

## Effect of Toll-Like Receptor 3 Agonists on the Functionality and Metastatic Properties of Breast Cancer Cell Model

Nastaran Alizadeh <sup>1</sup>, Mohammad Mehdi Amiri <sup>2</sup>, Alireza Salek Moghadam <sup>1</sup>, Amir Hassan Zarnani <sup>1</sup>, Farshid Saadat <sup>3</sup>, Farnaz Safavifar <sup>4</sup>, Azar Berahmeh <sup>4</sup>, and Mohammad Reza Khorramizadeh <sup>4,5,6</sup>

<sup>1</sup> Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

<sup>2</sup> Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

<sup>3</sup> Department of Immunology, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran

<sup>4</sup> Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences, Tehran, Iran

<sup>5</sup> Biosensor Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

<sup>6</sup> Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

Received: 10 April 2012; Received in revised form: 23 July 2012; Accepted: 20 August 2012

### ABSTRACT

There exists compelling evidence that Toll-like receptor 3 (TLR3) agonists can directly affect human cancer cells. The aim of this study was to investigate anti-cancer effects of TLR3 agonist in human breast cell line.

We assessed potential effects of poly (A:U) on human breast cell line (MDA-MB-231) on a dose-response and time-course basis. Human breast cell line MDA-MB-231 was treated with different concentrations of poly (A:U) and lipopolysaccharide (LPS). Then, the following assays were performed on the treated cells: dose-response and time-course cytotoxicity using colorimetric method; matrix metalloproteinase-2 (MMP-2) activity using gelatin zymography method; apoptosis using annexin-v flowcytometry method; and relative expression of TLR3 and MMP-2 mRNA using reverse transcriptase polymerase chain reaction (RT-PCR) method. Following treatments, dose-response and time-course cytotoxicity using a colorimetric method, (MMP-2) activity (using gelatin zymography), apoptosis (using annexin-v flowcytometry method) assays and expression of TLR3 and MMP-2 genes (using PCR method) were performed.

Cytotoxicity and flowcytometry analysis of poly (A:U) showed that poly (A:U) do not have any cytotoxic and apoptotic effects in different concentrations used. MMP-2 activity analysis showed significant decrease in higher concentrations (50 and 100 µg/ ml) between treated and untreated cells. Moreover, poly A:U treated cells demonstrated decreased expression of MMP-2 gene in higher concentrations.

Collectively, our data indicated that human breast cancer cell line (MDA-MB-231) was highly responsive to poly (A:U). The antimetastatic effect of direct poly (A:U) and TLR3 interactions in MDA-MB-231 cells could provide new approaches in malignant tumor therapeutic strategy.

**Keyword:** MDA-MB-231; MMP-2; Poly (A:U); TLR3

**Corresponding Author:** Mohammad Reza Khorramizadeh; PhD; Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Research Institute, Tehran University of Medical

Sciences, P.O. Box 14114-13137, Tehran, Iran. Tel: (+98) 8822 0037-8; Fax: (+98 21) 8822 0052, E-mail: khoramza@sina.tums.ac.ir

## INTRODUCTION

As a part of pattern recognition receptor of human immune cells, toll like receptors (TLRs) are considered as a link between innate and adoptive immunity.<sup>1</sup> TLR family consists of a germ line encoded set of molecules thought to be crucially involved in the identification of pathogens and the triggering of an immune response versus microbial infections.<sup>2,3</sup> Ligation of TLRs by their relevant ligands triggers well-characterized signaling cascades that result in activation of downstream effectors, resistance against pathogens and occasionally cell death, which is another way of protecting the host against microbes spreading.<sup>4,6</sup> It is now obvious that TLR expression is not restricted to immune cells, but also shared with many different cell types, including endothelial cells, epithelial cells<sup>7,8</sup> and several tumors which display increased and altered TLR expression.<sup>9,10</sup> Induction of TLRs on tumor cells may directly promote proliferation, such as in human prostate, head and neck cancer. This time- and dose-dependent response is believed to occur by NF- $\kappa$ B induction and the subsequent up-regulation of oncoprotein c-Myc.<sup>11,12</sup> However, there are a number of reports suggesting a protective role of TLR stimulation and potential therapeutic options exploiting TLR ligation.<sup>13</sup> As TLRs comprising various members with their nearly defined ligands, certain TLR agonists appear to promote cell death in tumors. TLR3-mediated cell death involves the activation of caspases and engages both the extrinsic and intrinsic apoptotic pathways.<sup>14</sup> This finding suggests that TLR3 agonists may be suitable for cancer vaccines due to their cytostatic and cytotoxic effects on tumor cells. TLR3 agonists have been used in the past, with variable efficiency, as an adjuvant to treat cancer patients, with the aim of inducing an IFN-mediated anticancer immune response.<sup>15,16</sup> Synthetic analogue of double-stranded RNA (dsRNA) and a ligand of TLR3 has been shown as potent adjuvants in melanoma and human cervical cancer xenograft murine tumor models.<sup>17-19</sup> These agonist components induce TLR3 pathway that was shown to directly activate natural killer cells, to strongly enhance antigen-specific CD8+ T cell responses and to promote antigen presentation.<sup>20,21</sup> A reported result has highlighted the adjuvant role of analog of dsRNA in experimental models of cancer, most notably through the promotion of co-stimulatory molecules on Ag-presenting dendritic cells.<sup>22</sup> More

recent investigation has also indicated less undesirable and more specific effects of poly (A:U), as compared to polyinosinic-polycytidylic acid (poly (I:C)) in nasopharyngeal carcinoma. In this study, the authors postulated that polyadenylic-polyuridylic acid (poly (A:U)) might be better tolerated than poly (I:C) in human medical use, owing to the fact that poly (I:C) is not as strictly specific to TLR3 as poly (A:U) and it can also cross react with other RNA double-strand cellular receptors retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene-5 (MAD5).<sup>23</sup> Since TLR3 is also expressed on non-immune cells, the question of a putative expression and role of this receptor in metastatic cells needs to be investigated. In this study, we have assessed the effects of poly (A:U), a synthetic dsRNA, on metastatic aspects of breast cancer cell line and analyzed the expression of TLR3 and MMP-2.

## MATERIALS AND METHODS

### Cell line and Reagents

The intestinal epithelial cell line MDA-MB-231 was purchased from National Cell Bank of Tehran, Iran. Poly (A:U) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (Germany).

### Cell Culture

The cell line MDA-MB-231 was seeded at an initial density of 10,000 cells/well in 96-well tissue culture plates. Cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 100 u/ml penicillin, 100  $\mu$ g/ml streptomycin, with 5% CO<sub>2</sub>, at 37°C and saturated humidity.

### Dose-Response and Time-Course Treatments

Triplicate, poly (A:U) preparations at concentrations of 10, 50, 100  $\mu$ g/ml were transferred to overnight cultures of MDA-MB-231 cells. Non-treated cells were used as controls. Cell were cultured for 24 hours then subjected to colorimetric cytotoxicity assay. A sample of each media was also used for gelatinase zymography. We assessed effects of polymixin (Daru Pakhsh, Tehran, Iran) on poly A:U effects. The cells were treated with 100  $\mu$ g/ml polymixin for 1 h, then the cells were treated with various concentration of poly (A:U) (10, 50, 100  $\mu$ g/ml) incubated for 24 hours and then colorimetric cytotoxicity, gelatin zymography and RT-PCR were performed. The effect of LPS on MDA-MB-231 cell line was also evaluated at concentration of 100  $\mu$ g/ml.

### Colorimetric Cytotoxicity Assay

This method was carried out according to the published data [19]. Briefly, after each experiment, cells were washed three times with ice-cold phosphate buffered saline (PBS), pH 7.2, followed by fixation in a 5 % formaldehyde solution. Fixed cells were washed three times and stained with 1 % crystal violet. The stained cells were washed, lysed and solubilized with 33.33% acetic acid solution. The density of developed purple color was read at 580 nm using a conventional ELISA reader instrument.

### Gelatinase Zymography

This technique is mainly used for the detection of gelatinases, MMP-2 and MMP-9. The zymography procedure was adopted according to our previous publication.<sup>24</sup> Detection limit of this method was 10 pg for MMP-2. Briefly, protein-content adjusted (normalized) aliquots of conditioned media, in triplicate, were subjected to sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) containing 2 mg/ml gelatin. Electrophoresis was performed for 3 hours at a constant voltage of 80 volts. After electrophoresis, gels were gently washed three times with 2.5 % Triton X-100 solution to remove SDS. The gel slabs were then incubated at 37°C overnight in 0.1 M Tris HCL gelatinase activation buffer (pH 7.4) containing 10 mM CaCl<sub>2</sub>. Staining was performed with 0.5% coomassie brilliant blue followed by intensive destaining. MMP-2 proteolysis areas appeared as clear bands against a blue background in the gels. Using a UVI pro gel documentation system (GDS-8000, Cambridge, UK), quantitative evaluation of both surface and intensity of lysing bands, on the basis of grey levels, were compared relative to non-treated control wells and expressed as relative expression of gelatinolytic activity. To determine the poly A:U mode of action, a set of treatments and analyses were performed on cell-free media.

### Cell Apoptosis Assay

The cells were treated with various concentrations of poly A:U (10, 50, 100 µg/ml) and incubated for 24 hours and harvested cells were stained with FITC-conjugated annexin-v (IQ Products Germany) and propidium iodide. The cell death was assessed by flow cytometry. Apoptotic and total cells were counted by flowcytometry instrument (Partec PAS Germany). The results were expressed as percentage of apoptotic cells.

### RT-PCR

MDA-MB-231 cells were plated in six-well plates at 10<sup>6</sup> cells/well. Total RNA was isolated from tumor cells using TRIzol reagent according to manufacturer's guide (Invitrogen, Carlsbad, CA). Cellular RNA (1 µg) was reversibly transcribed into cDNA in a reaction mixture containing 5 mM MgCl<sub>2</sub>, 1 mM dNTP, 2.5 µM oligo dT primer, 1U RNase inhibitor, and 2.5U reverse transcriptase (Invitrogen). After incubation at 37°C for 50 min, the reaction was terminated by heating at 70°C for 15 min. PCR primers for detecting TLR3 and β-actin mRNA sequences were as follows: human TLR3, sense, 5'-AAC GAT TCC TTT GCT TGG CTT C-3', antisense 5'-GCT TAG ATC CAG AATGGTCAAG-3'; human MMP-2, sense, 5'-ACGAAGACCCACAGGAGGAG-3', antisense, 5'-TAGCCAGTCGGATTGATGC-3'. The PCR reaction buffer (25 µl), consisting of 2 mM MgCl<sub>2</sub>, 0.5 µM of each primer, and 1U Ampli Taq DNA polymerase, was added to the amplification tubes. PCR was run for 35 cycles. Each cycle consisted of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

### Statistical Analysis

All the data shown in the figures were representative of at least triplicate experiments. Data were expressed as mean±SD. The statistical differences in data obtained from cell proliferation, gelatinase activity and apoptosis were compared using ANOVA and Student's t-test. Student's t-test was used for between-group comparisons while analysis of variance was applied when more than 2 groups were compared. A P-value less than 0.05 was considered statistically significant.

## RESULTS

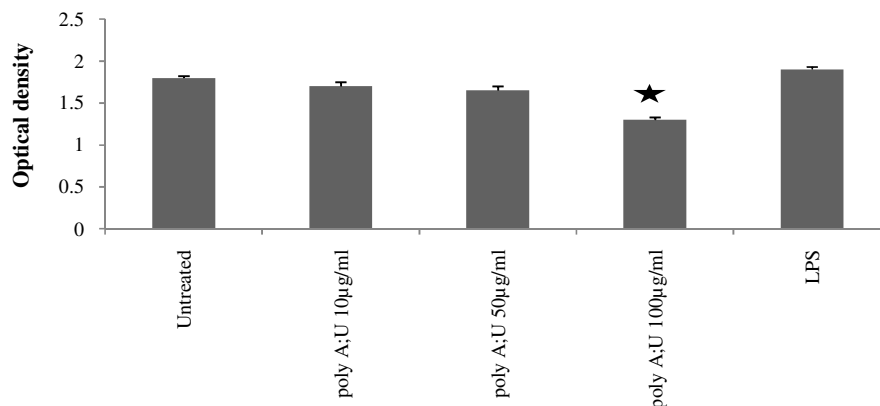
### Cytotoxic Analysis of Poly A:U and LPS

Figure 1a shows the cytotoxic effects poly (A:U) at various concentrations (10, 50, 100 µg/ml) for 24 hours on human breast cell line (MDA-MB-231). At the highest concentration (100 µg/ml), poly (A:U) had significant cytotoxic effects compared to control but lower concentrations showed no significant cytotoxicity compared to controls. Figure 1b shows inhibitory effect of polymixin on poly (A:U), verifying the specificity of poly (A:U) effects in cytotoxicity assay.

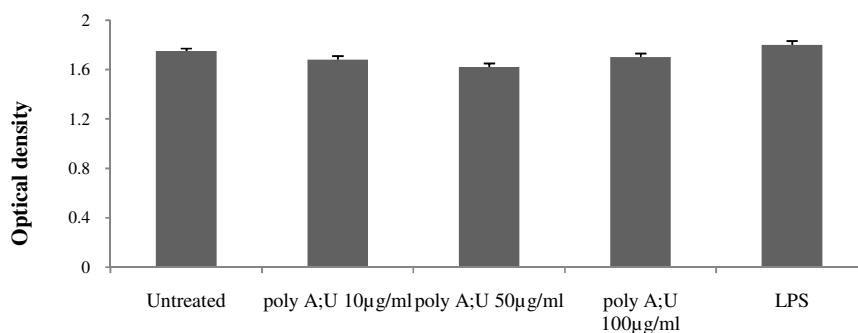
**Effects of Poly (A:U) and LPS on MMP-2 Activity**

Dose response analysis of poly (A:U) on MMP-2 activity of cell line MDA-MB-231 is presented in figure 2. Overall MMP-2 activity analysis showed

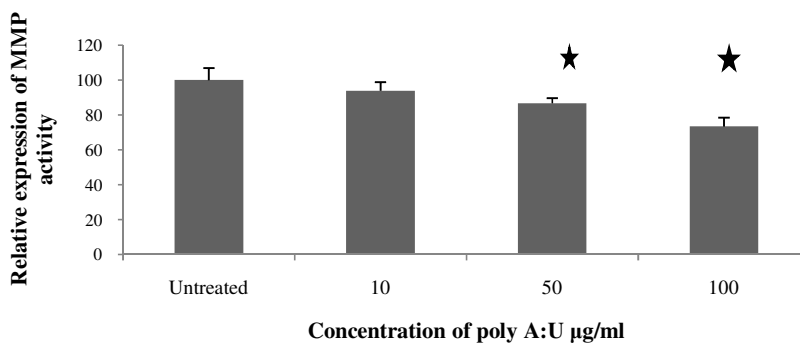
significant differences between different groups of treated and untreated cells. However, minimal changes were observed when MMP-2 activity was assessed per cell.



**Figure 1a.** Cytotoxic analysis of poly A:U. Proliferative response of human breast cell line (MDA-MB\_231) to poly A:U at different doses compared to LPS as described in Materials and Methods. Asterisk denote significant differences between Poly A:U 100µg/ml and untreated cells (\*P ≤ 0.05).



**Figure 1b.** Effects of poly A:U on human breast cell line (MDA-MB\_231) treated with 100 µg/ml polymixin for 1 hour. MDA-MB\_231 as a cell line showed high tolerability against increasing amounts of poly A:U. Each bar represents the mean ± S.D.



**Figure 2.** Inhibitory effect of poly A:U on MMP-2 activity. Human breast cell line (MDA-MB\_231) was incubated overnight with increasing doses of poly A:U as described in Materials and Methods. Analyses were performed using UVI Pro Gel Documentation system. Surface and intensity of lysis bands on the basis of grey levels were analysed. Asterisk denote significant differences between poly A:U 50 and 100µg/ml compared to untreated cells (\*P ≤ 0.05).

Assessments of cell-free media showed no difference in treated versus untreated supernatant, indicating that poly (A:U) affects directly on cells rather than on enzyme alone.

**Effect of Poly (A:U) on Apoptosis**

As depicted in figure 3, the rates of apoptosis for poly A:U at different concentrations with or without polymixin illustrated that programmed cell death was not significantly altered in poly A:U treated cells, as compared to untreated cells.

**Effect of Poly (A:U) on Expression of TLR-3 and MMP-2**

As shown in figure 4a, TLR3 was expressed on MDA-MB-231 cell line and by increasing poly (A:U) concentration (50 to 100 µg/ml), TLR3 expression increased. By increasing the poly A:U concentration, the expression of MMP-2 was decreased. This data parallel with zymography analysis indicated that decrease in MMP-2 activity is result in decrease in MMP-2 expression.

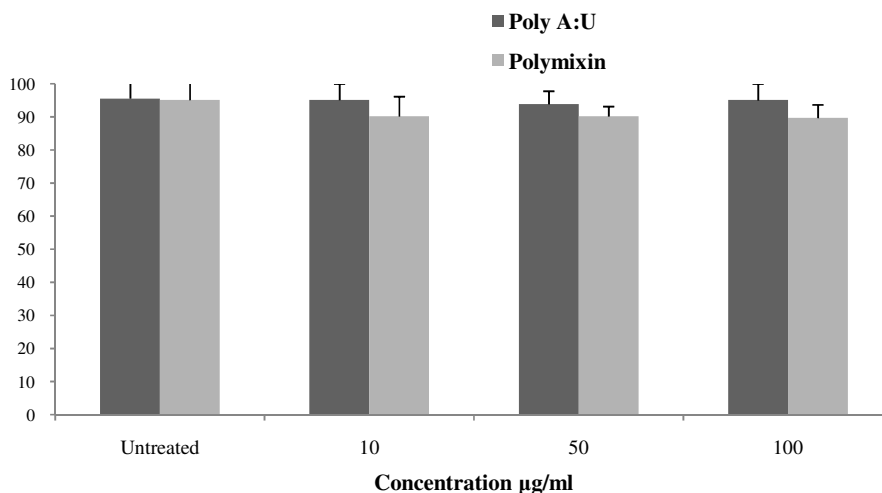


Figure 3. Apoptotic effect of poly A:U on human breast cell line (MDA-MB\_231) treated with 100 µg/ml polymixin for 1 hour. MDA-MB\_231 as a cell line showed high tolerability against increasing amounts of poly A:U. Each bar represents the mean ± S.D.

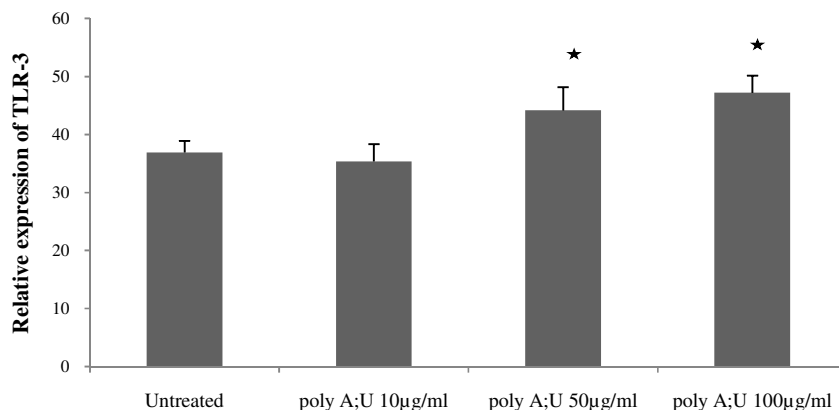
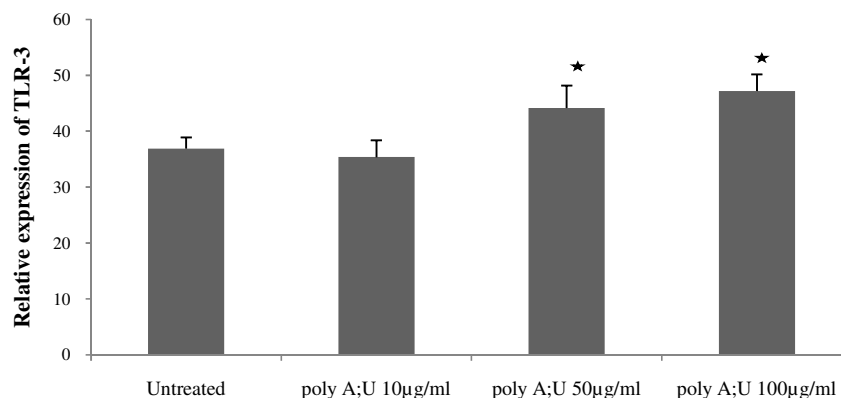


Figure 4a. Effect of poly A:U on expression of TLR-3. The relative amount of TLR-3 of human breast cell line (MDA-MB\_231) treated with poly A:U increased in a dose dependent manner. Asterisk denote significant differences between poly A:U at concentration of 50 µg/ml and 100 µg/ml versus untreated cells (\*P ≤ 0.05).



**Figure 4b.** Effect of poly A:U on expression of MMP-2. The relative amount of MMP-2 of MDA-MB\_231 treated with poly A:U decreased in a dose dependent manner. Asterisk indicates considerable differences between poly A:U at concentration of 50 µg/ml and 100 µg/ml versus untreated cells (\* $P \leq 0.05$ ).

## DISCUSSION

TLR3 was thought to be mainly expressed in immune cells, keratinocytes and some endothelial cells. Recently, it has been reported that certain human tumor cells also express this receptor.<sup>6,25</sup> Our work showed that TLR3 was widely expressed in human breast tumor cells lines, suggesting that TLR3 activation may play important functions in tumor biology. Ligation of TLR3 by its ligand dsRNA triggers well-characterized signaling cascades that result in activation of downstream effectors, such as NF- $\kappa$ B, p38, JNK, and IFN regulatory factors (IRFs) 4.<sup>2,26</sup>

MMP-2 activity was also assessed in this study. One of the critical steps for tumor invasion and metastasis is the destruction of extracellular matrix, which is catalyzed mainly by the MMPs.<sup>27</sup> According to our findings, poly (A:U) significantly decreased MMP-2 activity in this model. Although, some studies found that MMP-2 was over-expressed in tumor cells, our data showed that using an analog of dsRNA in cell culture media caused a significant decrease in MMP-2 expression. In addition, assessments of cell-free media showed no difference in treated versus untreated supernatants, indicating that poly (A:U) affected directly on cells rather than on enzymes alone. Thus; inhibition of MMPs could be beneficial in preventing tumor metastasis.

On the other hand, it has been recently speculated that the anti-apoptotic agents inhibit tumoral cell growth under *in vivo* and *in vitro* conditions. The effect of poly (A:U) on inducing apoptotic DNA fragmentation was examined in cultured cell line

MDA-MB-231. Data obtained from our assay showed no induction of cell apoptosis by poly (A:U) at various concentrations. This finding might imply why TLR-3 stimulation alone had no therapeutic influence.<sup>13</sup>

In conclusion, we showed that human breast cell line (MDA-MB-231) can express functional TLR3 protein and that its engagement by TLR3 agonists (poly (A:U)) can lead to decreased MMP-2 expression and activity. These data thereby showed that TLR3 agonists might represent very promising adjuvants for cancer vaccines not only based on their well-recognized immunostimulatory properties, but also due to decreasing metastatic effect of human breast tumor cells.

## ACKNOWLEDGEMENTS

Authors would like to appreciate Deputy of Research, Tehran University of Medical Sciences (Grants no. 8547 and 10141), and Neuroscience Research Centre, Shaheed Beheshti University of Medical Sciences for financial supports.

## REFERENCES

1. Kawai T, Akira S. TLR signaling. *Cell Death Differ* 2006; 13(5):816-25.
2. Huan Yang, Huiqin Zhou, Ping Feng, Xiaoni Zhou, Huiyan Wen, Xiaofang Xie, et al. Reduced expression of Toll-like receptor 4 inhibits human breast cancer cells proliferation and inflammatory cytokines secretion. *J Exp Clin Cancer Res* 2010; 29:92.

## Toll-Like Receptor 3 Agonists and Breast Cancer

- Beutler B, Hoebe K, Du X, Ulevitch RJ. How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J Leukoc Biol* 2003; 74(4):479-85.
- Matijevic T, Pavelic J. Toll-like receptors: cost or benefit for cancer? *Curr Pharm Des* 2010; 16(9):1081-90.
- Mikula I Jr, Pastoreková S, Mikula I Sr. Toll-like receptors in immune response to the viral infections. *Acta Virol* 2010; 54(4):231-45.
- Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. *J Immunol* 2006; 176(8):4894-901.
- Kaiser WJ, Kaufman JL, Offermann MK. IFN- $\alpha$  sensitizes human umbilical vein endothelial cells to apoptosis induced by double-stranded RNA. *J Immunol* 2004; 172(3):1699-710.
- Ritter M, Mennerich D, Weith A, Seither P. Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR 3 ligand poly (I: C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. *J Inflamm (Lond)* 2005; 2:16.
- Fukata M, Chen A, Vamadevan AS, Cohen J, Breglio K, Krishnareddy S, et al. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. *Gastroenterology* 2007; 133(6):1869-81.
- Swann JB, Vesely MD, Silva A, Sharkey J, Akira S, Schreiber RD, et al. Demonstration of inflammation-induced cancer and cancer immunoeediting during primary tumorigenesis. *Proc Natl Acad Sci U S A* 2008; 105(2):652-6.
- Kundu SD, Lee C, Billips BK, Habermacher GM, Zhang Q, Liu V, et al. The toll-like receptor pathway: a novel mechanism of infection-induced carcinogenesis of prostate epithelial cells. *Prostate* 2008; 68(2):223-9.
- Pries R, Hogrefe L, Xie L, Frenzel H, Brocks C, Ditz C, et al. Induction of c-Myc-dependent cell proliferation through toll-like receptor 3 in head and neck cancer. *Int J Mol Med* 2008; 21(2):209-15.
- Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P. Toll-like receptor 3 expressed by melanoma cells as a target for therapy? *Clin Cancer Res* 2007; 13:4565-74.
- Wolska A, Lech-Mara da E, Robak T. Toll-like receptors and their role in carcinogenesis and anti-tumor treatment. *Cell Mol Biol Lett* 2009; 14(2):248-72.
- Khan AL, Heys SD, Eremin O. Synthetic polyribonucleotides: current role and potential use in oncological practice. *Eur J Surg Oncol* 1995; 21(2):224-7.
- Lacour J, Lacour F, Spira A, Michelson M, Petit JY, Delage G, et al. Adjuvant treatment with polyadenylic-polyuridylic acid (Polya. Polyu) in operable breast cancer. *Lancet* 1980; 2(8187):161-4.
- Kim H, Yang E, Lee J, Kim SH, Shin JS, Park JY, et al. Double-stranded RNA mediates interferon regulatory factor 3 activation and interleukin-6 production by engaging Toll-like receptor 3 in human brain astrocytes. *Immunology* 2008; 124(4):480-8.
- Salem ML, Kadima AN, Cole DJ, Gillanders WE. Defining the antigen-specific T-cell response to vaccination and poly (I: C)/TLR3 signaling: evidence of enhanced primary and memory CD8 T-cell responses and antitumor immunity. *J Immunother* 2005; 28(3):220-8.
- Cui Z, Qiu F. Synthetic double-stranded RNA poly (I: C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model. *Cancer Immunol Immunother* 2006; 55(10):1267-79.
- Sivori S, Falco M, Della Chiesa M, Carlomagno S, Vitale M, Moretta L, et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci U S A* 2004; 101(27):10116-21.
- Schulz O, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 2005; 433(7028):887-92.
- Pulko V, Liu X, Krco CJ, Harris KJ, Frigola X, Kwon ED, et al. TLR3-Stimulated Dendritic Cells Up-regulate B7-H1 Expression and Influence the Magnitude of CD8 T Cell Responses to Tumor Vaccination. *J Immunol* 2009; 183(6):3634-41.
- Khorramizadeh MR, Aalizadeh N, Pezeshki M, Ghahary A, Zeraati H, Berahmeh A, et al. Determination of gelatinase A using a modified indirect hemagglutination assay in human prostate cancer screening and assessment of its correlation with prostate-specific antigen parameters. *Int J Urol* 2005; 12(7):637-43.
- Khvalevsky E, Rivkin L, Rachmilewitz J, Galun E, Giladi H. TLR3 signaling in a hepatoma cell line is skewed towards apoptosis. *J Cell Biochem* 2007; 100(5):1301-12.
- Jiang Q, Wei H, Tian Z. Poly I: C enhances cycloheximide-induced apoptosis of tumor cells through TLR 3 pathway. *BMC Cancer* 2008; 8:12.
- Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* 2006; 25(1):9-34.