

Toll-like receptor 3-mediated modulation of umbilical cord mesenchymal stem cell phenotype and pancreatic cancer cell responses during coculture

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Abstract

Mesenchymal stem cells (MSCs) are progenitor cells isolated from various tissues and are crucial for tissue repair, immune support, and anticancer therapies. MSC functions such as migration, immunomodulation, and regeneration are regulated through Toll-like receptors (TLRs). In particular, TLR3 activation enhances the immunosuppressive and therapeutic capabilities of MSCs. This research employed human umbilical cord-derived MSCs (UCMSCs) and investigated the effects of TLR3 stimulation on their viability, phenotype-associated gene expression, and during co-culture with *Panc-1* pancreatic cancer cells. UCMSCs were cultured and characterized for mesenchymal markers by flow cytometry. TLR3-based signaling was modulated using Poly(A:U) (an agonist) and CU-CPT4a (an antagonist). Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay, and relative gene expression was measured employing quantitative reverse transcription polymerase chain reaction. *Panc-1* cells were co-cultured with UCMSCs to evaluate TLR3-mediated effects. Data are presented as the means $\pm$ standard error of the mean, with statistical significance determined by analysis of variance ($p \leq 0.05$). The TLR3 agonist improved cell viability, whereas the antagonist reduced it. Additionally, both regulated the expression of *CD44*, *CDH1*, and *VIMs*. When UCMSCs and *Panc-1* cells were cocultured at 10:1, TLR3 affected the expression of MSC-related genes, including *CD44*, *CDH1*, *CLDN1*, *VIM*, *ZEB1*, *MMP9*, *MMP2*, *TIMP1*, *VEGFR2*, and *PLAU*. Thus, TLR3-based signaling influenced the viability, maintenance of the mesenchymal phenotype, and *Panc-1* coculture-associated phenotype in UCMSCs. These results underscore the crucial role of TLR3-based signaling in modulating UCMSC function

Özet

Mezenkimal kök hücreler (MSC'ler), doku onarımı, bağışıklık desteği ve kanser karşıtı tedaviler açısından kritik öneme sahip progenitor hücrelerdir. Farklı dokulardan izole edilen MSC'lerin göç, immünomodülasyon ve rejenerasyon gibi fonksiyonları Toll-benzeri reseptörler (TLR'ler) aracılığıyla düzenlenmektedir. Özellikle, TLR3 aktivasyonu MSC'lerin immünosupresif ve terapötik özelliklerini artırmaktadır. Bu çalışmada, TLR3 uyarımının insan göbek kordonu kaynaklı MSC'lerin (UK-MSC'ler) canlılığı, fenotip ile ilişkili gen ekspresyonu üzerindeki etkileri ile bu hücrelerin *Panc-1* pankreas kanseri hücreleriyle ortak kültürdeki etkileri araştırılmıştır. UK-MSC'ler kültür ortamında çoğaltıldı ve mezenkimal yüzey belirteçleri açısından akım sitometrisi ile karakterize edildi. TLR3 sinyali, Poly(A:U) agonisti ve CU-CPT4a antagonisti ile modüle edildi. Hücre canlılığı 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromür testi ile değerlendirildi ve gen ekspresyonu gliseraldehit-3-fosfat dehidrogenaz referans geni kullanılarak kantitatif gerçek zamanlı ters transkripsiyon polimeraz zincir reaksiyonu ile ölçüldü. *Panc-1* pankreas kanseri hücreleri UK-MSC'lerle ko-kültüre edilerek TLR3 aracılı etkiler analiz edildi. Tüm veriler ortalama $\pm$ ortalamasının standart hatası olarak sunuldu ve istatistiksel analiz varyans analizi veya t-test ile yapıldı ($p \leq 0,05$). TLR3 agonistlerinin hücre canlılığını artırdığı, TLR3 antagonistlerinin ise azalttığı gözlemlenmiştir. Ayrıca, hem agonist hem de antagonistlerin *CD44*, *CDH1* ve *VIM* genlerinin ekspresyonunu düzenlediği bulunmuştur. UK-MSC ve *Panc-1* hücreleri 10:1 oranında ortak kültüre alındığında, TLR3 aktivasyonu ve inhibisyonunun *CD44*, *CDH1*, *CLDN1*, *VIM*, *ZEB1*, *MMP9*, *MMP2*, *TIMP1*, *VEGFR2* ve *PLAU* gibi MSC ile ilişkili genlerin ekspresyon profillerini etkilediği gösterilmiştir. Sonuç

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and suggest its potential utility in enhancing MSC-based therapeutic strategies. We believe that these results can help elucidate the role of TLR3-based signaling on UCMSC functions and provide a basis for future research.

olarak, TLR3 sinyal iletiminin UK-MSC'lerin canlılığını, mezenkimal fenotipin korunmasını ve *Panc-1* kanser hücrelerine yanıtla ilişkili fenotipini etkilediği gözlemlenmiştir. Bu bulgular, TLR3 sinyal iletiminin UK-MSC fonksiyonlarını modüle etmedeki önemini vurgulamakta ve özellikle tümörle ilişkili uygulamalarda MSC tabanlı tedavi stratejilerini geliştirmede potansiyel bir araç olabileceğini düşündürmektedir. Bu çalışmanın, TLR3 sinyal iletiminin UK-MSC'lerin biyolojik işlevleri üzerindeki etkilerini aydınlatmaya yardımcı olacağına ve gelecekteki araştırmalara temel oluşturacağına inanmaktayız.

Keywords: mesenchymal stem cell, TLR3, UCMSC, pancreatic ductal adenocarcinoma, cancer biology

Introduction

Mesenchymal stem cells (MSCs), essential for maintaining tissue homeostasis, constitute the cell-level foundation of the stromal microenvironment (Zhang et al., 2024). Within biological systems, MSCs exert a variety of beneficial effects, including supporting antiviral immune responses, promoting tissue repair through antifibrotic activity, and secreting proangiogenic growth factors (Golchin et al., 2020). These cells can be isolated from embryonic and adult tissues and comprise a heterogeneous population with unique regenerative and immunomodulatory properties, making them highly valuable for therapeutic applications (Chandrasekar et al., 2023; El Omar et al., 2014; Gholizadeh-Ghaleh Aziz et al., 2021). MSCs have potential in cancer therapy due to their ability to target tumor sites, modulate immune responses, and serve as carriers for anticancer agents. They can be polarized through different toll-like receptors (TLRs), creating tumor-suppressing and -promoting effects (Chandrasekar et al., 2023).

TLRs are pattern recognition receptors that detect microbe-associated molecular patterns. Their expression in MSCs varies with tissue origin, including umbilical cord, adipose tissue, and dental pulp (Liu et al., 2023; Raicevic et al., 2011). Human umbilical cord-derived MSCs (UCMSCs) play crucial roles in tissue repair due to their higher regenerative and immunomodulatory capacities compared to MSCs from other sources. Thus, they attract considerable interest in experimental and clinical studies as they are used as carrier cells in cancer treatment. Notably, MSCs express various TLRs and are capable of targeted migration to inflammation sites, demonstrating anticancer responses, and secrete immunomodulatory molecules in response to TLR activation (Tomchuck et al., 2008). Therefore, the regulation of MSC responses via TLRs is of great importance in tumor therapies, by modulating immune cells to affect the tumor microenvironment (TME) and directly impact tumor cells.

TLR signaling modulates MSC behavior: TLR2 maintains the undifferentiated state of bone marrow MSCs, while TLR3 and TLR4 influence stress responses, migration, and immunoregulation, especially with activated T cells (Hwang et al., 2023; Najar et al., 2017, 2019). TLR3-primed MSCs facilitate the suppression of NK cell activity by reducing the susceptibility of infected/damaged

cells to NKs and increasing immunosuppressive capacity of MSCs (Hwang et al., 2023; Najar et al., 2019). TLR3 signaling shapes MSC phenotype and affects tumor cell responses. Activation by dsRNA analogs, such as Poly(I:C), triggers the NF- κ B and IRF3 pathways, altering cytokine secretion, adhesion molecule expression, and extracellular matrix (ECM) remodeling (Najar et al., 2017). Importantly, several downstream genes regulated by TLR3 signaling play pivotal roles in determining MSC phenotype and influencing tumor biology. Claudin-1 (CLDN1) is a tight junction protein involved in maintaining epithelial barrier integrity; its dysregulation is linked to epithelial-mesenchymal transition (EMT) and metastasis in various cancers, including gastric, pancreatic, colon, and hepatocellular carcinoma (Bhat et al., 2020). Zinc finger E-box-binding homeobox 1 (ZEB1) is a transcription factor (TF) that represses E-cadherin expression but promotes EMT and invasiveness. Matrix metalloproteinase-9 (MMP9) degrades ECM components, aiding MSC migration and tumor invasion/angiogenesis (Zheng & Ma, 2022). These proteins collectively link TLR3-mediated MSC behavior to the modulation of the TME; the MSC phenotype alters during this process. Determining the role of MSCs is particularly critical in aggressive tumors such as pancreatic cancer, which have a microenvironment dense in immune cells and MSCs.

A literature review indicated that TLR3 activation regulates migration, regeneration responses, immunomodulation capacity, differentiation, and inflammation-related responses in MSCs. Understanding the mechanisms by which TLR activation influences MSC characteristics and the responses of tumor cells is critical for optimizing their therapeutic use. This study investigated whether TLR3 stimulation affects viability and the expression of phenotype-associated genes in UCMSCs. It also examined the effect of these genes during coculture with *Panc-1* cells.

Materials and Methods

UCMSC Cell Culture and Immunophenotyping

UCMSCs were purchased from STEM BIO, İstanbul, Türkiye, and cultured in T-75 flasks at 37°C in a humidified atmosphere containing 5% CO₂. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (Capricorn Scientific GmbH,

Ebsdorfergrund, Germany) supplemented with 20% fetal bovine serum (Capricorn Scientific GmbH, Ebsdorfergrund, Germany), 1% L-glutamine (Sigma-Aldrich, Milan, Italy), and 1% penicillin + streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel). The culture medium was renewed every 2–3 days until the cells reached 70–80% confluence. These optimized culture conditions supported UCMSC proliferation while preserving their characteristics, useful for subsequent analysis. To evaluate the expression of the mesenchymal cell surface markers cluster of differentiation 73 (CD73), CD90, CD44, and CD105, 50,000 cells at passage 3 were used. An MSC Marker Validation Kit (Catalog # FMC020; R&D Systems, MN, USA) was used for phenotypic characterization.

The cells were incubated with 10 μ L of a cocktail containing 10 μ L each of anti-CD73, anti-CD90, anti-CD44, and anti-CD105 positive marker antibodies + 10 μ L of a cocktail containing 10 μ L each of anti-CD45, anti-CD34, anti-CD11b, anti-HLA-DR, and anti-CD79 negative marker antibodies + 10 μ L of an isotype control antibody. Staining was performed in the dark at incubated at 37 °C for 30–45 min. After incubation, the cells were washed with a staining buffer, and the pellet was resuspended in 100 μ L of the staining buffer for flow cytometry. Cell debris was excluded utilizing forward scatter and side scatter dot plots. Fluorescence data were analyzed using CellQuest Pro software (BD Biosciences, CA, USA), and the marker expression proportion was determined relative to the isotype-matched controls.

Method of Preparing TLR3 and TLR3 Solutions

The TLR3 agonist, polyadenylic-polyuridylic acid Poly(A:U) (Cat No: tlr1-pau) was obtained from InVivogen, CA, USA¹⁸. The TLR3 antagonist, CU-CPT4a (Cas No: 1279713-77-7), was obtained from Cayman Chemical Company Inc., MI, USA. The antagonist was dissolved in dimethyl sulfoxide (DMSO) (Serva, Germany) to achieve a 1 mg/mL stock solution. The agonist was dissolved in the physiological water provided along with it to obtain a 1 mg/mL stock solution. The agonist and antagonist stocks were diluted to 0.01, 0.1, 1, 10, and 100 μ g/mL, according to the manufacturer's protocol. For viability analysis, these concentrations were applied for 24 and 48 h. For gene expression analysis, the cells were seeded in 24-well plates at a density of 50,000 cells/well. They were treated with 0.1, 1, 5, and 10 μ g/mL of TLR3 or 0.01, 0.1, 0.5, and 1 μ g/mL of CU-CPT4a for 24 h. Each experimental group consisted of six wells, the contents of which were later pooled for analysis. For co-culture experiments, 1 μ g/mL of TLR3 and 0.5 μ g/mL of CU-CPT4a were added. After 24 h of polarization, the coculture experiments were started.

Cell Viability Analysis

To determine the role of TLR3 signaling in cell viability, UCMSCs were seeded in 96-well plates at 3,000 cells per well. TLR3 agonist and antagonist solutions were prepared in a new medium to obtain concentrations of 0.01, 0.1, 1, 10, and 100 μ g/mL; each concentration was added to a separate well. Each concentration group included three replicates. After 24 and 48 h of incubation, cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyl tetrazolium bromide (MTT) assay. To each well, 10 μ L of 0.5 mg/mL MTT solution (Biotium, CA, USA) and 90 μ L of DMEM were added. The plates were then dark-incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere to allow the formation of formazan crystals. Next, 100 μ L of DMSO was added to each well to dissolve the crystals. OD₅₇₀ was measured using a Synergy H1 microplate reader (BioTek Instruments, Inc., VT, USA). The OD of the untreated control group was considered to represent 100% viability, and those of the experimental groups were calculated relative to the control.

Panc-1 Cell Culture and Coculture Design

Panc-1 cells (ATCC:CRL-1469™) were purchased and cultured in high-glucose DMEM medium supplemented with 10% serum, 1% antibiotics, and 1% L-glutamine. The cells were seeded into the lower chamber of a 24-well plate at a density of 30,000 cells/well, in a 1:10 *Panc-1*:UCMSC ratio, and incubated at 37°C under 5% CO₂ for 24 h to allow cell adhesion. In parallel, UCMSCs were also seeded in transwell inserts (0.4 μ m pore size) at 300,000 cells/insert. The control UCMSCs were cultured in standard DMEM/F-12 medium, while the experimental UCMSCs were cultured in the same medium supplemented with either TLR3 agonist or antagonist. The inserts were then placed above the *Panc-1* cells containing wells to establish the coculture conditions; six wells/experimental group were used to ensure replicates. The UCMSCs were incubated for 24 h, washed with phosphate-buffered saline, and then placed on the *Panc-1* cells. After 72 h, the cells in the insert were collected, and DNA was isolated with Trizol, which was used for polymerase chain reaction (PCR).

Gene Expression Analysis

The expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *CD44*, cadherin 1 (*CDH1*), and vimentin (*VIM*) was evaluated in TLR3 agonist and antagonist-treated UCMSCs. The expression of *GAPDH*, *CD44*, *CDH1*, *CLDN1*, *VIM*, *ZEB1*, *MMP9*, matrix metalloproteinase-2 (*MMP2*), tissue inhibitor of metalloproteinase-1 (*TIMP1*), vascular endothelial growth factor receptor 2 (*VEGFR2*) and plasminogen activator urokinase (*PLAU*) was ascertained in UCMSCs co-cultured with *Panc-1* cells. For gene expression analysis, the Lightcycler® 96 system (Roche Diagnostics GmbH, Penzberg, Germany) and its compatible software were used. For the one-step PCR reactions, ABT™ 2X qPCR SYBR-Green Master Mix (ATLAS Biotechnology, Ankara, Türkiye) was used. Primers specific to *GAPDH*, *CD44*, *VIM*, and *CDH1* were employed for one set of analyses. Primers for a broader panel of genes, including *GAPDH*, *CD44*, *VIM*, *CDH1*, *CLDN1*, *ZEB1*, *MMP2*, *MMP9*, *PLAU*, *VEGFR2*, and *TIMP1* were used for the other set. The sequences of the primers and ENSEMBL transcript IDs are presented in Table 1. The reaction mix for each sample comprised 10 μ L of master mix, 45 ng/mL of RNA, 3 μ L of H₂O, 10 mM each of sense and antisense primers; 20 μ L of the mix was pipetted into each octet strip well. The octuplicate strips were centrifuged at 1000 $\times$ g for 30 s. The Lightcycler® 96 system was run employing the program recommended by the

Table 1. Primers used for qRT-PCR expression analysis.

Gene name	Forward sequences	Reverse sequences	ENSEMBL transcript IDs
<i>GAPDH</i>	5'-GTCTCCTCTGACTTCAACAGCG-3'	5'-ACCACCCTGTTGCTGTAGCCAA -3'	ENST00000229239.10
<i>MMP2</i>	5'- TTCATTTGGCGGACTGTGAC -3'	5'-GTGCTGGCTGAGTAGATCCA -3'	ENST00000219070.9
<i>PLAU</i>	5'-GCCACACACTGCTTCATTGA-3'	5'-TATACATCGAGGGCAGGCAG-3'	ENST00000372764.4
<i>VEGFR2</i>	5'- ATCTGTGACTTTGGCTTGGC -3'	5'-TCCCACAGCAAAACACCAAA-3'	ENST00000263923.4
<i>MMP9</i>	5'-GACGAGGGCCTGGAGTGT-3'	5'-TGTGCTGTAGGAAGCTCATCTC-3'	ENST00000372330.3
<i>TIMPI</i>	5'-ACCCCTGGAGCACGGCT-3'	5'-CCCACCTTCCAAGTTAGTGACA-3'	ENST00000218388.9
<i>CD44</i>	5'- CACACGAAGGAAAGCAGGAC -3'	5'- CCAGAGGTTGTGTTTGCTCC -3'	ENST00000263398.11
<i>CDH1</i>	5'-TTAGAGGTCAGCGTGTGTGA-3'	5'- CTTCTCCGCCCTCCTTCTTCA -3'	ENST00000261769.10
<i>VIM</i>	5'-CTGCCAACCGGAACAATGAC-3'	5'- TAGTTAGCAGCTTCAACGGC-3'	ENST00000544301
<i>ZEB1</i>	5'-AGGAGCCACAAAAGGACAGT- 3'	5'- TGGGGAATCAGAATCGTTTGC-3'	ENST00000318451.11
<i>CLDN1</i>	5'- TGCTTGGAAGACGATGAGGT- 3'	5'- GAGCCTGACCAAATTCGTACC-3'	ENST00000358432.9

manufacturer. The threshold cycle (C_t) value of *GAPDH* was used for data normalization, and the relative gene expression levels were calculated utilizing the $2^{-\Delta\Delta C_t}$ method. All samples were analyzed in triplicate.

Statistical Analysis

All data are expressed as the mean $\pm$ standard error of the mean (SEM). Graphs were created using GraphPad Prism version 8.1 (GraphPad Software, CA, USA). Statistical evaluations of cell viability were conducted via one-way analysis of variance using the same software. The untreated cells served as the control group in all experiments. The changes in *CDH1*, *VIM*, and *CD44* expression levels were analyzed utilizing two-tailed t-tests. The *Panc-1* and UCMSC coculture data were also examined using two-tailed t-tests by comparing with untreated groups. A $*p \leq 0.05$ was considered statistically significant. Additional levels were $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Results

Characterization of Umbilical Cord MSCs

The cultured cells were characterized morphologically for surface markers. Flow cytometry results of UCMSCs are shown in Figure 1a as staining-based histogram plots. Specifically, 43,155/50,000 stained cells were included, and the passage interval was 86.3%. Of the stained cells within the passage range, 99.7% expressed CD73, 99.9% CD90, 99.3% CD44, and 99.8% CD105, confirming their identity as MSCs. The cultured cells exhibited a characteristic fibroblast-like morphology (Figure 1b).

TLR3 Agonist and Antagonist Affect the Viability of UCMSCs in a Dose-dependent Manner

Treatment with the TLR3 agonist resulted in statistically significant variations in viability at all doses. In the 24-hour group, treatment with 0.01 μ g TLR3 agonist decreased cell viability by 15%, by 17% at 0.1 μ g, 7% at 1 μ g, but a 2% increase at 10 μ g, compared to the control. However, at 100 μ g, the cell viability declined by 17%.

At 48 h, the cell viability was reduced by 23% at 0.01 μ g, by 30% at 0.1 μ g, by 14% at 1 μ g, and by 25% at 100 μ g, demonstrating a dose-dependent cytotoxic effect (Figure 2a).

At 24 h treatment with 0.01 μ g of the TLR3 antagonist increased viability by 15% and by 20% at 0.1 μ g. However, the viability decreased by 24% at 1 μ g, 30% at 10 μ g, and by 44% at 100 μ g, re-affirming a dose-dependent response. At 48 h, the antagonist suppressed the viability by 16% at 0.01 μ g, by 35% at 0.1 μ g, by 30% at 1 μ g, by 25% at 10 μ g, and by the most pronounced effect of 80% reduction with 100 μ g (Figure 2b). Thus, the TLR3 agonist and antagonist affect the viability of UCMSCs in a dose- and time-dependent manner.

TLR3 Agonist and Antagonist Influence the Expression of Phenotypic Markers in UCMSCs

The treatment of UCMSCs with a TLR3 agonist markedly suppressed *CDH1* expression (RQ: 0.79) but significantly increased *VIM* expression (RQ: 0.04) (Figure 3a, c). With *CD44*, expression was enhanced up to a 5 μ g dose (RQ: 0.009), whereas it was reduced at 10 μ g (RQ: 0.001) (Figure 3e, f). Following TLR3 antagonist treatment, *CDH1* (RQ: 24.82) and *VIM* (RQ: 0.75) expression elevated significantly at 1 μ g (Figure 3b, d). However, the expression levels of both markers declined markedly at other concentrations. *CD44* expression was markedly downregulated at 0.1 (RQ: 0.01) and 10 μ g (RQ: 0.014) of antagonist compared to the control. Thus, it can be concluded that an enhancement in TLR3 signaling improves the maintenance of the mesenchymal phenotype, whereas its inhibition alters cell responses in a dose-dependent manner. Overall, these results highlight the critical role of TLR3 in modulating the UCMSC phenotype and underline its potential as a target for regulating stem cell behavior in therapeutic applications.

TLR3-Modulated the Influence of UCMSCs During Co-culture with *Panc-1* Cells

Panc-1 and UCMSCs were cocultured at a 1:10 ratio, and the gene expression profiles in *Panc-1* cells were analyzed following

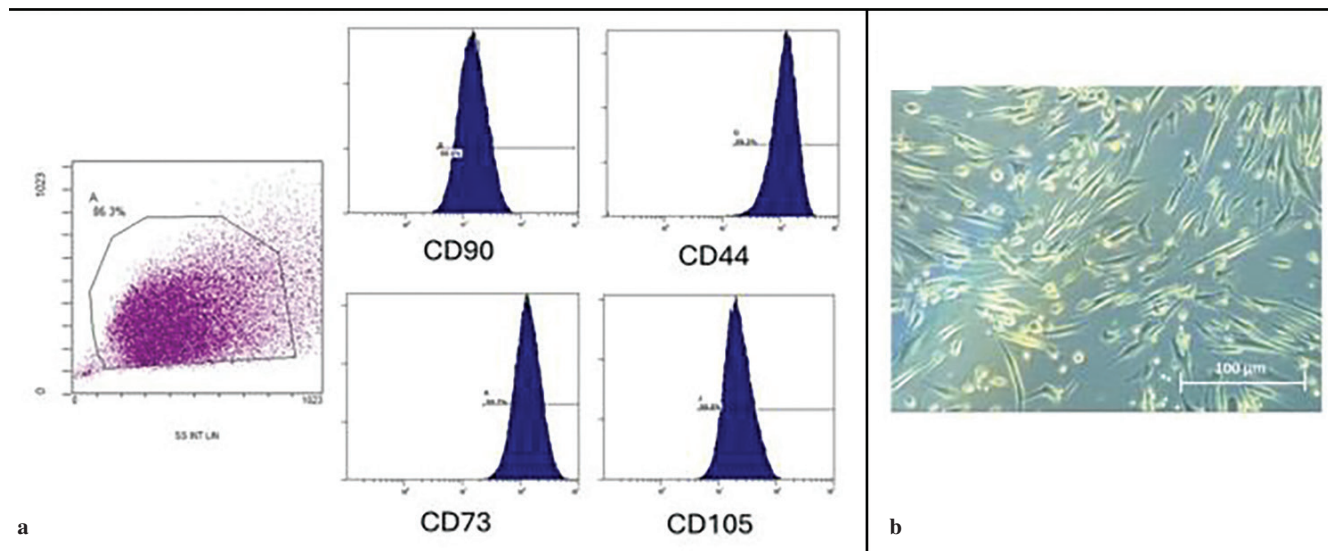


Figure 1. Morphological and immunophenotypic characterization of UCMSCs during culture. (a) Representative gate dot plot and flow cytometry histograms showing the expression levels of MSC surface markers CD90, CD73, CD44, and CD105; (b) fibroblast-like morphology of UCMSCs was observed under light microscopy. MSC = mesenchymal stem cells; UCMSCs = umbilical cord-derived MSCs.

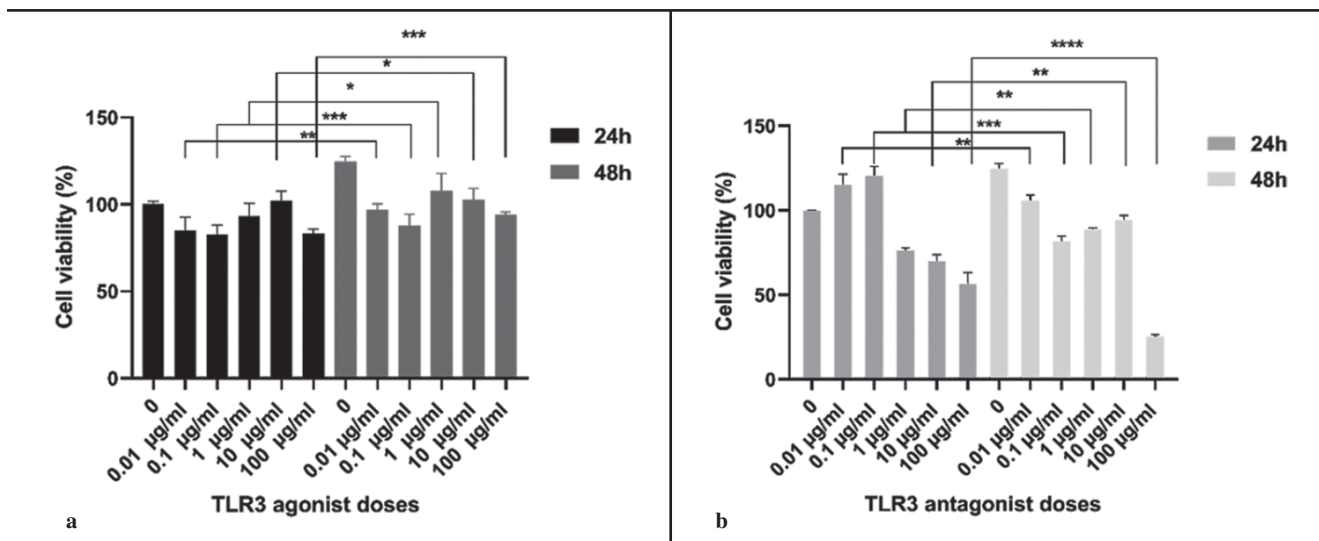


Figure 2. Viability changes in UCMSCs following 24 h and 48 h of treatment with different doses of a TLR3 agonist or antagonist. (a) Graph illustrating the viability changes of TLR3 agonists UCMSCs after 24 h of treatment; (b) graph illustrating the viability changes of TLR3 antagonists UCMSCs after 48 h of treatment. Data are displayed as the mean $\pm$ SEM. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ were considered statistically significant. MSC = mesenchymal stem cells; UCMSCs = umbilical cord-derived MSCs; TLR = Toll-like receptors; SEM = standard error of the mean.

the treatment of UCMSCs with the TLR3 agonist or antagonist. In such co-cultures, a significant increase in the expression of *CD44* (RQ: 6.46), *CDH1* (RQ: 2.86), and *ZEB1* (RQ: 0.03) was observed, while *CLDN1* expression (RQ: 4.42) was markedly decreased (Figure 4a–e). In contrast, the expression levels of *CD44* (RQ: 3.62), *VIM* (RQ: 0.004), and *MMP9* (RQ: 0.0001) were elevated, suggesting that activation and inhibition of TLR3 modulate the expression profiles of tumor-associated genes in

distinct ways (Figure 4a, d, f). During UCMSCs and *Panc-1* co-culture, TLR3 activation and inhibition affected the expression profiles of UCMSC phenotype- and function-relevant genes, including *CD44*, *CDH1*, *CLDN1*, *VIM*, *ZEB1*, *MMP9*, *MMP2*, *TIMP1*, *VEGFR2*, and *PLAU* (Figure 4g, h, i, j). These results underscore the dual regulatory role of TLR3 signaling in shaping pancreatic cancer cells through its impact on UCMSC-mediated gene expression dynamics.

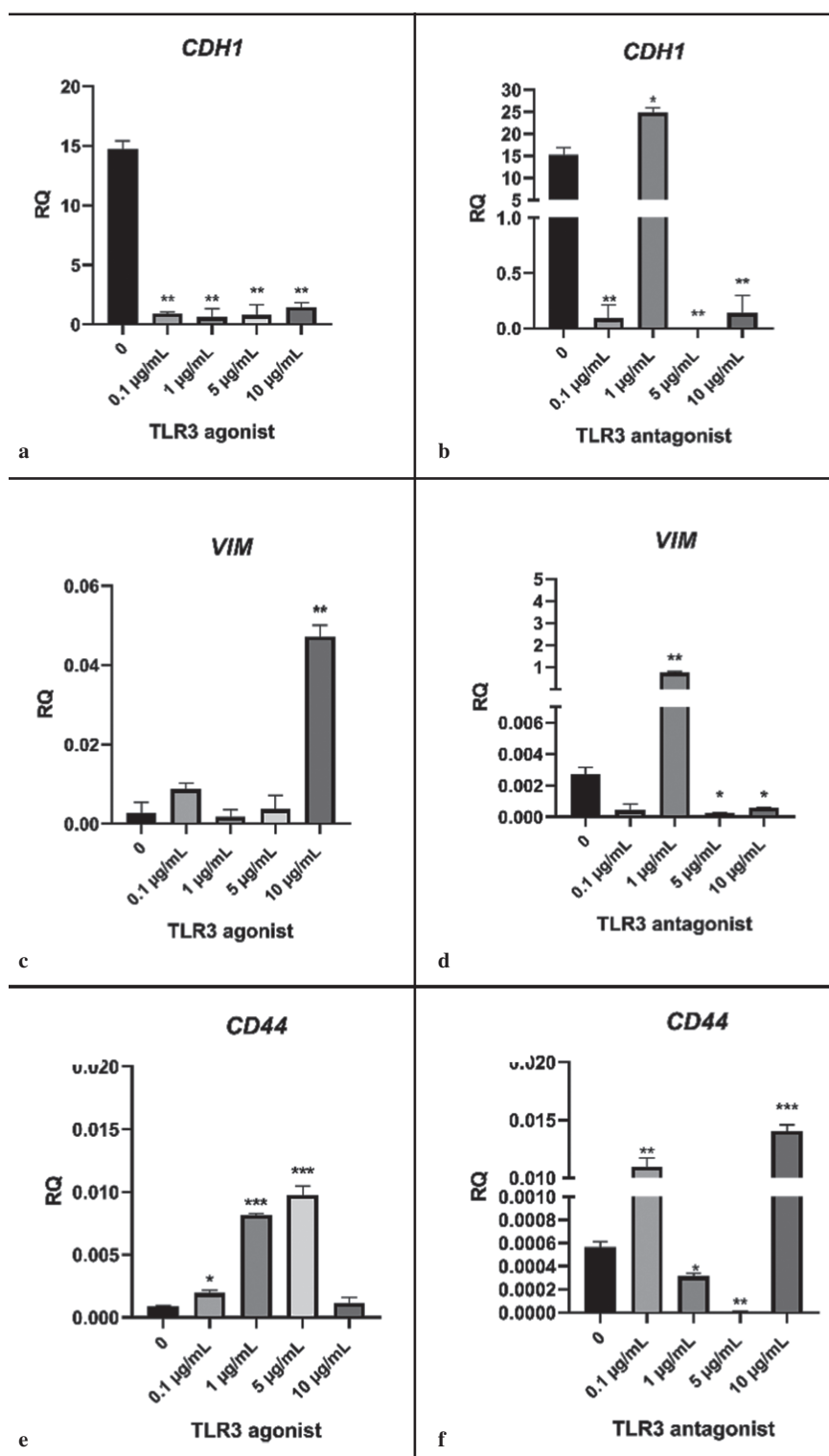


Figure 3. Changes in *CDH1*, *VIM*, and *CD44* expression in UCMSCs following TLR3 agonist and antagonist treatment. Expression changes following TLR3 agonist treatment at doses of 0.1–10 µg/mL (a, c, e); (b) expression changes following TLR3 agonist treatment at doses of 0.1–10 µg/mL (b, d, e). Data are presented as mean ± SEM, and RQ values were normalized using *GAPDH*. In statistical tests, the significance level was accepted as $p \leq 0.05$ and this was represented by an asterisk (*) sign. The significance levels are presented by ranking as follows: * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. RQ: Represents relative quantification. MSC = mesenchymal stem cells; UCMSCs = umbilical cord-derived MSCs; TLR = Toll-like receptors; SEM = standard error of the mean.

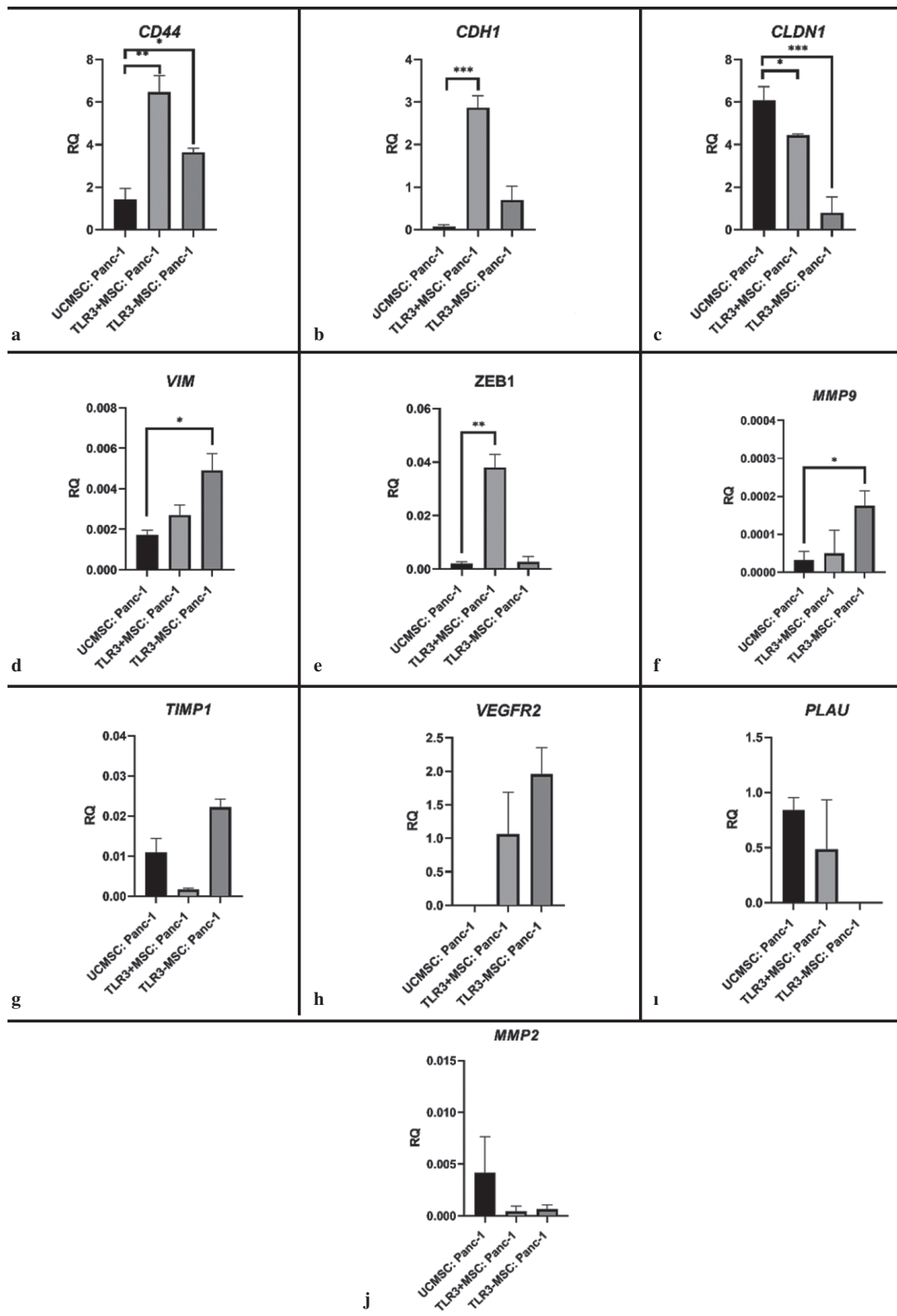


Figure 4. Gene expression changes in UCMSC and Panc-1 cocultures (1:10 Panc-1:UCMSC ratio) after 72 hours. Experimental groups included: Panc-1 + untreated UCMSC, Panc-1 + TLR3-activated UCMSC, and Panc-1 + TLR3-inhibited UCMSC. Genes analyzed: CD44, CDH1, CLDN1, VIM, ZEB1, MMP9, MMP2, TIMP1, VEGFR2, and PLAU (a-j). Data are presented as mean $\pm$ SEM, and RQ values were normalized using GAPDH. p -value ≤ 0.05 was considered statistically significant and represented by an asterisk (*). Levels of statistical significance were also described as follows: * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. MSC = mesenchymal stem cells; UCMSCs = umbilical cord-derived MSCs; TLR = Toll-like receptors; SEM = standard error of the mean.

Discussion

MSCs are essential for regeneration, tissue homeostasis, and anti-tumor responses. This study examined the effects of TLR3 activation/inhibition on the viability and phenotype-associated gene expression in UCMSCs, as well as their impact during coculture with *Panc-1* pancreatic cancer cells. UCMSC viability is modulated by the TLR3 agonist and antagonist in a dose- and time-dependent manner. Both regulated the expression of mesenchymal phenotype-related genes. During co-culture, TLR3 modulation influenced the expression profiles of various UCMSC phenotype- and function-associated genes. These findings suggest that TLR3 signaling not only governs intrinsic UCMSC functions but also dynamically alters their interactions with tumor cells, highlighting a bidirectional crosstalk between the stromal and cancer cell compartments.

Numerous studies have demonstrated that MSCs express various TLRs. Evidence from mesenchymal and hematopoietic stem cell research suggests that TLR activation may play a critical role in modulating stem cell functions, particularly by enhancing their migratory capacity. MSC phenotype is substantially impacted by their diverse functions, such as multilineage differentiation potential, hematopoietic supporting capacity, and immunomodulatory properties. Furthermore, TLR activation plays a vital role in the pathogenesis of various inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. The underlying reason is that sustained exposure to TLR ligands can either initiate or sustain chronic inflammation (Huang & Chen, 2016; Ishihara et al., 2006; Yamamoto-Furusho & Podolsky, 2007). Our data are consistent with such a view, as in this study, prolonged TLR3 activation promoted EMT-like changes in the UCMSCs, which may create a pro-inflammatory and pro-tumorigenic niche.

Understanding the impacts of TLR activation on MSC immunobiology is essential for optimizing their therapeutic potential. Among various MSCs, UCMSCs have gained considerable attention in regenerative medicine owing to a non-invasive isolation procedure, robust proliferative capacity, and pronounced immunomodulatory functions (El Omar et al., 2014). In comparison to MSCs obtained from adult tissues, UCMSCs exhibit a more primitive phenotype and enhanced differentiation potential, positioning them as a highly advantageous cell population for clinical applications. This study investigated the dose-dependent effects of a TLR3 agonist and antagonist on UCMSC viability, phenotype-associated gene expression, and tumor-related gene profiles during coculture with *Panc-1* pancreatic cancer cells. Our findings indicate that the modulation of TLR3 signaling not only affects MSC survival but also significantly alters the expression of EMT markers and TME-associated genes. These results align with those of a previous study, demonstrating that TLR3 activates NF- κ B in response to dsRNA, supporting its broader role in regulating cell fate and the expression of inflammation-related genes (Alexopoulou et al., 2001).

The results of the viability assays indicated a biphasic response to

the TLR3 agonist, where low doses reduced the viability, while intermediate doses had minimal or slightly proliferative effects. However, high concentrations, especially 100 μ g, consistently suppressed viability at 24 and 48 h of treatment. This dose-dependent cytotoxicity is in line with prior studies, which reported TLR3-mediated apoptosis in various cell types through the activation of downstream pathways such as TRIF- and caspase-based signaling (Salaun et al., 2006; Eskandari et al., 2023). In contrast, the TLR3 antagonist enhanced viability at lower doses but demonstrated cytotoxicity at higher concentrations, suggesting that delicately balanced TLR3 signaling may regulate stem cell survival (Eskandari et al., 2023).

In terms of gene expression, TLR3 activation markedly downregulated *CDH1* but upregulated *VIM* in UCMSCs. These alterations may reflect changes in cell plasticity or shifts in migratory, adhesive, or differentiation-related properties rather than a phenotypic transition. Additionally, *CD44* expression increased at lower doses of the agonist but declined at elevated concentrations, suggesting a dose-dependent effect on cell-surface-marker encoding genes. These findings imply that TLR3 activation modulates the mesenchymal features of UCMSCs, potentially enhancing their responsiveness to microenvironmental cues (Waterman et al., 2010). Conversely, TLR3 inhibition exhibited a variable effect: while low doses slightly increased *CDH1* and *VIM* expression, higher doses generally suppressed them, indicating an altered regulation of cell characteristics under TLR3-suppressed conditions (Eskandari et al., 2023; Zhao et al., 2015). Overall, TLR3 signaling appears to influence the phenotypic dynamics of UCMSCs in a dose-dependent manner, which could impact their behavior in therapeutic or pathological contexts.

MSCs have been extensively studied as potential vectors for targeted anti-cancer therapy, primarily due to their intrinsic tendency to migrate toward tumors (Fayyad-Kazan et al., 2016; Chulpanova et al., 2018; Hmadcha et al., 2020). The TME, often likened to a “non-healing wound,” mimics the inflamed and damaged states of injured tissues, thereby facilitating MSC recruitment and migration. This homing behavior is typically induced in response to stress-induced conditions, including mechanical trauma, inflammation, infection, or neoplastic processes (Huerta et al., 2023; Nwabo Kamdje et al., 2020; Monguió-Tortajada et al., 2021). While the precise molecular mechanisms governing MSC tumor tropism remain to be fully elucidated, it is widely believed that their transendothelial migration resembles that of leukocytes, encompassing steps such as rolling, adhesion, and transvascular migration. Our results build upon this concept by indicating that TLR3 modulation alters the expression of adhesion molecules (e.g., CD44 and CLDN1) and EMT-associated TFs (e.g., ZEB1), thereby potentially redefining MSC migratory behavior in tumor contexts.

This study has several limitations that should be acknowledged. First, the interpretations regarding potential changes in migration or differentiation capacities are based solely on gene expression data, but no functional assays were performed to directly assess and confirm these behaviors. Second, the absence of *in vivo*

validation limits the translational relevance of the findings, as the observed *in vitro* effects may not fully reflect UCMSC behavior within a particular physiological microenvironment. Third, donor-dependent variability in UCMSC populations could influence gene expression responses, potentially affecting the reproducibility and generalizability of the results. Lastly, although levels of key markers such as E-cadherin, vimentin, and CD44 were assessed, the evaluation of a more comprehensive panel of mesenchymal and epithelial markers would be necessary to more robustly characterize the phenotypic changes observed. Future studies incorporating functional, *in vivo*, and broader molecular analyses are needed to more definitively elucidate the impacts of TLR3 modulation on UCMSC biology.

Collectively, our findings highlight the dual and context-dependent roles of TLR3 signaling in MSCs, particularly in shaping their interactions with cancer cells. These results contribute to a growing body of evidence suggesting that TLR3 is not only an immune sensor but also a modulator of stem cell phenotype and TME dynamics. By integrating gene expression profiles and functional outcomes in a co-culture model, our study provides novel insights into the mechanisms by which TLR3 acts as a molecular switch—modulating UCMSC plasticity and tumor-modulatory capacity. Further studies are warranted to elucidate the downstream pathways involved and to determine whether the targeting of TLR3 signaling can offer therapeutic benefits in pancreatic cancer or other malignancies involving the stromal compartment.

Ethics

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: Data are available on reasonable request.

Footnotes

Authorship Contributions: Conceptualization: A.Y. and D.K.; Design/methodology: D.K.; Execution/investigation: A.Y.; Resources/materials: D.K.; Data acquisition: A.Y.; Data analysis/interpretation: A.Y. and D.K.; Writing – original draft: A.Y.; Writing – review & editing/critical revision: D.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

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