and MCF-7 breast tumor cells cultured with MSCs have been shown to increase the expression of potent inhibitory molecules such as PD-L1 and HLA-G.^[26] In our findings, we observed that there was PD-L1 expression in about 50% of MSCs and 15-20% of MDA-MB-231 cells (Fig. 1). The presence of MSCs may have caused a relative increase in presence of PD-L1, in addition, it may have increased PD-L1 expression in MDA-MB-231 cells. This may have resulted in a more efficient suppression in NK-92 cells. Additionally, in our findings, we found that there were significantly higher IDO levels than other groups in the presence of MSCs (Fig. 2). IDO is an enzyme that metabolizes tryptophan to kynurenine, causing tryptophan starvation in lymphocytes and indirect metabolic inhibition.^[27] Özdemir et al. reported that MSCs co-cultured with MDA-MB-231 and MCF-7 cells could be the main source of IDO in the environment.^[26] In accordance with the literature, we observed that IDO levels increased only in co-culture groups with MSCs. It has been shown that MSCs preconditioned with pro-inflammatory cytokines such as IFN- γ and TNF- α have a significant increase in immune cell suppression properties.^[28] It has been shown that MSCs stimulated with Toll-like receptor (TLR)3 and TLR9 agonists can suppress the anti-tumor effects of NK-92 cells more effectively. It has been pointed out that one of the reasons may be that TLR agonists simultaneously cause NK-92 activation and the increase in cytokines such as IFN- γ and TNF- α stimulates the inhibitory properties of MSCs.^[29] Although MSCs can provide immunosuppression in different ways, these effects were decreased with the anti-PD-L1 antibodies we applied and, in a dose-dependent manner. However, additional combinations such as IDO inhibitors may be required for full efficacy (Fig 3).

Conclusion

MSCs are strong immunosuppressive cells that express PD-L1, and the PD-1/PD-L1 interaction is critical in the inhibition of lymphocytes. In our study, we showed that the antitumor activity of NK-92 cells on MDA-MB-231 cells was significantly suppressed in the presence of MSCs. However, we observed that this effect of MSCs was significantly reduced by anti-PD-L1 antibodies, although not completely. Our findings suggested that in addition to blocking the PD-L1 pathway, inhibition of suppressor molecules such as IDO secreted by MSCs could be a more effective approach. However, these in vitro data need to be supported by in vivo studies.

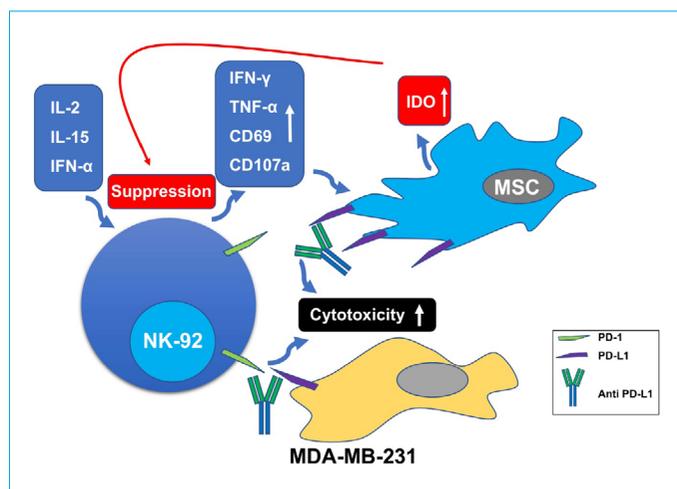


Figure 3. Blocking the PD-1/PD-L1 axis with anti PD-L1 antibodies may increase the anti-tumor effect of NK-92 cells on MDA-MB-231 tumor cells. However, the presence of mesenchymal stem cells (MSCs) in the environment may reduce the effectiveness of this blockage due to the indoleamine 2,3-dioxygenase (IDO) they secrete.

Disclosures

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Financial Disclosure: The Scientific Research Projects Commission of Manisa Celal Bayar University financially supported the project whose reference number is 2018/072.

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

References

1. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, et al. Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Semin Cancer Biol* 2015;35:185–98. [CrossRef]
2. Driessens G, Kline J, Gajewski TF. Costimulatory and coinhibitory receptors in anti-tumor immunity. *Immunol Rev* 2009;229:126–44. [CrossRef]
3. Özgül Özdemir RB, Özdemir AT, Oltulu F, Kurt K, Yiğittürk G, Kırmaz C. A comparison of cancer stem cell markers and nonclassical major histocompatibility complex antigens in colorectal tumor and noncancerous tissues. *Ann Diagn Pathol* 2016;25:60–3. [CrossRef]
4. Ferns DM, Kema IP, Buist MR, Nijman HW, Kenter GG, Jordano-va ES. Indoleamine-2,3-dioxygenase (IDO) metabolic activity is detrimental for cervical cancer patient survival. *Oncoimmunology* 2015;4:e981457. [CrossRef]

5. Larrain MTI, Rabassa ME, Lacunza E, Barbera A, Cretón A, Segal-Eiras A, et al. IDO is highly expressed in breast cancer and breast cancer-derived circulating microvesicles and associated to aggressive types of tumors by in silico analysis. *Tumor Biol* 2014;35:6511–9. [\[CrossRef\]](#)
6. Swart M, Verbrugge I, Beltman JB. Combination approaches with immune-checkpoint blockade in cancer therapy. *Front Oncol* 2016;6:233. [\[CrossRef\]](#)
7. Navarrete-Bernal MGC, Cervantes-Badillo MG, Martínez-Herrera JF, Lara-Torres CO, Gerson-Cwilich R, Zentella-Dehesa A, et al. Biological landscape of triple negative breast cancers expressing CTLA-4. *Front Oncol* 2020;10:1206. [\[CrossRef\]](#)
8. Sun JY, Zhang D, Wu S, Xu M, Zhou X, Lu XJ, et al. Resistance to PD-1/PD-L1 blockade cancer immunotherapy: mechanisms, predictive factors, and future perspectives. *Biomark Res* 2020;8:35. [\[CrossRef\]](#)
9. Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC. Tumor-associated stromal cells as key contributors to the tumor microenvironment. *Breast Cancer Res* 2016;18:84.
10. Goswami KK, Ghosh T, Ghosh S, Sarkar M, Bose A, Baral R. Tumor promoting role of anti-tumor macrophages in tumor microenvironment. *Cell Immunol* 2017;316:1–10. [\[CrossRef\]](#)
11. Franquesa M, Hoogduijn MJ, Bestard O, Grinyó JM. Immunomodulatory effect of mesenchymal stem cells on B cells. *Front Immunol* 2012;3:212. [\[CrossRef\]](#)
12. Özdemir AT, Özgül Özdemir RB, Kırmaz C, Sarıboyacı AE, Ünal Halbutoğlları ZS, Özel C, et al. The paracrine immunomodulatory interactions between the human dental pulp derived mesenchymal stem cells and CD4 T cell subsets. *Cell Immunol* 2016;310:108–15. [\[CrossRef\]](#)
13. Özdemir RBÖ, Özdemir AT, Sarıboyacı AE, Uysal O, Tuğlu Mİ, Kırmaz C. The investigation of immunomodulatory effects of adipose tissue mesenchymal stem cell educated macrophages on the CD4 T cells. *Immunobiology* 2019;224:585–94.
14. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* 2006;107:1484–90. [\[CrossRef\]](#)
15. Weiss ARR, Dahlke MH. Immunomodulation by mesenchymal stem cells (MSCs): mechanisms of action of living, apoptotic, and dead MSCs. *Front Immunol* 2019;10:1191. [\[CrossRef\]](#)
16. Haque N, Ramasamy TS, Kasim NHA. Mechanisms of mesenchymal stem cells for autoimmune disease treatment. In: Pham PV, editor. *Stem cell transplantation for autoimmune diseases and inflammation*. Cham: Springer International Publishing; 2019. p. 27–44.
17. Riley JL. PD-1 signaling in primary T cells. *Immunol Rev* 2009;229:114–25.
18. Pesce S, Greppi M, Grossi F, Del Zotto G, Moretta L, Sivori S, et al. PD-1/PD-Ls checkpoint: insight on the potential role of NK Cells. *Front Immunol* 2019;10:1242.
19. Park JE, Kim SE, Keam B, Park HR, Kim S, Kim M, et al. Antitumor effects of NK cells and anti-PD-L1 antibody with antibody-dependent cellular cytotoxicity in PD-L1-positive cancer cell lines. *J Immunother Cancer* 2020;8:e000873. [\[CrossRef\]](#)
20. Oyer JL, Gitto SB, Altomare DA, Copik AJ. PD-L1 blockade enhances anti-tumor efficacy of NK cells. *Oncoimmunology* 2018;7:e1509819. [\[CrossRef\]](#)
21. Williams L, Bradley L, Smith A, Foxwell B. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol* 2004;172:567–76.
22. Trandem K, Zhao J, Fleming E, Perlman S. Highly activated cytotoxic CD8 T cells express protective IL-10 at the peak of coronavirus-induced encephalitis. *J Immunol* 2011;186:3642–52. [\[CrossRef\]](#)
23. Cacalano NA. Regulation of Natural Killer Cell Function by STAT3. *Front Immunol* 2016;7:128.
24. Lee HH, Cho H. Attenuated anti-tumor activity of NK-92 cells by invasive human breast carcinoma MDA-MB-231 cells. *Mol Cell Toxicol* 2020;16:139–47. [\[CrossRef\]](#)
25. Davies LC, Heldring N, Kadri N, Blanc KL. Mesenchymal stromal cell secretion of programmed death-1 ligands regulates T Cell mediated immunosuppression. *Stem Cells* 2017;35:766–76.
26. Özdemir RBÖ, Özdemir AT, Kırmaz C, Tuğlu Mİ, Şenol Ö, Özverel CS, et al. The effects of mesenchymal stem cells on the IDO, HLA-G and PD-L1 expression of breast tumor cells MDA-MB-231 and MCF-7. *Arch Clin Exp Med* 2019;4:132–7.
27. Munn DH, Mellor AL. IDO in the tumor microenvironment: inflammation, counter-regulation, and tolerance. *Trends Immunol* 2016;37:193–207. [\[CrossRef\]](#)
28. Hu C, Li L. Preconditioning influences mesenchymal stem cell properties in vitro and in vivo. *J Cell Mol Med* 2018;22:1428–42. [\[CrossRef\]](#)
29. Ozdemir AT, Kırmaz C, Özgül Ozdemir RB, Degirmenci P, Öztatlıcı M, Degirmenci M. In-vitro evaluation of effects of mesenchymal stem cells on TLR3, TLR7/8 and TLR9-activated natural killer cells. *EJMO* 2021;5:71–9. [\[CrossRef\]](#)