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Research Article

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

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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

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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

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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 µg/ml streptomycin (Biosera, USA) and 1% 2 mM L-glutamate (Biosera, USA) at 37°C and 5% CO_2 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 µg/ml streptomycin, 1% 2 mM L-glutamate and 500 IU/ml IL-2 (Proleukin®, Prometheus Theraputics, USA) at 37°C and 5% CO_2 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 Theraputics, USA) and 50 ng/ml IL-15 (Reprokine, Israel) for 24 hours. $5x10^4$ 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 µg/ml, 5 µg/ml and 10 µg / 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, $5x10^3$ 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 $5x10^3$+$5x10^3$ 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, 5x104 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 µg/ml, 5 µg/ml, and 10 µg/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 µl 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 µg/ml $p<0.001$, for 5 µg/ml $p<0.001$, for 10 µg/ml $p<0.001$ respectively). However, in the presence of MSCs, IFN-γ expressions were significantly decreased in all groups (without anti PD-L1 $p<0.001$, for 1 µg/ml $p<0.001$, for 5 µg/ml $p<0.001$ respectively) except for the 10 µg/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 µg/ml $p=0.03$, for 5 µg/ml $p<0.001$, for 10 µg/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 ug/ml dose ($p<0.001$). There was no significant difference at the 5 µg/ml dose ($p>0.99$), but a significant increase at the 10 µg/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-231cells 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 µg/ml ($p=0.70$) antibody doses did not show a significant change, however, there was a significant increase at the 5 µg/ml ($p=0.03$) and 10 µg/ml ($p=0.003$) doses. There was a significant increase in IDO expressions at all doses in the presence of MSCs (for all groups $p<0.001$).

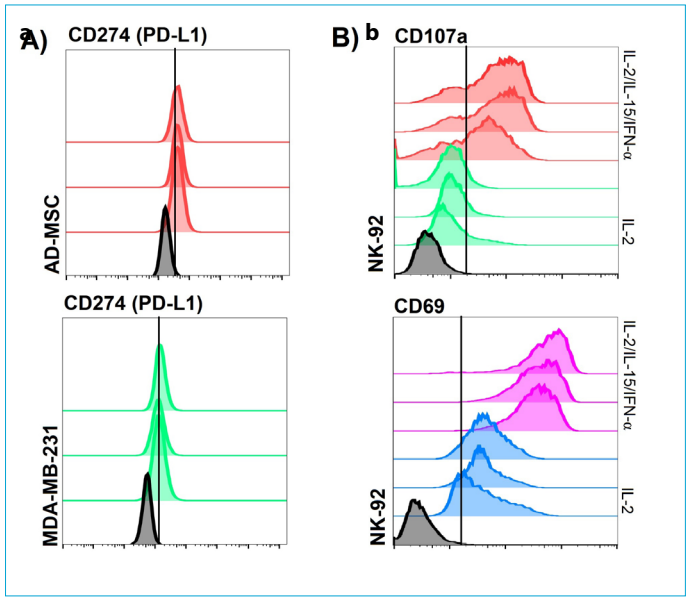

Figure 1. Histogram graphs obtained from flow cytometry analysis. Comparison histograms of adipose derived mesenchymal stem cells (AD-MSCs) and MDA-MB-231 tumor cells analyzes made from three different cell cultures and positive for CD274/PD-L1 (a). Histograms of CD69 and CD107a expression of NK-92 cells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

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

p>0.99 respectively) TNF-α (for 1 µg/ml p=0.43, for 5 µg/ml p>0.99, for 10 µg/ml p=0.52 respectively), IL-10 (for 1 µg/ml p>0.99, for 5 µg/ml p>0.99, for 10 µg/ml p>0.99 respectively) and IDO (for 1 µg/ml p>0.99, for 5 µg/ml p>0.99, for 10 µg/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 µg/ml (p>0.99) and 5 µg/ml (p=0.07) PD-L1 antibody, but a significant increase occurred with 10 µg/ml (p<0.001). TNF-α expression of NK-92 cells cultured with MDA-MB-231 cells did not change significantly with 1 µg/ml PD-L1 antibody (p=0.97), but a significant increase occurred with 5 (p<0.001) and 10 µg/ml (p<0.001). In contrast, IL-10 expressions were not significantly altered with 1 µg/ml PD-L1 antibody (p>0.99), but a significant reduction occurred with 5 (p=0.04) and 10 µg/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 µg/ml p>0.99, for 5 µg/ml p=0.23, for 10 µg/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 µg/ml PD-L1 antibody (p>0.99, p>0.99 and p=0.75 respectively), but administration of 5 µg/ml (p<0.001, p=0.003 and p<0.001 respectively) and 10 µg/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 µg/ml PD-L1 antibody (p>0.99), but a significant reduction occurred with 5 (p=0.003) and 10 µg/ml (p<0.001). The measurements of ELISA analysis of the groups are presented in γe-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 significant effect on the proliferation of MDA-MB-231 cells (for 1 µg/ml p=0.38, for 5 µg/ml p=0.87, for 10 µg/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 µg/ml p>0.99, for 5 µg/ml p>0.99, for 10 µg/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 µg/ml antibody (p>0.99), however, there was a significant decrease at the 5 µg/ml (p=0.01) and 10 µg/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 µg/ml (p>0.99) and 5 µg/ml (p=0.10) dose, but a significant increase occurred at the 10 µg/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

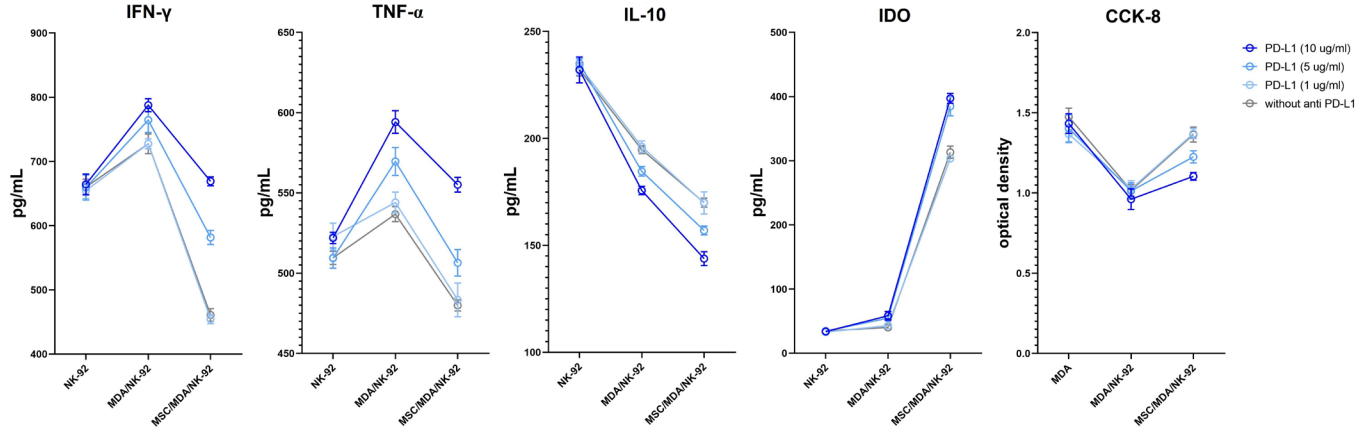

Figure 2. Comparison graphics of IFN-γ , TNF-α, IL-10 and IDO ELISA measurements and CCK-8 optical densities of experiments with different doses of anti PD-L1 antibody (without, 1 µg/ml, 5 µg/ml, and 10 µg/ml) added. Data are presented as mean and standard deviation.

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.

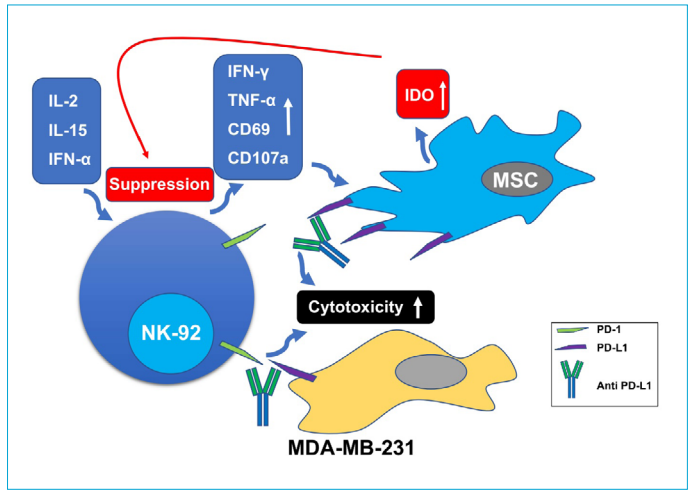

Figure 3. Blocking the PD-1/PD-L1 axis with anti PD-L1 antibodies may increase the anti-tumor effect of NK-92 cells on MDA-MB-231 tumor cells. However, the presence of mesenchymal stem cells (MSCs) in the environment may reduce the effectiveness of this blockage due to the indoleamine 2,3-dioxygenase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

Disclosures

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