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EFFECT OF ANTI-TSLPR MONOCLONAL ANTIBODY ON VIABILITY, PROAPOPTOTIC GENES EXPRESSION, AND PRODUCTION OF PRO-INFLAMMATORY CYTOKINES IN MCF-7 AND A549 CELLS

Background. Thymic stromal lymphopoietin (TSLP) and its receptor (TSLPR) are expressed in various cancer cells. However, their role in cancer development is not well defined. **Aim.** To investigate the effects of anti-TSLPR antibody on the viability, proapoptotic genes expression, and production of pro-inflammatory cytokines in MCF-7 and A549 cancer cells. **Materials and Methods.** MCF-7 and A549 cells were exposed to anti-TSLPR monoclonal antibody for 24, 48, and 72 h. The effect on cell viability was examined by MTT assay. The expression levels of *TP53*, *BAX*, and *CASP3* genes were evaluated by the quantitative reverse transcription polymerase chain reaction (qRT-PCR). Levels of interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and transforming growth factor (TGF-β1) were measured by the enzyme-linked immunosorbent assay (ELISA). **Results.** The treatment of MCF-7 cells with anti-TSLPR antibody slightly stimulates cell proliferation after 48 h and 72 h following initial cytotoxicity in 24 h with a significant reduction in IL-6 and TNF-α production. A significant increase in the *BAX* expression in anti-TSLPR treated cells at a concentration of 2.5 μg/ml at 24-h point was evident. In anti-TSLPR-treated A549 cells, no decrease in cell count was observed, and slight dose-dependent stimulation of cell proliferation was evident in 48 h and 72 h of culture. A significant increase in *TP53*, *BAX*, and *CASP3* expression upon treatment with 2.5 μg/ml of anti-TSLPR was evident in A549 cells. Conclusion. The effects of anti-TSLPR on cell viability, proapoptotic gene expression, and production of pro-inflammatory cytokines (IL-6 and TNF-α) vary in MCF-7 and A549 cells.

Keywords: TSLP, breast cancer, lung cancer, viability, inflammation, apoptosis.

Thymic stromal lymphopoietin (TSLP) is an interleukin (IL)-7–like cytokine. It is mainly expressed by epithelial cells and keratinocytes in response to viruses, bacteria, or allergens [1, 2]. Although the main function of TSLP is to regulate type 2 inflammatory responses, its dysregu-

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lation was found in several inflammatory diseases and malignancies [3, 4]. TSLP was found to be expressed in various cancer cell types, including breast [5] and lung [6] cancer. Moreover, it plays a crucial role in the induction and progression of solid tumors such as cervical [7], gastric cancer [8], and leukemia [9]. On the contrary, it was reported to have an inhibiting role in other types, such as colon [10], skin [3], and pancreatic cancer [11].

The functional TSLP receptor (TSLPR) is composed of the TSLP-R chain (also known as cytokine receptor-like factor 2 (CRLF2)) and IL-7Rα. It is expressed mainly on the surface of immune cells, including monocytes, B cells, and dendritic cells (DCs) [12]. It was also found on the surface of other cell types, such as colonic and airway epithelium. Its expression level was found to differ depending on the local inflammation [10, 13]. Besides normal cells, TSLPR is expressed on several types of cancer cells, including breast [14], lung [15], pancreatic [16], colon [10], cervical [7], gastric [8], skin cancer cells [3], and leukemia [9]. However, its effect on cancer development is not well defined yet.

Arguably data concerning the role of TSLP in breast cancer have been documented. Some studies suggested that TSLP had an expanding role in carcinogenesis [5, 14, 17]. Furthermore, a significant association was reported between single nucleotide polymorphisms (SNP) in TSLP and breast cancer progression in Saudi female patients [18]. A recent study by Shi et al. [19] assumed that reducing TSLP levels using arctigenin (a bioactive compound from *Arctiumlappa L.*) could slow down breast cancer progression. On the other hand, a suppressing role of TSLP in breast cancer was demonstrated in [11]. Other studies reported that TSLP does not affect breast cancer development [20, 21]. Concerning lung cancer, only a few researchers looking into the role of TSLP in lung cancer found it to be stimulating [6, 15, 22, 23]. To evaluate the role of TSLP in cancer cells, this study was designed to investigate the effect of anti-TSLPR antibody treatment on the proliferation, apoptosis, and inflammation in breast and lung cancer cell lines.

Materials and Methods

Cell culture. MCF-7 and A549 cells were obtained from the American Type Culture Collection (ATCC). They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (200 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (50 μg/ml), and HEPES buffer (all from Biowest, France). Cells were maintained in the logarithmic growth phase at 37 °C in humidified atmosphere containing 5% CO_2 by routine sub-culturing every two days in T75 and T25 tissue culture flasks. When reaching 95% confluency, monolayer cells were rinsed with normal saline and harvested by trypsin/EDTA (Biowest, France) treatment.

Anti-TSLPR antibody treatment of MCF-7 and A549 cells. About 2.5×10^5 cells/ml were cultured in flat-bottom 96 well-microtiter plates (200 μl/well) in complete RPMI-1640 media at 37 °C to allow cells to settle down. On the following day, monolayer cells were treated with monoclonal anti-TSLPR antibody (produced by the Monoclonal Antibody Core Facility, University of Texas, M.D. Anderson Cancer Centre, USA) at different concentrations (5, 2.5 and 1.25 μg/ml) for 24, 48, and 72 h. Cells supplemented with complete culture media served as untreated negative controls. Nine wells were used for each concentration.

Cytotoxicity assay. To assess the potential effect of anti-TSLPR antibody treatment on cancer growth, cell viability was measured using the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described by Talaat et al. [24]. At the end of the incubation period, the medium was removed, and 40 μl/well MTT was added. Purple formazan crystals were solubilized by dimethylsulfoxide

(DMSO) (160 µl/well). The absorbance was measured at 570 nm (SunriseTM, Tecan Group Ltd., Switzerland). The mean absorbance of treated cells/mean absorbance of negative control cells x100 was used to compute the viability percentage. Basing on the results of the MTT assay, we performed the following experiments using the optimal antibody concentrations and time intervals.

Apoptotic gene expression level. In flat bottom 6 well-microtiter plates (Greiner, USA), cells were treated with anti-TSLPR antibody using two concentrations (2.5 and 1.25 µg/ml). Triplicate wells were used for each concentration and two plates were used, either being examined in different time interval (24 h and 48 h). Cells with the culture media were used as the negative control. According to the manufacturer's instructions, the total RNA was isolated from treated and untreated MCF-7 and A549 cells using the TRIzol reagent kit (Life Technologies Ltd., UK). NanoDrop 2000/2000c (Thermo Fisher Scientific, USA) was used to determine the purity and concentration of RNA, and 1% agarose gel electrophoresis was used to ensure RNA integrity. One of the RNA samples was used for quantitative reverse transcription polymerase chain reaction (qRT-PCR). RNA was reverse-transcribed into cDNA using the TOPscript™ cDNA synthesis kit (Enzynomics, Life Technologies, India) according to the manufacturer's instructions. The detection of *TP53, BAX,* and *CASP3* gene expression was carried out in 20 µL PCR reactions using TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics, Life Technologies, India) PCR Kit according to the manufacturer's protocol. The primer sequences are listed in Table 1. The PCR cycles were as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s 60 °C for 34 s and 72 °C for 30 s. β-actin served as the endogenous control. The $2^{-\Delta\Delta Ct}$ method was used for analyzing the expression level [25]. All experiments were made on the AriaMx Real-Time PCR System (Agilent Technologies, USA). The relative fold change (RFC) was used to express the data.

Assessment of inflammatory cytokine levels. MCF-7 and A549 cells suspended in complete RPMI-1640 were dispensed in triplicates in 96-well flat-bottom cell culture plates (Greiner, USA) and treated with different concentrations of anti-TSLPR antibody (2.5 and 1.25 µg/ml) at 24 h and 48 h. Supernatants were collected and stored at −80 °C to measure inflammatory mediators. Duoset enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc., USA) was used for the detection of IL-6 tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β1) levels in the supernatant of treated and control MCF-7 and A549 cells as previously described by Talaat et al. [26]. The intensity of the developed color was measured by reading optical absorbance at 450 nm using a microplate reader (Sunrise; Tecan Group Ltd,

Table 1. **PCR primer sequences**

Gene	Primers	References
TP53	F: 5'- TCAGATCCTAGCGTCGAGCCC -3' R: 5'- GGGTGTGGAATCAACCCACAG -3'	[34]
BAX	F: 5'- CCCAGTTGAAGTTGCCGTCA -3' R: 5'-ATGGACGGGTCCGGGGAGCA -3'	[35]
CASP3	F:5'-GCAGCAAACCTCAGGGAAAC-3' R: 5'-TGTCGGCATACTGTTTCAGCA-3'	[36]
β-actin	F:5'-GAGACCTTCAACAACCCAGCC-3' R: 5'-GGATCTTCATGAGGTAGTCAG-3'	[24]

Switzerland). The ELISA reader-controlling software (Softmax; Molecular Devices, USA) processed the digital data of the raw absorbance values into a standard curve, from which the cytokine concentrations were derived. The results were expressed as picograms of cytokine per millilitre of supernatant (pg/ml).

Statistical analysis. All statistical analyses were performed using SPSS (version 19) (SPSS, Inc., USA). The data are presented as means with corresponding SE. The comparisons between different studied groups were performed using Student's t-test or one-way analysis of variance (ANOVA). *P*-values less than 0.05 were considered statistically significant.

Results

Effect of anti-TSLPR antibody treatment on viability of MCF-7 and A549 cells. The treatment of MCF-7 cells with the anti-TSLPR antibody slightly stimulates cell proliferation in a dose- and time-dependent mode following initial cytotoxicity as shown in Fig. 1. Especially noticeable is the decrease in the count of viable cells treated with the anti-TSLPR antibody at 24-h time point, especially in cells treated with an antibody dose of 1.25 μg/ml (57.16% viable cells).

As shown in Fig. 2, in anti-TSLPR-treated A549 cells, no decrease in cell count was observed. Slight dose-dependent stimulation of cell proliferation was evident after 48 h and 72 h of culture.

Effect of anti-TSLPR antibody treatment on apoptotic gene expression level. The effect of MCF-7 cell treatment with the anti-TSLPR antibody on the expression of pro-apoptotic genes (*TP53, BAX,* and *CASP3*) is presented in Table 2. The anti-TSLPR antibody treatment did not significantly affect *CASP3* in response to both doses (1.25 and 2.5 μg/ml) after 24 h and 48 h. In contrast, a significant increase in the *BAX* expression in anti-TSLPR treated cells at a dose of 2.5 μg/ml at 24-h point was evident. Only slight changes in *TP53* expression were detected at 48-h point in cells treated with anti-TSLPR at a dose of 1.25 μg/ml.

As shown in Table 3, similar changes in the *BAX* expression were observed in anti-TSLPR treated A549 cells. Nevertheless, in this cell line, the expression of *TP53* and *CASP3* was affected significantly as well. Meanwhile, by comparing the two time intervals, no significant change was noted in the expression level of all tested apoptotic genes in cells treated with 2.5 μg/ml anti-TSLPR.

Effect of anti-TSLPR antibody treatment on cytokine levels. As illustrated in Fig. 3, reduction in the IL-6 and TNF-α levels was notified in MCF-7 cells in both anti-TSLPR concentrations at 24 h. On the contrary, a gradual increase in the TNF-α level was observed at 48 h post-treatment with maximum significant elevation 1.25 μg/ml compared to the control ($p < 0.01$) and 2.5 μg/ml dose ($p < 0.05$). No change in TGF-β1 level was demonstrated after treating MCF-7 cells with both concentrations of the antibody at both time intervals. Compared to the control, the only remarkable reduction ($p < 0.001$) in TGF-β1 was detected after 48 h with 1.25 μg/ml anti-TSLPR. Consequently, at both time intervals, the levels of pro- and anti-inflammatory cytokines were consistent with cell viability data.

As shown in Fig. 4, A549 cells treated with 1.25 μg/ml of anti-TSLPR showed a significant elevation ($p < 0.01$) in the IL-6 levels. The TGF-β1 level was changed insignificantly. Concerning TNF-α its level was significantly ($p < 0.001$) decreased after 24 h then significantly elevated ($p < 0.001$) after 48 h compared to the control.

Discussion

Several studies have discussed the role of TSLP in tumor development and progression seeking to identify its role as a potential prognostic marker or therapeutic target [7, 10, 16]. However, the mechanisms by which TSLP is expressed in lung and breast cancer cells and their association with oncogenic events remain to

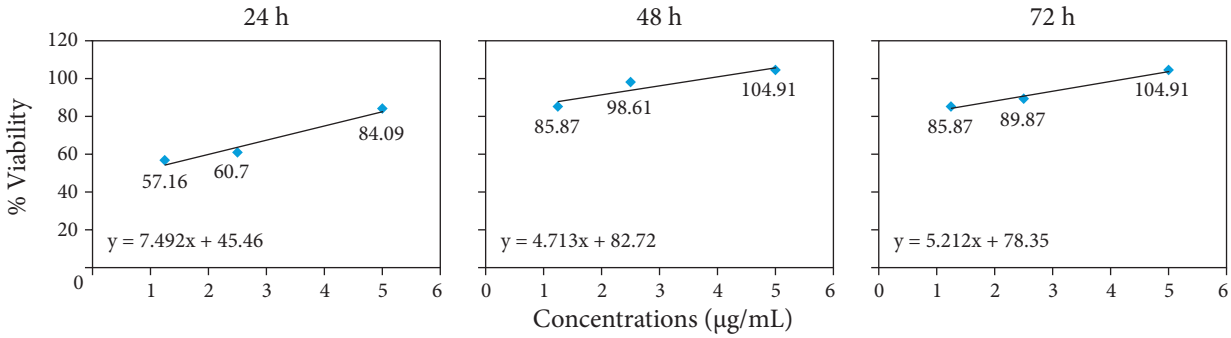

Fig. 1. Viability of MCF-7 cells treated with anti-TSLPR antibodies after 24, 48, and 72 h

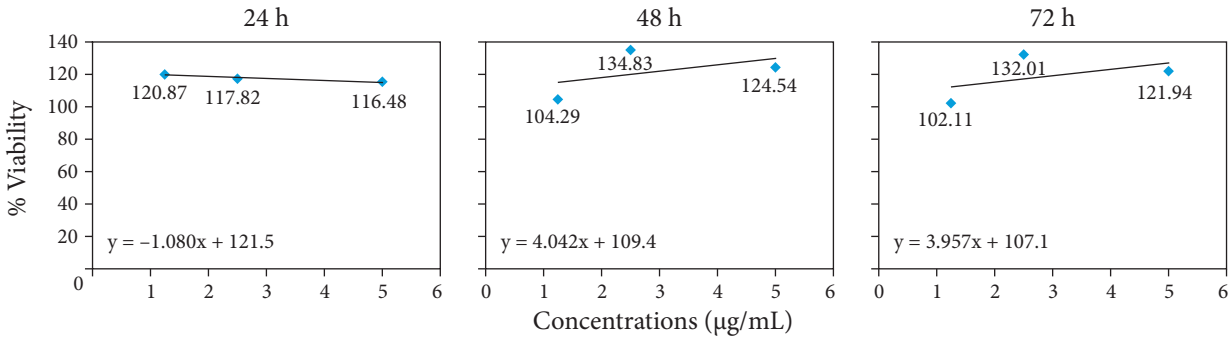

Fig. 2. Viability of A549 cells treated with anti-TSLPR antibodies after 24, 48, and 72 h

Table 2. **Relative fold change (RFC) of *TP53*, *BAX*, and *CASP3* expression in MCF-7 cells after 24 and 48 h of treatment with anti-TSLP-R antibodies**

Genes	Time	2.5 μg/ml	1.25 μg/ml	*p*
TP53	24 h	4.91 ± 1.33	4.92 ± 0.11	NS
	48 h	4.09 ± 0.59	9.34 ± 1.99	< 0.05
BAX	24 h	277.80 ± 43.62	2.88 ± 1.29	< 0.01
	48 h	78.57 ± 6.88	3.73 ± 1.02	< 0.001
CASP3	24 h	2.83 ± 0.48	1.89 ± 1.04	NS
	48 h	2.41 ± 0.98	4.18 ± 1.86	NS

Table 3. **Relative fold change (RFC) of *TP53*, *BAX*, and *CASP3* expression in A549 cells after 24 and 48 h of treatment with anti-TSLP-R antibodies**

Genes	Time	2.5 μg/ml	1.25 μg/ml	*p*
TP53	24 h	139.16 ± 61.78	0.78 ± 0.06	< 0.05
	48 h	60.41 ± 4.44	93.31 ± 2.51	< 0.001
BAX	24 h	399.72 ± 213.15	19.93 ± 4.14	NS
	48 h	405.07 ± 37.84	113.48 ± 20.32	< 0.001
CASP3	24 h	150.18 ± 26.47	4.77 ± 0.88	< 0.01
	48 h	115.85 ± 47.29	182.29 ± 74.42	< 0.001

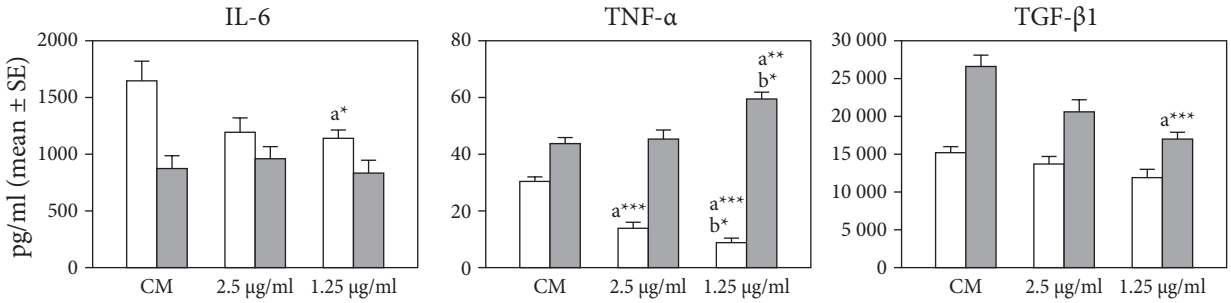

Fig. 3. Levels of cytokines (IL-6, TGF-β1, and TNF-α) produced by MCF-7 cells treated with anti-TSLPR antibodies after 24 and 48 h: (a) Significant difference compared to CM, (b) Significant difference compared to concentration 2.5 μg/ml. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, □ 24 h, ■ 48 h. Results are expressed as mean ± SE

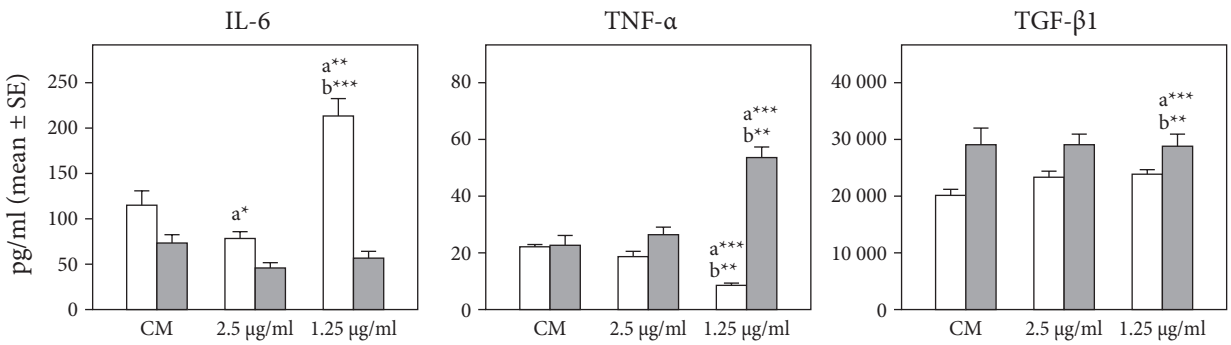

Fig. 4. Levels of cytokines (IL-6, TGF-β1, and TNF-α) produced by A549 cells treated with anti-TSLPR antibody after 24 and 48 h: (a) Significant difference compared to CM, (b) Significant difference compared to concentration 2.5 μg/ml. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, □ 24 h, ■ 48 h. Results are expressed as mean ± SE

be established. This cytokine was found to have a contradictory effect on several cancer cell types [9, 10]. Consequently, in this study, we aimed to investigate this argument by using anti-TSLPR monoclonal antibodies and elucidate the impact of this antibody-receptor interaction on MCF-7 and A549 cells.

The anti-TSLPR treatment of MCF-7 cells slightly stimulate their proliferation after 48 h and 72 h following the initial decrease in the count of the viable cells after 24 h as compared to the control culture. This effect was accompanied by the increase in *BAX* expression at 24-h point in cells treated with 2.5 μg/ml of anti-TSLPR antibody.

Our data are consistent with the aforementioned study on mice, which verified that TSLP could block early stages of breast cancer development via enhancing $CD4^+$ Th2 cells, which play a role in the anti-tumor immune response [11]. Moreover, another study by Yu et al. [27] reported that TSLP expression in early-stage breast cancer is associated with the efficacy of adjuvant chemotherapy and prognosis. Previously, Li et al. [15] have demonstrated the decreased levels of TNF-α and increased levels of TGF-β1 secreted from human dendritic cells in response to TSLP, which was supposed to enhance human lung cancer progression.

We notified that treating MCF-7 cells with a lower dose of anti-TSLPR antibody (1.25 μg/ml) after 24 h of incubation exerts slight cytotoxicity with accompanying reduction in IL-6 and TNF-α production. Surprisingly, no significant change was reported in the apoptotic genes suggesting that the reported cell death

might be caspase-independent. These findings agree with a previous study demonstrating that breast cancer cells receive the endogenous TSLP signal to enhance their growth [11]. Oakland et al. [17] suggested a supporting role for TSLP in tumor growth via direct signaling by $CD4^+$ T cells and disabling TSLP signaling inhibited cancer progression and metastasis. In agreement, Kuan and Ziegler [14] reported that TSLP produced by tumor-infiltrating myeloid cells is a critical survival factor for breast tumor cells. In addition, IL-13–TSLP pathway showed an ability to induce tumor growth by promoting Th2 cells in the tumor microenvironment [5].

Hartgring et al. [28] reported decreased concentrations of IL-6 in TSLPR-deficient mice. Moreover, a significant increase in IL-6 expression was demonstrated in HeLa and CaSki via recombinant human TSLP [2], in human mast cells (HMC-1) treated with TSLP [29], and after TSLPR activation on human airway smooth muscle cells [30]. In agreement, Ito et al. [31] reported that human TSLP can activate dendritic cells, which induces the naive $CD4^+$ T cells to differentiate into Th2 cells producing high amounts of TNF-α. Another study indicated that human TSLP can increase levels of TNF-α in HMC-1 cells [29].

In A549 lung cancer cells, the anti-TSLPR antibody causes slight dose-dependent stimulation of cell proliferation without cell toxicity with a significant elevation in the IL-6 levels and decrease in TNF-α after 24 h. In agreement with Li et al. [15], the significant elevation in IL-6 was found to be associated with lung cancer progression. On the contrary, decreased levels of TNF-α were found, which is consistent with previous studies by Ito et al. [29] and Yoou et al. [31]. The diminished IL-6 and TGF-β1 levels are consistent with previously reported studies [2, 15, 28—29]. Moreover, the expression levels of pro-apoptotic *TP53, BAX,* and *CASP3* genes increased in A549 cells treated with anti-TSLPR antibody.

A recent study reported that high levels of TSLP promoted the progression of advanced human lung adenocarcinoma [22]. Another study by Lau et al. [23] revealed that the increased TSLP levels might cause a primary resistance to the anti-programmed death factor (PD-1) inhibitors in human metastatic small-cell lung cancer. Moreover, Burkard-Mandel et al. [32] hypothesized that TSLP enhances lung metastasis by stimulating tissue-resident alveolar macrophage pro-tumorigenic population in mice. DCs stimulated by human TSLP could induce regulatory T cells (Tregs), playing an important role in suppressing anti-tumor immune responses, which may be the leading cause of lung tumor progression [15]. Despite the results of previously reported data, none were obtained on the A549 lung cancer cell line. To the best of our knowledge, this is the first study to demonstrate a stimulating role of anti-TSLPR antibody on A549 lung cancer cells growth.

To sum up, the effects of anti-TSLPR on cell viability, proapoptotic genes expression, and production of pro-inflammatory cytokines (IL-6 and TNF-α) vary in cancer cells of different genesis.

Conflict of interest

Authors declared no conflict of interest

Acknowledgments

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ВПЛИВ МОНОКЛОНАЛЬНИХ АНТИТІЛ ДО TSLPR НА ЖИТТЄЗДАТНІСТЬ КЛІТИН MCF-7 ТА A549, ЕКСПРЕСІЮ В НИХ ПРОАПОПТОТИЧНИХ ГЕНІВ ТА ПРОДУКЦІЮ ПРОЗАПАЛЬНИХ ЦИТОКІНІВ

Стан питання. Стромальний лімфопоетин тимуса (TSLP) і його рецептор (TSLPR) експресуються в різних злоякісно трансформованих клітинах, але їх роль у розвитку раку залишається повністю не з'ясованою. **Мета.** Оцінити вплив антитіл до TSLPR на життєздатність, експресію проапоптичних генів і продукцію прозапальних цитокінів у злоякісно трансформованих клітинах ліній MCF-7 і A549. **Матеріали та методи.** Клітини MCF-7 і A549 культивували в присутності моноклональних антитіл до TSLPR впродовж 24, 48 і 72 годин. Вплив на життєздатність клітин досліджували методом MTT. Показники експресії генів *TP53, BAX* і *CASP3* оцінювали методом кількісної зворотно-транскрипційної полімеразної ланцюгової реакції (qRT-PCR). Рівні інтерлейкіну-6 (IL-6), фактора некрозу пухлин-альфа (TNF-α) і трансформуючого фактора росту (TGF-β1) вимірювали за допомогою імуноферментного аналізу (ELISA). **Результати.** Дія антитіл до TSLPR на клітини MCF-7 незначно стимулює клітинну проліферацію через 48 год і 72 год після початкової цитотоксичності через 24 год на фоні значного зниження продукції IL-6 і TNF-α. У клітинах лінії MCF-7 через 24 год під дією антитіл до TSLPR у концентрації 2,5 мкг/мл, встановлено зростання експресії *BAX*. Встановлено, що культивування клітин лінії A549 у присутності антитіл до TSLPR, приводило до незначної дозозалежної стимуляції проліферації клітин через 48 і 72 години. Виявлено значне зростання рівнів експресії *TP53, BAX* і *CASP3* під дією антитіл до TSLPR у концентрації 2,5 мкг/мл у клітинах лінії A549. **Висновок.** Дія антитіл до TSLPR на життєздатність клітин, експресію проапоптичних генів і продукцію прозапальних цитокінів (IL-6 і TNF-α) характеризується різноспрямованими ефектами у клітинах ліній MCF-7 і A549.

Ключові слова: TSLP, рак молочної залози, рак легені, життєздатність, запалення, апоптоз.