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Research Article

In-vitro Evaluation of Effects of Mesenchymal Stem Cells on TLR3, TLR7/8 and TLR9-activated Natural Killer Cells

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Abstract

Objectives: In this study, it was aimed to investigate the immunomodulatory effects of Mesenchymal stem cells (MSCs) on Natural Killer (NK) cells activated by Toll-like receptor (TLR) agonists.

Methods: MDA-MB-231, MCF-7 and NK-92 cells were induced with TLR3, TLR7/8 and TLR9 agonists and co-cultured with MSCs. Alterations in IFN-γ, TNF-α, Granzyme-b and Perforin expressions were determined by qPCR method, CD69 and CD107a expressions were determined by flow cytometry, and cytotoxicity was determined by MTT-assay.

Results: All TLR agonists significantly increased the expressions of the IFN-γ, TNF-α, Granzyme-b, Perforin, CD69 and CD107a in-vitro. We determined that the cytokine, cytotoxic molecules, and activation markers of NK-92 cells interacting with breast tumor cells significantly increased by TLR3 and TLR9 agonists. However, suppression rather than activation occurred on the NK-92 cells due to the simultaneous induction of the immunosuppressive effects of MSCs by these agonists. On the other hand, the TLR7/8 agonists provided a low NK-92 induction, however, the inhibitory effects of MSCs were not triggered. Therefore, it provided a more significant activation than TLR3 and TLR9 agonists.

Conclusion: Our findings suggested that TLR7/8 agonists may be a better choice to induce antitumor effects of NK cells in a tumor tissue rich in MSCs.

Keywords: Toll like receptor agonists, mesenchymal stem cells, natural killer cells, immunomodulation, anti-tumor immunity

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Toll like receptors (TLRs) are expressed on the immune cells and interact with the structural components of pathogenic microorganisms. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell membrane and interacts with molecules of microorganisms such as hemagglutinin, lipopolysaccharide and flagellin. TLR3, TLR7, TLR8 and TLR9 are intracellular receptors and interact with CpG-DNA and ss/ds RNA molecules of bacteria and viruses.[1] Interaction of TLRs with specific ligands leads to activation of myeloid differentiation primary response 88 protein, TIR-domain-containing adapter-inducing interferon-β and nuclear factor kappa B transcription factors, in this way inflammatory cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), cytolytic molecules perforin and gran-

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zyme-b are expressed from various of immune cells such as macrophages, CD4/CD8 T cells, B cells and natural killer (NK) cells.[2,3] Because of these effects, synthetic TLR agonists have become potential drugs for cancer immunotherapy. The TLR2/4 agonist Bacillus Calmette–Guérin, TLR4 agonist monophosphoryl lipid A, and TLR7 agonist Imiquimod are synthetic agonists approved by the U.S. Food and Drug Administration. TLR3 agonist polyriboinosinic-polyribocytidylic acid [poly(I:C)], TLR7/8 agonist Resiquimod (R-848), and TLR9 agonist CpG ODNs are molecules in clinical trial phase.[4] Although they have been shown to be effective in certain types of cancer, it is remarkable that most TLR agonists have failed clinical trials. Safety and efficacy issues are the most important reasons for the termination of these studies.[5] Another reason for the failure in clinical trials may be that TLRs biology is not well understood.

Mesenchymal stem cells (MSCs) are multipotent cells that stand out with their immunomodulation properties. They non-specifically and non-selectively suppress immune cells by secreting bioactive molecules such as interleukin (IL) 10, transforming growth factor beta (TGF-β), indoleamine-pyrrole 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), and human leukocyte antigen G (HLA-G).[6] In in vitro studies, it has been shown that MSCs can effectively suppress the CD4 T cell subsets Th1, Th2 and Th17 cells, but activate regulatory T (Treg) cells.[7,8] In different studies, it has been reported that the interaction of MSCs with macrophages leads to permanent changes in the phenotype and functions of macrophages and the immunosuppressive aspects of these cells become evident.[9,10] The programmed death-ligand 1 (PD-L1), IDO and PGE2 molecules expressed by MSCs induce differentiation of B lymphocytes into the IL-10 producer regulatory B cells.[11] On the other hand, it has been reported that MSCs can suppress the expression of inflammatory cytokines like as IFN-γ of natural killer (NK) cells but not their cytotoxic effects.[12] TLRs also play an important role in regulating the immunomodulatory effects of MSCs. It has been reported that TLR4 agonists increase the expression of pro-inflammatory molecules IL-1β, IL-6, IL-8 and monocyte chemotactic protein (MCP)-1 from MSCs, while TLR3 agonists increase the expression of anti-inflammatory molecules IDO, PGE2, IL-10, TGF-β, hepatocyte growth factor and hemoxygenase.[13]

The tumor microenvironment (TME) surrounding the tumor cells plays an active role in all stages of tumor development. This bidirectional interaction significantly reduces the success rates of cancer treatments.[14] There are many studies showings that the cellular actors of TME, like as tumor associated macrophages (TAMs), myeloid-derived suppressive cells (MDSCs), cancer-associated fibroblasts (CAFs) and MSCs contribute significantly to the development of treatment resistance and tumor progression.[15] Particularly, the contribution of MSCs in critical processes such as angiogenesis, epithelial-mesenchymal transition and metastasis in breast cancer is remarkable.[16–18] Given the effects of TLR agonists on MSCs, the type of TLR agonist administered may be an important determinant of the treatment response. However, there are not enough studies in the current literature revealing the effects of the interaction of MSCs with TLR agonists on tumor immunity. Therefore, in this study, we aimed to investigate the effects of MSCs on the NK-92 cells that cultured with breast tumor cells MDA-MB-231 and MCF-7, and how these effects alter by the synthetic TLR3, TLR7/8 and TLR9 agonists.

Methods

Cell culture

Adipose derived MSCs (PCS-500-011), MCF-7 (HTB-22), MDA-MB-231 (HTB-26) and NK-92 (CRL-2407) cells were purchased from the American Type Culture Collection. Adherent 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 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 µg/ml streptomycin, 2 mM L-glutamate and 500 IU/ml IL-2 (Proleukin®, Prometheus Theraputics, USA) at 37 ˚C and 5% CO2 incubator.

First, TLR agonist mixtures were prepared for cell stimulation by purchasing the Human TLR3-7-9 Agonist kit (Invivogen, USA). The mixture containing synthetic analog of double-stranded RNA (dsRNA) high molecular weight polyinosinic-polycytidylic acid [Poly (I:C) HMW] 10 µg/ml, low molecular weight (LMW) Poly (I:C) 10 µg/ml, and polyadenylic–polyuridylic acid [Poly (A:U)] 25 µg/ml was prepared to generate TLR3 stimulation. The mixture containing imidazoquinoline amine analog to guanosine Imiquimod (R837) 5 µg/ml, R848 (Resiquimod) 10 µg/ml, thiazoloquinolone derivative CL075 (3M002) 5 µg/ml, and 20-mer phosphorothioate protected single-stranded RNA oligonucleotide containing a GU-rich sequence ssRNA40/LyoVec™ 5 µg/ml was prepared to generate TLR7/8 stimulation. The mixture containing unmethylated CpG dinucleotides in specific sequence contexts (CpG motifs) ODN2006 5 µM, ODN2216 5 µM and ODN2395 5 µM was prepared to generate TLR9 stimulation.

To evaluate the effects of TLR agonists on MSCs, 3x105 cells were seeded into each well of 6 well culture dishes and incubated for 24 hours to adhere. Then, TLR3, TLR7/8

and TLR9 agonist mixtures were added to the designated wells and incubated for 24 hours. At the end of the incubation, the medium was removed, and the cells washed three times with sterile PBS (Biosera, USA). MSCs were harvested by cell-scraper and stored at -80 ˚C for qPCR analysis. To evaluate the effects of TLR agonists on NK-92 cells, 1x106 cells were seeded into each well of 24 well culture dishes and then, TLR3, TLR7/8 and TLR9 agonist mixtures were added to the designated wells and incubated for 24 hours. Next, the medium was removed, and the cells washed three times with sterile PBS and stored at -80 ° C for quantitative PCR (qPCR) analysis.

Two-cell co-culture experiments were performed to observe how the effects of TLR agonists on NK-92 cells changed in the presence of MSCs. 5x104 MSCs were seeded in upper part of 0.4 µm pore size inserts (Thermo Fisher Scientific, USA) in a 24-well culture plate and cultured for 24 hours to adhere. At the end of the incubation, 5x105 NK-92 cells were seeded in the bottom section of wells and TLR agonist mixtures were added to the designated wells and incubated at 37 ˚C and 5% CO_2 for 24 hours. Three-cell co-culture experiments were performed to evaluate how NK-92 cells' effects on tumor cells were affected by TLR agonists and MSCs. 5x104 MDA-MB-231 and 5x104 MCF-7 cells were seeded into appropriate wells of a 24-well culture plate, 5x104 MSCs were seeded in upper part of 0.4 µm pore size inserts in another culture plate, and all cells were cultured for 24 hours to adhere. Then, 5x105 NK-92 cells were added on the tumor cells, the inserts contained MSCs were placed upper chamber of the wells and TLR agonist mixtures were added to the designated wells and plates were incubated at 37 ˚C and 5% CO_2 for 24 hours. At the end of the incubation, non-adherend NK-92 cells were carefully harvested and stored at -80 ˚C for qPCR analysis.

Quantitative PCR

To observe the effects of TLR agonists on MSCs, IL-10, TGF-β and HGF expressions of MSCs were evaluated. To observe the effects of TLR agonists and MSCs on the NK-92 cells, IFN-γ, TNF-α, granzyme-b, perforin expressions of NK-92 cells were evaluated. For this, the total RNA was isolated from the collected NK-92 cells (Total RNA purification kit, Jena Bioscience, Germany cat# PP-210L). Subsequently, cDNAs were synthesized from the isolated RNAs by using the SCRIPT cDNA synthesis kit (Jena Bioscience, Germany cat# PCR-511L). Changes in mRNA expressions were detected with CFX Connect Real-Time PCR Detection System (Biorad, USA) by using qPCR SybrMaster mix (Jena Bioscience, Germany cat# PCR-372L). GAPDH was used as reference gene, and relative gene expression differences were calculated by ΔΔ CT method. The primers of genes are shown in Table 1.

Table 1. Table of primer sequences used for the studied genes

Gene	Primer	Base count
Human IFN-γ	F- GAACTCATCCAAGTGATGGCT	
	R- GACCTCGAAACAGCATCTGA	41
Human TNF-α	F- CTGCTGCACTTTGGAGTGAT	
	R- GCCAGAGGGCTGATTAGAGA	40
Human TGF-β1	F- TTATTGAGCACCTTGGGCACT	
	R- TCCCTGCATCTCAGAGTGTTG	42
Human IL-10	F- TGAGAACCAAGACCCAGACA	
	R- AAGAAATCGATGACAGCGCC	40
Human Granzyme-b	F- CAGGGCAGATGCAGACTTTTC	
	R- AAGATAAGCCATGTAGGGGCG	42
Human HGF	F-GACGCAGCTACAAGGGAACA	
	R-AGCTCGAAGGCAAAAAGCTG	40
Human Perforin	F- GGCCACCTAAAATTCCGCTA	
	R- CAGAAGCATTTGGGGGACAT	40
Human GADPH	F- GCCGCATCTTCTTTTGCGTC	
	R- GACGAACATGGGGGCATCAG	40

F: forward; R: reverse.

Flow Cytometry

Flow cytometry analyzes were performed to evaluate the activation status of NK-92 cells and the increase in cytotoxic molecule secretions. For this, NK-92 cells collected from the experiments were incubated in the dark and at room temperature with Phycoerythrin (PE) labeled anti-human CD69 (clone: FN50, Biolegend, USA) and CD107a (clone: H4A3, Biolegend, USA) antibodies for 30 minutes. Then, cells were washed with PBS to remove unbound antibodies and changes in fluorescence signals were measured by using BD Accuri C6 cytometer.

Cytotoxicity Assay

MTT assays (Abcam, USA cat#ab211091) were performed to evaluate the cytotoxic effects of NK-92 cells on the MCF-7 and MDA-MB-231 cells. For this purpose, 2x104 MCF-7 and MDA-MB-231 cells were seeded to the appropriate wells of 96 well culture plate, 2x104 MSCs were seeded in upper part of 0.4 µm pore size inserts (Corning® HTS Transwell®, USA) in another 96 well culture plate, and all cells were cultured for 24 hours to adhere. After incubation 2x105 NK-92 cells were on the tumor cells, the inserts contained MSCs were placed upper chamber of the wells and TLR agonist mixtures were added to the designated wells and plates were incubated at 37 ˚C and 5% CO_2 for 24 hours. Next, non-adherend NK-92 cells and the inserts containing MSCs were removed and wells washed with PBS for three times. Then serum free medium containing equal volume of MTT reagent was added and cultured for 3 hours. After incubation, the medium was removed and 150 µl of MTT solvent

was added, after shaking for 15 minutes with an orbital shaker, the absorbances of the plate were read at 590 nm.

Statistical Analysis

The data obtained from the experimental groups were statistically compared by PRISM v7 (GraphPad, California, USA) software. First, the column analyzes were performed to determine whether the data were in normal distribution. Since the standard deviations of the groups were not similar, Brown-Forsythe and Welch ANOVA test was used for statistical comparisons. Results of $p<0.05$ were considered significant.

Results

The Direct Effects of TLR Agonists on MSC and NK-92 Cells

In the first stage, we evaluated the effects of TLR agonists on MSC and NK-92 cells. We found that IL-10, TGF-β and HGF expressions of MSCs stimulated with TLR3 were significantly higher than TLR7/8 ($p=0.001$, $p<0.001$ and $p=0.001$, respectively) and TLR9 ($p<0.001$, $p=0.003$ and $p=0.001$, respectively) stimuli. In addition, we observed that TLR9 stimulation resulted in a significant increase in the same cytokines compared to TLR7/8 stimulation ($p=0.002$, $p<0.001$ and $p<0.001$, respectively). IFN-γ, TNF-α, granzyme-b and perforin expressions of NK-92 cells stimulated with TLR3 mix were significantly higher than TLR7/8 ($p=0.001$, $p<0.001$, $p=0.02$ and $p<0.001$, respectively) and TLR9 ($p<0.001$, $p<0.001$, $p= 0.01$ and $p<0.001$, respectively) mixes. IFN-γ expression induced by TLR9 were significantly higher compared to TLR7/8 ($p=0.04$), in contrast, TNF-α expressions of NK-92 cells stimulated with TLR7/8 were significantly higher than that of TLR9-stimulated cells ($p=0.01$). There was no significant difference between granzyme-b and perforin expressions formed by TLR7/8 and TLR9 stimuli ($p=0.18$ and $p=0.12$, respectively). The comparison graphs of changes in gene expression of MSCs and NK-92 cells are shown in Figure 1.

The Effects of TLR Agonists and MSCs on Antitumor Response

We compared IFN-γ expressions of NK-92 cells co-cultured with MDA-MB-231 cells and found that TLR3 and TLR9 agonists provided a significant increase compared to TLR7/8 ($p=0.004$ and $p=0.005$, respectively). In contrast, in the presence of MSCs, TLR7/8 agonists led to a significant increase compared to TLR3 ($p<0.001$) and TLR9 ($p<0.001$) agonists. In addition, the increases in IFN-γ induced by TLR agonists decreased significantly in the presence of MSCs (for TLR3 $p<0.001$, for TLR7/8 $p=0.03$ and for TLR9 $p=0.002$ respectively). In NK-92 cells co-cultured with MCF-7, TLR3 agonists significantly increased IFN-γ expression compared to TLR7/8 and TLR9 agonists ($p=0.003$ and $p=0.004$, respectively). However, in the presence of MSCs, the increase induced by TLR3 and TLR9 agonists was significantly suppressed ($p<0.001$ and $p=0.004$ respectively). Interestingly, the increase in IFN-γ caused by TLR7/8 agonists was not affected by the presence of MSCs ($p=0.20$).

The TNF-α expressions of NK-92 cells co-cultured with MDA-MB-231 cells were significantly increased by TLR3 agonist compared with TLR7/8 and TLR9 ($p=0.004$ and $p=0.005$, respectively). In addition, TLR9 agonists led to a significant increase compared to TLR7/8 agonists ($p=0.01$). TNF-α increases induced by TLR agonists were significantly suppressed in the presence of MSCs (for TLR3 $p=0.002$, for TLR7/8 $p=0.02$ and for TLR9 $p<0.001$ respectively). However, in the presence of MSCs, TLR3 agonists caused significant suppression compared to TLR7/8 and TLR9 agonists ($p<0.001$ and $p=0.005$, respectively), and the suppression

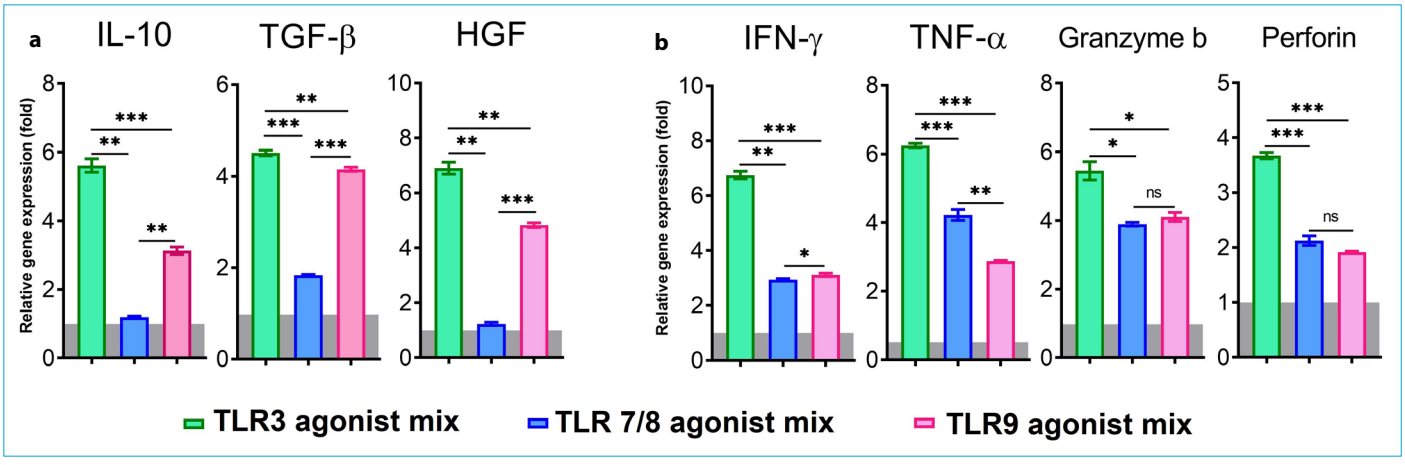

Figure 1. TLR agonists-induced gene expression alterations in mesenchymal stem cells **(a)** and NK-92 cells (b). Data are presented as mean and standard deviation. Gray area represents the gene expression of the control group. (*= statistically significant $p<0.05$)

created by TLR9 agonists was significantly higher than that of TLR7/8 agonists (p=0.009). In NK-92 cells co-cultured with MCF-7, TLR3 agonists significantly increased TNF-α expression compared to TLR7/8 and TLR9 agonists (p<0.001 and p=0.002, respectively). Similar to IFN-γ expressions, MSCs significantly suppressed the increase induced by TLR3 (p<0.001) and TLR9 (p=0.009) agonists, but there was no significant change in TNF-α expressions formed by TLR7/8 agonists (p=0.34).

The granzyme-b and perforin expressions of NK-92 cells co-cultured with MDA-MB-231 cells were significantly increased by TLR3 agonist compared with TLR7/8 and TLR9 (for granzyme-b p=0.008 and p=0.01; for perforin p<0.001 and p=0.005 respectively). Granzyme-b and perforin increases induced by TLR3 agonists were significantly suppressed by MSCs (p=0.006 and p<0.001, respectively). However, MSCs had no significant effect on TLR7/8 and TLR9-induced increases, except for the significant suppression of perforin expressions induced by TLR9 agonists (p<0.001). In NK-92 cells co-cultured with MCF-7 cells, granzyme-b increases induced by TLR7/8 agonists were significantly lower than those of TLR3 and TLR9 agonists (p=0.007 and p=0.02, respectively). For perforin, there was only a significant difference between TLR9 and TLR7/8 agonists (p=0.02). Granzyme-b increases induced by TLR3 (p<0.001) and TLR9 (p<0.001) were significantly suppressed by MSCs, but there was no difference in the effects of TLR7/8 agonists (p=0.11). TLR9-induced increases in perforin were significantly suppressed by MSCs (p=0.002), but there was no difference in the effects of TLR3 (p=0.18) and TLR7/8 (p=0.94) agonists. The comparison graphs of changes in IFN-γ, TNF-α, granzyme-b and perforin gene expressions of NK-92 cells are shown in Figure 2.

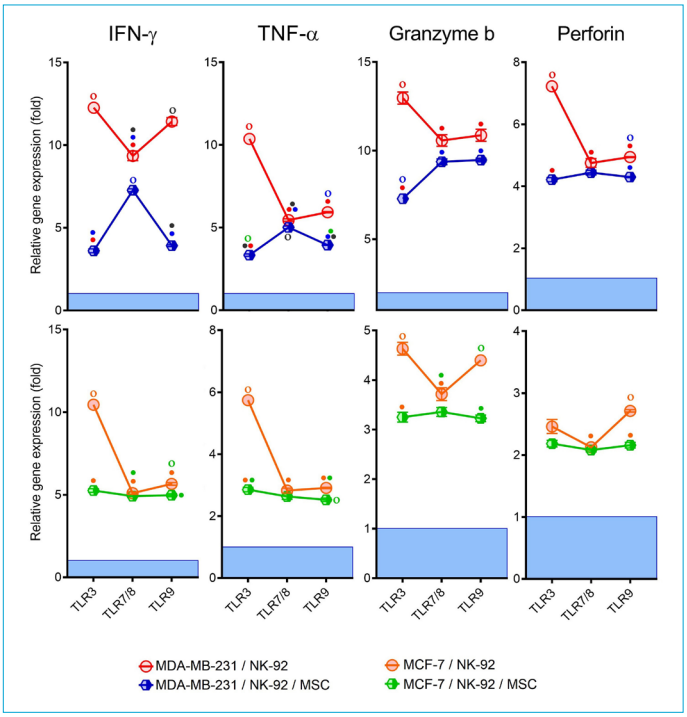

Figure 2. The comparison graphs of the effects of MSCs and TLR agonists on the gene expressions of NK-92 cells co-cultured with MDA-MB-231 and MCF-7 cells. Data are presented as mean and standard deviation. Blue area represents the gene expression of the control group. There is a statistically significant difference between groups with "round" and "dot" symbols of the same color.

Alterations in NK-92 Activation Markers

We evaluated how CD69 and CD107a, markers used for activation of NK-92 cells, change with TLR agonists and MSCs by flow cytometry analysis. We found that NK-92 cells' CD69 and CD107a expressions were significantly increased by TLR agonists. TLR3 agonists induced a significant increase compared to TLR7/8 and TLR9 agonists (for CD69 p<0.001 and p<0.001; for CD107a p<0.001 and p<0.001 respectively), however, the increase caused by TLR7/8 agonists was significantly lower than that of TLR9 agonists (for CD69 p=0.003, for CD107a p<0.001). CD69 and CD107a expressions of NK-92 cells co-cultured with MDA-MB-231 cells with/without MSCs were significantly higher compared to those that were not (for CD69 induced by TLR3 p<0.001/p<0.001; TLR7/8 p=0.002/p=0.003 and TLR9 p<0.001/p=0.003; for CD107a induced by TLR3 p<0.001/p<0.001; TLR7/8 p<0.001/p<0.001 and TLR9 p<0.001/p<0.001 respectively). In addition, increases in CD69 and CD107a induced by TLR agonists were significantly suppressed by MSCs (for CD69 induced by TLR3 p<0.001, TLR7/8 p<0.001 and TLR9 p<0.001; for CD107a induced by TLR3 p<0.001, TLR7/8 p=0.04 and TLR9 p<0.001 respectively). CD69 and CD107a expressions of NK-92 cells co-cultured with MCF-7 cells with/without MSCs were significantly higher compared to those that were not (for CD69 induced by TLR3 p<0.001/p<0.001; TLR7/8 p=0.003/p=0.01 and TLR9 p=0.003/p<0.001; for CD107a induced by TLR3 p<0.001/p=0.001; TLR7/8 p<0.001/p<0.001 and TLR9 p<0.001/p=0.001 respectively). CD69 and CD107a expressions of NK-92 cells co-cultured with MDA-MB-231 cells and MSCs and induced with TLR3 agonists were significantly higher compared to TLR7/8 (for CD69 p<0.001 and p<0.001; for CD107a p<0.001 and p<0.001 respectively) and TLR9 (for CD69 p<0.001 and p<0.001; for CD107a p<0.001 and p<0.001 respectively) agonists, however, the increase caused by TLR7/8 agonists was significantly lower than that of TLR9 agonists (for CD69 p=0.006 and p<0.001; for CD107a p<0.001 and p<0.001 respectively). In addition, increases in CD69 and CD107a induced by TLR agonists were significantly suppressed by MSCs (for CD69 induced by TLR3 p<0.001, TLR7/8 p=0.03; for CD107a induced by

TLR3 p<0.001, TLR7/8 p=0.01 and TLR9 p<0.001 respectively) excluding CD69 expressions induced by TLR9 agonist (TLR9 p=0.06). Histogram graphs of CD69 and CD107a expressions of NK-92 cells and comparison graphs of median fluorescence intensity values between groups are shown in Figure 3.

Alterations in Cytotoxic Activity

We performed an MTT assay to observe how the cytotoxic effects of NK-92 cells changed in the presence of TLR agonists and MSCs. Proliferations of MDA-MB-231 and MCF-7 cells were not affected by TLR agonists. The cytotoxic effect of NK-92 cells on MDA-MB-231 cells was significantly increased by TLR3 (p=0.02), TLR7/8 (p=0.003) and TLR9 (p=0.004) agonists. Similarly, TLR agonists also provided a significant increase in cytotoxic effect in the presence of MSCs (for TLR3 p=0.004, for TLR7/8 p<0.001 and for TLR9 p=0.002). The cytotoxic effect of NK-92 cells on MCF-7 cells was significantly increased by TLR3 (p=0.006), and TLR9 (p=0.001) agonists, but was not by TLR7/8 (p>0.99). However, in the presence of MSCs, the cytotoxic effects of NK-92 cells on MCF-7 cells were not affected by TLR agonists (for TLR3 p=0.16, for TLR7/8 p=0.22 and for TLR9 p=0.29). Optical density (OD) of unstimulated (p=0.03) and TLR3-induced (p=0.01) MDA-MB-231/NK-92 cells increased significantly in the presence of MSCs, however, there was no significant difference in TLR7/8 (p=0.16) and TLR9-induced cells (p=0.06). ODs of TLR3 (p<0.001) and TLR9-induced (p<0.001) MCF-7 / NK-92 cells increased significantly in the presence of MSCs, however, there was no significant difference in unstimulated (p=0.06) and TLR7/8-induced cells (p=0.23). Comparison graphs of optical densities obtained from MTT assays are shown in Figure 4.

Discussion

In this study, we detected that IFN-γ, TNF-α, granzyme-b and perforin expression of NK-92 cells co-cultured with MDA-MB-231 and MCF-7 cells significantly increased with TLR3, TLR7/8 and TLR9 agonists. However, the effects of TLR3 and TLR9 agonists were significantly inhibited by MSCs. Interestingly, the effects formed by TLR7/8 agonists was not affected by the presence of MSCs, except for IFN-γ and TNF-α expressions of NK-92 cells cultured with MDA-MB-231 cells. We also found similar effects for the expression of the activation markers CD69 and CD107a, and the lowest activation was formed by TLR7 agonists. Expressions of activation markers were also significantly reduced in the presence of MSCs. Finally, we detected that the cytotoxic effect induced by TLR3 agonists in MDA-MB-231 and MCF-7 cells and induced by TLR9 agonists in MCF-7 cells was significantly suppressed by MSCs (Figure 5).

NK cells identify non-self-cells through many receptors on their cell surface. Major histocompatibility antigen I specific interactions with receptors such as Killer cell immunoglobulin-like receptors and lectin-like CD94-NKG2A heterodimers are critical in making this distinction.[19] As a result of this mechanism, it is expected that NK-92 cells are activated against non-self-cells such as MDA-MB-231 and MCF-7. In in-vivo tumor models, interactions of tumor cells

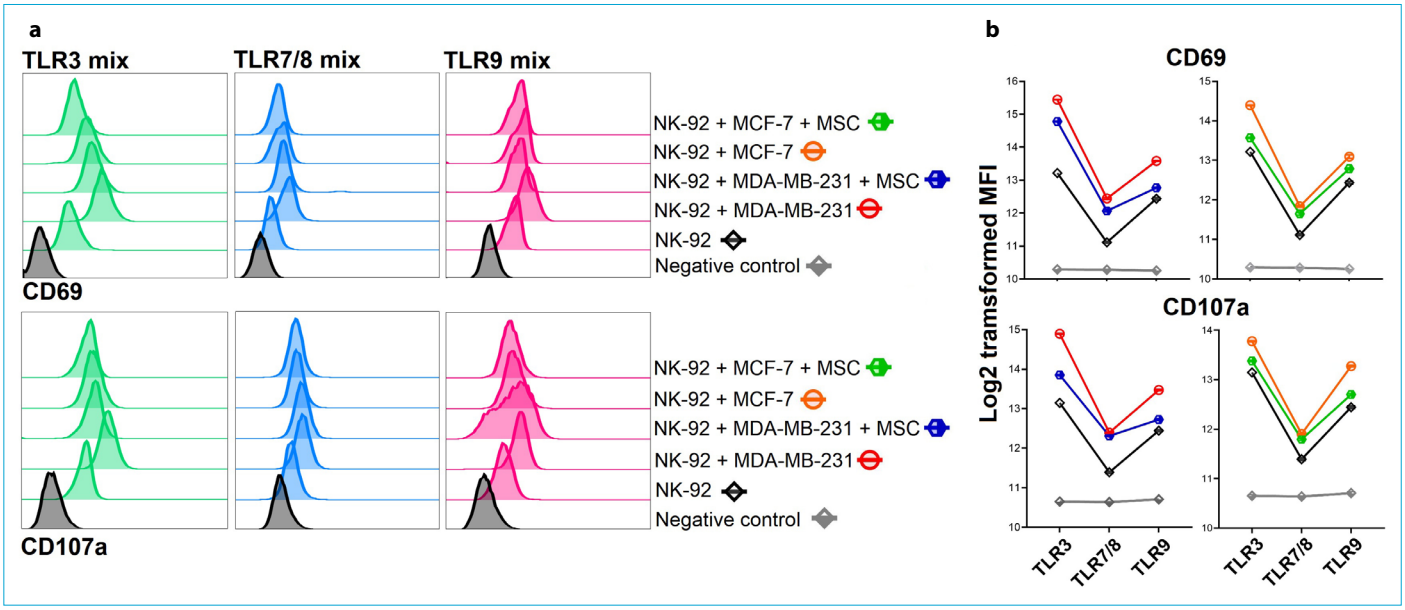

Figure 3. The effects of MSCs and TLR agonists on activation markers of NK-92 cells co-cultured with MDA-MB-231 and MCF-7 cells. Representative histogram graphs of flow cytometry analyses of CD69 and CD107a molecules **(a)**, and comparison graphics of MFI data obtained from these analyzes **(b)**.

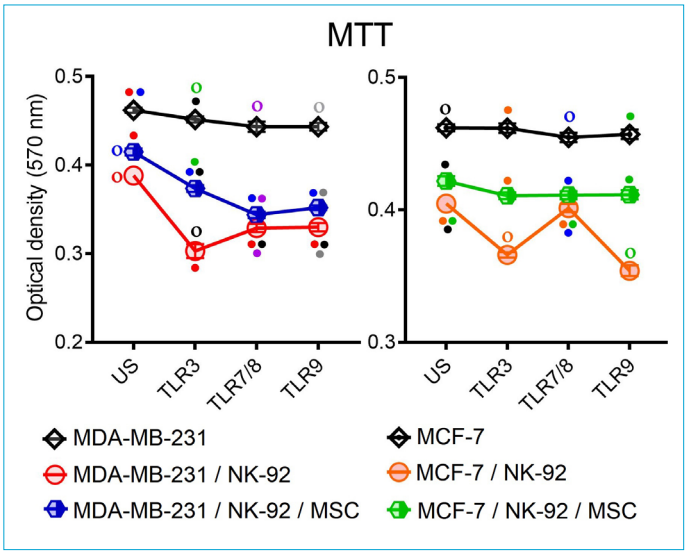

Figure 4. The comparison graphs of optical density values obtained from MTT assays of groups. Data are presented as mean and standard deviation. There is a statistically significant difference between groups with "round" and "dot" symbols of the same color.

and immune cells lack pathogen-induced stimuli such as bacteria and viruses. In addition, in this environment where intense cell death occurs, genetic materials such as mutated DNA and RNA are likely to interact with specific TLRs in immune cells.[20,21] Stimulants such as TLR agonists to be applied externally will make a positive contribution to the activation of immune cells. In this respect, cytotoxic NK cells can be considered as a good antitumor cell. It has been reported that NK cells have high TLR3 low TLR7, TLR8 and TLR9 expression.[22] In our study, we detected that IFN-γ, TNF-α, granzyme-b and perforin expressions of NK-92 cells induced by TLR3 agonists were significantly higher than cells induced by TLR7/8 and TLR9 (Fig. 1b). TLR3 agonists also led to a significant increase compared to other TLR agonists for NK-92 cells cultured with both MDA-MB-231 and MCF-7 cells. Expressions of IFN-γ and TNF-α in NK-92 cells cultured with MDA-MB-231 cells, and granzyme-b and perforin expressions in those cultured with MCF-7 cells were significantly increased by TLR9 agonists compared to TLR7/8 agonists (Fig. 2). Similar situation was present with NK-92 activation markers, expressions of CD69 and CD107a induced by TLR3 and TLR9 agonists were significantly higher compared to TLR7/8 agonists. These findings suggested that TLR3 agonists were superior in inducing the anti-tumor effects of NK-92 cells in vitro.

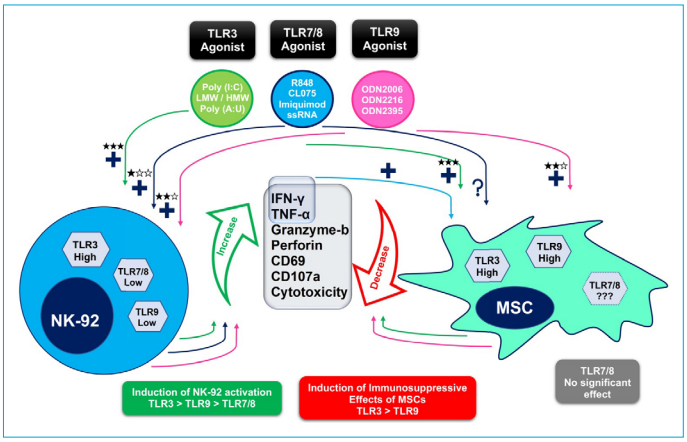

Figure 5. Expressions of IFN-γ, TNF-α, Granzyme-b, Perforin, CD69 and CD107a increased significantly in NK-92 cells stimulated with TLR mixes. In addition, its cytotoxic effects on tumor cells also increased. These effects of TLR mixes were TLR3> TLR9> TLR7/8. On the other hand, mixes TLR3 and TLR9 in the presence of MSCs significantly suppressed the resulting activation. TLR7/8 mixes did not have a significant effect.

In-vitro and in-vivo studies have shown that synthetically produced TLR agonists have powerful effects on immune elimination of cancer cells.[23] However, clinical studies with these agonists have not gone beyond Phase I/II stages, and interestingly, many studies have been terminated or withdrawn.[24] In our study where we investigated the possible reasons for this, we came to hints that MSCs could cause a total opposite effect than expected for TLR agonists. Tumor tissue consists of tumor cells and various cells surrounding them. One of these cells, MSCs, are cytokine-producing cells with strong immunomodulatory abilities. IDO is an enzyme that inhibits lymphocytes by causing tryptophan deprivation, and it has been reported that MSCs co-cultured with MDA-MB-231 and MCF-7 cells might be the main source of secreted IDO.[25] It has been shown that IDO, IL-10, TGF-β and PGE2 expressions are increased in MSCs preconditioned with Poly (I:C) (TLR3 agonist) and GpC ODNs (TLR9 agonist), and in this way they can suppress the proliferation and cytotoxic effects of NK cells.[26–28]. However, there are not enough studies in the current literature to demonstrate the effects of TLR7/8 agonists on the immunomodulation capabilities of MSCs. We used human adipose tissue MSCs in our study, and it has been reported that these cells express TLR3 and TLR9 but do not express TLR7 and TLR8.[13,29] TLR3 and TLR9 agonists are known to be activators for both NK and MSCs, but activation of MSCs results in effective suppression of NK cells.[3,13] In our study, we determined that IFN-γ, TNF-α, granzyme-b, perforin, CD69 and CD107a expressions of NK-92 cells induced with TLR3 and TLR9 agonists and cultured with both of tumor cells significantly increased. However, we also observed that these increases were significantly suppressed in the presence of MSCs (Figs. 2, 3). A similar situation was also present in the cytotoxic effect created by NK-92 cells (Fig. 4). There could be several potential explanations for this situation. First, in our findings, we detected that TLR3 and TLR9 agonists

activated NK-92 cells more strongly than TLR7/8 agonists. In particular, there was a significant increase in IFN-γ and TNF-α expressions. It is known that MSCs stimulated with IFN-and TNF-α produce a superior immunosuppression.[30] Consequently, increases in IFN-γ and TNF-α caused by TLR3 and TLR9 agonists may have increased the inhibitory properties of MSCs. On the other hand, another salient factor is that MSCs preconditioned with TLR3 and TLR9 agonists can exert a superior suppression on NK cells.[26–28] That is, the TLR3 and TLR9 agonists we applied might have induced the effects of MSCs on NK-92 cells in this way. Finally, an important reason why the inhibitory effects of MSCs are not affected by the TLR7/8 agonists we apply may be the lack of TLR7 and TLR8 expressions of adipose tissue MSCs.

Our study has several limitations. In this study, we do not have data on TLR expressions of NK-92, MDA-MB-231, MCF-7 and MSCs that we used in experiments. This situation may leave our conclusions about the findings we obtained due to some controversial findings of the current literature unfounded. We used a mixture of the most widely studied synthetic agonists in the literature when performing TLR stimulation. The data we detected may have been due to the synergistic effects of these agonists, or to someone more active among them. The correlation between expressed RNA and observed protein levels is known to be poor.[31] We could not show the alterations in protein level of critical molecules such as IFN-γ, TNF-α, granzyme-b and perforin whose gene expression we compared. This situation made it difficult for us to reach a definitive conclusion when interpreting our findings.

Conclusion

In conclusion, according to our findings, TLR3 and TLR9 agonists significantly increased the antitumor effects of NK-92 cells on breast tumor cells in-vitro. However, suppression rather than activation occurred in NK-92 cells due to the simultaneous induction of the immunosuppressive effects of MSCs by these agonists. On the other hand, the TLR7/8 agonists provided a low NK-92 induction, however, the inhibitory effects of MSCs were not triggered. Therefore, it provided a more significant activation than TLR3 and TLR9 agonists. Consequently, our findings suggest that the use of TLR7/8 agonists may be a better approach to induce antitumor effects of NK cells in a tumor tissue rich in MSCs.

Disclosures

Ethics Committee Approval: Our study is an in-vitro cell culture study with commercially available cell lines. No human or animal samples were used for data collection.

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