

Co-Expression of Membrane-Bound Tumor Necrosis Factor-Alpha Receptor Types 1 and 2 by Tumor Cell Lines

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Keywords

TNFα · Cellular immunology · Cytokine receptors
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Abstract

Introduction: Density and co-expression of tumor necrosis factor (TNF) receptors may vary among cell populations. However, the role and potential of these changes remain unclear. This study aimed to determine the density of expression and co-expression of TNFR1/2 and the dose-dependent effect of soluble TNF on these parameters. **Methods:** Epithelial-like (HEp-2, K-562, MCF-7, ZR-75/1) and lymphoblast-like (MOLT-4, HL-60, Raji, RPMI-8226, IM-9) cell lines were characterized for co-expression of TNFR1/2 using a modified flow cytometry protocol. The dose-dependent effects of rhTNF on TNF receptor expression in these lines were studied. **Results:** This study reports a protocol for the simultaneous quantitative evaluation of the of TNF receptor number and co-expression of membrane-bound TNFR1/2. Cells within one tumor cell line were found to differ regarding their ex-

pression of type 1 and 2 TNFα receptors; simultaneously, cells with all 4 variants of co-expression may be present in culture. **Conclusion:** We demonstrated a dose-dependent effect of TNF on changes in the expression of TNFR1/2 by the percentage of positive cells and by the number of receptors, which may be used to control TNF-mediated processes in target cells.

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Introduction

The pro-inflammatory cytokine tumor necrosis factor (TNF) is a key mediator of the immune system, which is involved in most immunomediated processes in the body under normal and pathological conditions. In addition to the blood serum level of cytokines and the soluble form of cytokine receptors, the presence of 2 specific mem-

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brane-bound cytokine receptors with various functions regulates the cytokine effect on cells. The interaction between TNFR1 and a mediator leads to the formation of 2 signal complexes. Complex I controls the expression of anti-apoptotic proteins that prevent cell death. In contrast, Complex II is responsible for the activation of cell death. TNFR2 primarily mediates the metabolic effects of TNF α via the regulation of fat and carbohydrate metabolism, and is also involved in the transcription of genes responsible for cell growth, proliferation, and differentiation, as well as the cellular secretion of various mediators [1–3]. Furthermore, these effects on cells might depend on the presence of a certain receptor type on the membrane as well as the expression density and ratio of receptors on the same cell [4]. The density and co-expression of TNF receptors vary among populations of healthy cells and change during pathological conditions [5–7]. For some immune mediators, researchers have already demonstrated a relationship between the density of specific receptors on cells and cytokine functions [8–10]. However, for most cytokines, the exact threshold level of receptor expression for switching biological effects has not been determined yet. Tumor cell lines are a versatile model for studying biological effects, including the influence of TNF α on cells with a different expression density of type 1 and 2 TNF receptors. The use of cell lines is advantageous because they can survive many passages, they are homogeneous, have relatively constant properties and characteristics, and can be used to investigate the biological effects of TNF α . Accordingly, the aim of this study was to investigate the density of expression and co-expression of TNF α receptor types 1 and 2 and the dose-dependent effect of soluble TNF on these parameters in tumor cell lines with different histogenesis. This will allow the selection of several lines for the further study of the dose-dependent biological effects of TNF α on cells with different expression characteristics.

Materials and Methods

Human cell lines characterized for the expression and co-expression of TNF α receptor types 1 and 2 were used to evaluate the level of TNF α receptor expression. Epithelial-like (HEp-2, K-562, MCF-7, ZR-75/1) and lymphoblast-like (MOLT-4, HL-60, Raji, RPMI-8226, IM-9) cell lines were chosen for these experiments. All cell lines were obtained from the Federal Budgetary Scientific Institution State Research Center of Virology and Biotechnology “VECTOR” of the Federal Service for Surveillance in Consumer Rights Protection and Human Well-Being.

After thawing, the cell lines were cultured in 25-mL flasks (TPP, Switzerland) in 5 mL of complete RPMI-1640 culture medium

containing 10% FCS (HyClone, USA), 2 mM L-glutamine (BioloT, Russia), 5×10^{-4} M 2-mercaptoethanol (Sigma-Aldrich, USA), 80 μ g/mL gentamicin (KRKA, Slovenia), 10 mM HEPES buffer (Sigma-Aldrich, USA), and 100 μ g/mL benzylpenicillin (Biosintez, Russia) in an incubator with a humidified atmosphere at a temperature of 37 °C and a CO₂ concentration of 5%.

To evaluate the effect of recombinant TNF α on the functional activity of cells, the cell lines were cultured in the presence and absence of TNF α , and the dose-dependent effect of TNF α was also evaluated. Cells were cultivated for 72 h with the addition of rhTNF at a dose of 0.01–250 ng/mL. The phenotypic characteristics were assessed by flow cytometry (FACSaria and FACSVersa cytofluorometers; BD Biosciences, Franklin Lakes, NJ, USA) using monoclonal antibodies: anti-hTNFR1-PE (IgG1 isotype, clone 16803), anti-hTNFR2-PE (IgG2a isotype, clone 22235), anti-hTNFR1-APC (IgG1 isotype, clone 16803), and anti-hTNFR2-APC (IgG2a isotype, clone 22235; R&D Systems, Minneapolis, MN, USA). Data processing and the calculation of fluorescence intensity parameters were performed using FACS Diva software (BD Biosciences).

A BD QuantiBRITE PE kit (BD Biosciences) containing lyophilized beads conjugated with 4 levels of phycoerythrin was used to generate a calibration curve and convert the fluorescence intensity of cells expressing an appropriate marker to absolute receptor counts. A plot of the logarithm of the phycoerythrin molecule number versus the logarithm of the fluorescence intensity was generated based on the analysis of beads according to the manufacturer's instructions and fit to a linear regression model, as described previously [11]. The obtained relationship was used to derive an equation for converting the fluorescence intensity in the PE channel into the number of PE molecules for each of the cell lines: the mean number of receptors per cell for each cell line was calculated. For the simultaneous determination of the number of TNF α receptor types 1 and 2 on cells in all 4 fractions of each cell line (TNFR1+TNFR2–, TNFR1+TNFR2+, TNFR1–TNFR2+, TNFR1–TNFR2–), double labeling of paired samples was carried out. Each sample with a certain dose of rhTNF (or control sample without TNF) was divided into 2 tubes stained with TNFR1-PE and TNFR2-APC or with TNFR2-PE and TNFR1-APC. For the calculations, we used a compensation matrix constructed by a cytometer program using fluorescence minus 2 controls.

After cytometry analysis, the number of type 1 receptors (for TNFR1+TNFR2– and TNFR1+TNFR2+ fractions) was calculated in tubes with TNFR1-PE and TNFR2-APC. The number of type 2 receptors (for TNFR1–TNFR2+ and TNFR1+TNFR2+ fractions) were calculated in tubes with TNFR2-PE and TNFR1-APC. The percentage of each fraction was determined as the mean between 2 samples.

Statistics

Statistical data analysis was performed using STATISTICA 7.0 software (StatSoft, USA). Independent samples were compared, and statistical significance was calculated using the Kruskal-Wallis test by ranks with multiple comparison of the median values (when comparing identical indicators for different subpopulations and revealing the differences between study subgroups). Correlations between analyzed parameters were determined using Pearson's correlation coefficient at $p < 0.05$.

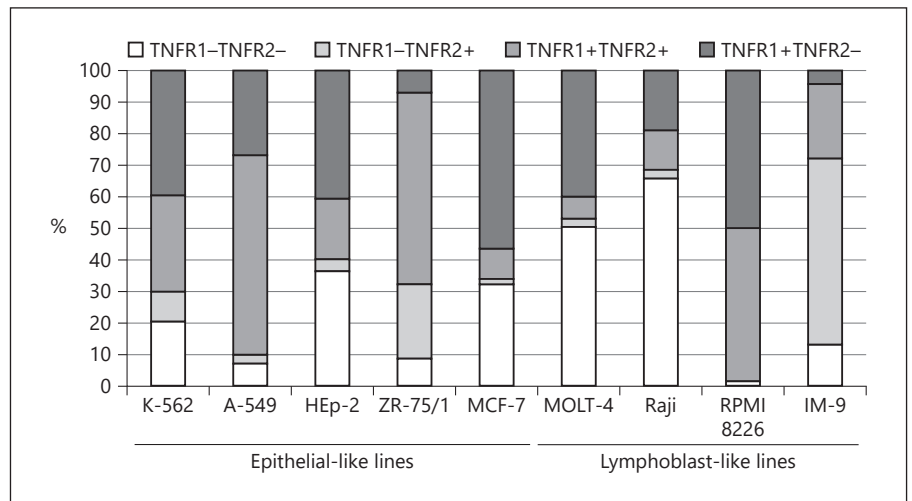


Fig. 1. TNFR1/2 co-expression on cell lines with a different genesis. For each cell line, at least 3 experiments were repeated.

Table 1. Quantitative expression of type 1 and 2 TNF α receptors in cell line cultures

Cell line	TNFR1 on TNFR1+TNFR2+ cells, <i>n</i>	TNFR1 on TNFR1+TNFR2- cells, <i>n</i>	TNFR2 on TNFR1+TNFR2+ cells, <i>n</i>	TNFR2 on TNFR1-TNFR2+ cells, <i>n</i>
A-549	5,131 $\pm$ 58 ^a	3,332 $\pm$ 32	5,155 $\pm$ 71 ^{a, b}	4,649 $\pm$ 44 ^{a, b}
HEp-2	4,289 $\pm$ 61	3,319 $\pm$ 21	3,461 $\pm$ 60	3,126 $\pm$ 26
ZR-75/1	5,554 $\pm$ 40 ^g	4,548 $\pm$ 25 ^{f, g}	3,630 $\pm$ 50	3,157 $\pm$ 27
MCF-7	3,964 $\pm$ 32 ^c	3,433 $\pm$ 57	1,176 $\pm$ 33 ^{b, c, h}	981 $\pm$ 22 ^{b, c, h}
MOLT-4	3,488 $\pm$ 32 ^{a, d, g}	1,180 $\pm$ 8 ^{d, g}	3,127 $\pm$ 27 ^{a, d}	2,610 $\pm$ 39 ^{a, d}
Raji	4,000 $\pm$ 37 ^e	2,647 $\pm$ 47 ^{e, f}	4,370 $\pm$ 58 ^h	3,340 $\pm$ 46 ^h
K-562	18,511 $\pm$ 299 ^{c, d, e}	16,190 $\pm$ 117 ^{d, e}	12,987 $\pm$ 326 ^{c, d}	10,944 $\pm$ 273 ^{c, d}

Data are given the mean $\pm$ error of the mean; *n* = 5 repeats for each cell line. Statistically significant (*p* < 0.05) differences between the same parameters of different cell lines are indicated by the following: ^a A-549 and MOLT-4 lines; ^b A-549 and MCF-7 lines; ^c K-562 and MCF-7 lines; ^d K-562 and MOLT-4 lines; ^e K-562 and Raji lines; ^f ZR-75/1 and Raji lines; ^g MOLT-4 and ZR-75/1 lines; ^h Raji and MCF-7 lines.

Results

Co-Expression of Different Receptor Types on Cell Lines

Investigation of tumor cell lines with a different genesis demonstrated significant variability in the indicators of the co-expression of type 1 and 2 TNF receptors both among cell lines and within a single cell line (Fig. 1). This allowed us to allocate cells within 1 line into 4 fractions:

1. Cells expressing only type 1 receptors (TNFR1+TNFR2-)
2. Cells expressing both receptor types (TNFR1+TNFR2+)
3. Cells not expressing TNF α receptors (TNFR1-TNFR2-)

4. Cells expressing only type 2 receptors (TNFR1-TNFR2+)

The K-562 cell line mainly expressed the type 1 receptor but also had a dual expression of TNFR1 and TNFR2. A-549 and ZR-75/1 cell lines had the highest expressions with regard to the relative number of cells bearing both receptors on the cell membrane. The remaining major fraction of A-542 cells expressed only the type 1 receptor, and the remaining major fraction of the ZR-75/1 cell line expressed only the type 2 receptor. The distribution of cell fractions in the HEp-2 line resembled that of the K-562 line. The MCF-7 cell line had the highest percentage of cells expressing only the type 1 receptor. In cultures of the lymphoblast-like cell lines MOLT-4 and Raji, the total

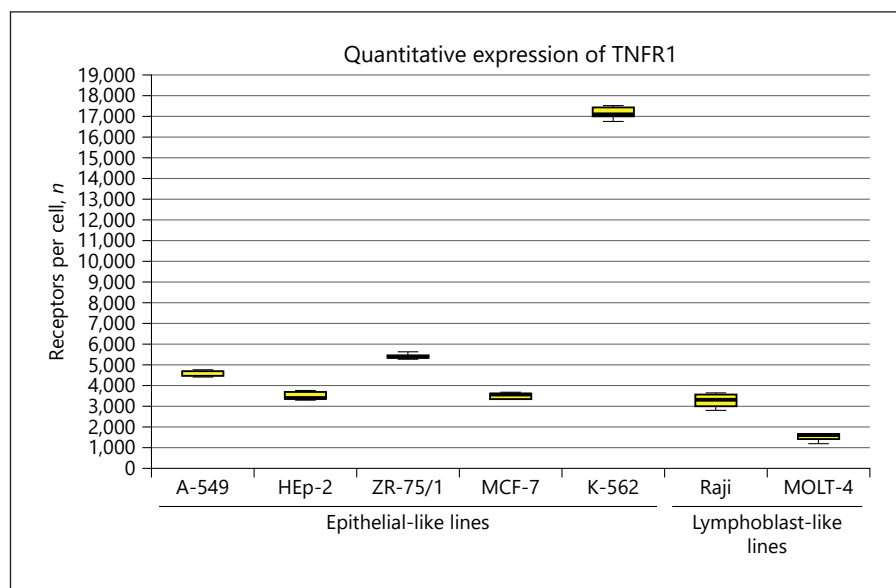


Fig. 2. Quantitative expression of type 1 receptors to TNF on cell lines; $n = 5$ repeats for each cell line.

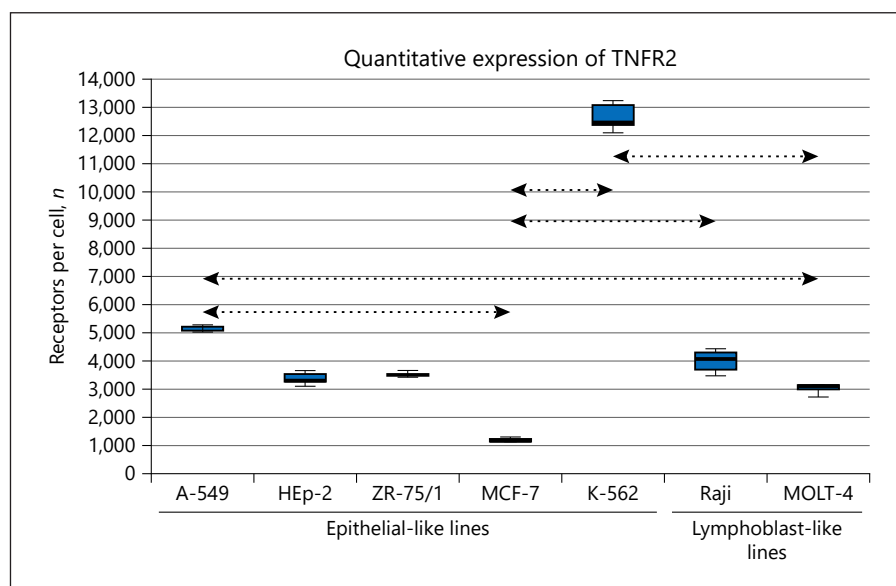


Fig. 3. Quantitative expression of type 2 receptors to TNF on cell lines; $n = 5$ repeats for each cell line. Statistically significant ($p < 0.05$) differences in the expression of TNFR2 between different cell lines are indicated by arrows.

relative number of cells that expressed any of the receptors was small. Among the remaining fraction of MOLT-4 and Raji cells expressing TNF α receptors, the expression of only TNFR1 was most common.

Cultures of cell lines with a common morphology were found to differ regarding the type of TNF α receptor co-expression. Among cells expressing only the type 1 receptor, significant differences ($p < 0.05$) were observed for the ZR-75/1 and MCF-7 lines. Among TNFR1+TNFR2+ cells, significant differences ($p < 0.05$) were found for A-549 and MCF-7 lines. Among cells expressing only

TNFR2, significant differences ($p < 0.05$) were found for ZR-75/1 and MCF-7 lines.

In addition, differences were found among cell lines of different morphological groups. Significant differences ($p < 0.05$) in the TNFR1+TNFR2 fraction were observed for the MCF-7 and Raji lines. Among cells expressing both receptors, significant differences ($p < 0.05$) were observed for A-549 and MOLT-4, A-549 and Raji, and ZR-75/1 and MOLT-4 lines. Among cells expressing only the type 2 receptor, significant differences ($p < 0.05$) were found for the K-562 and MCF-7 lines.

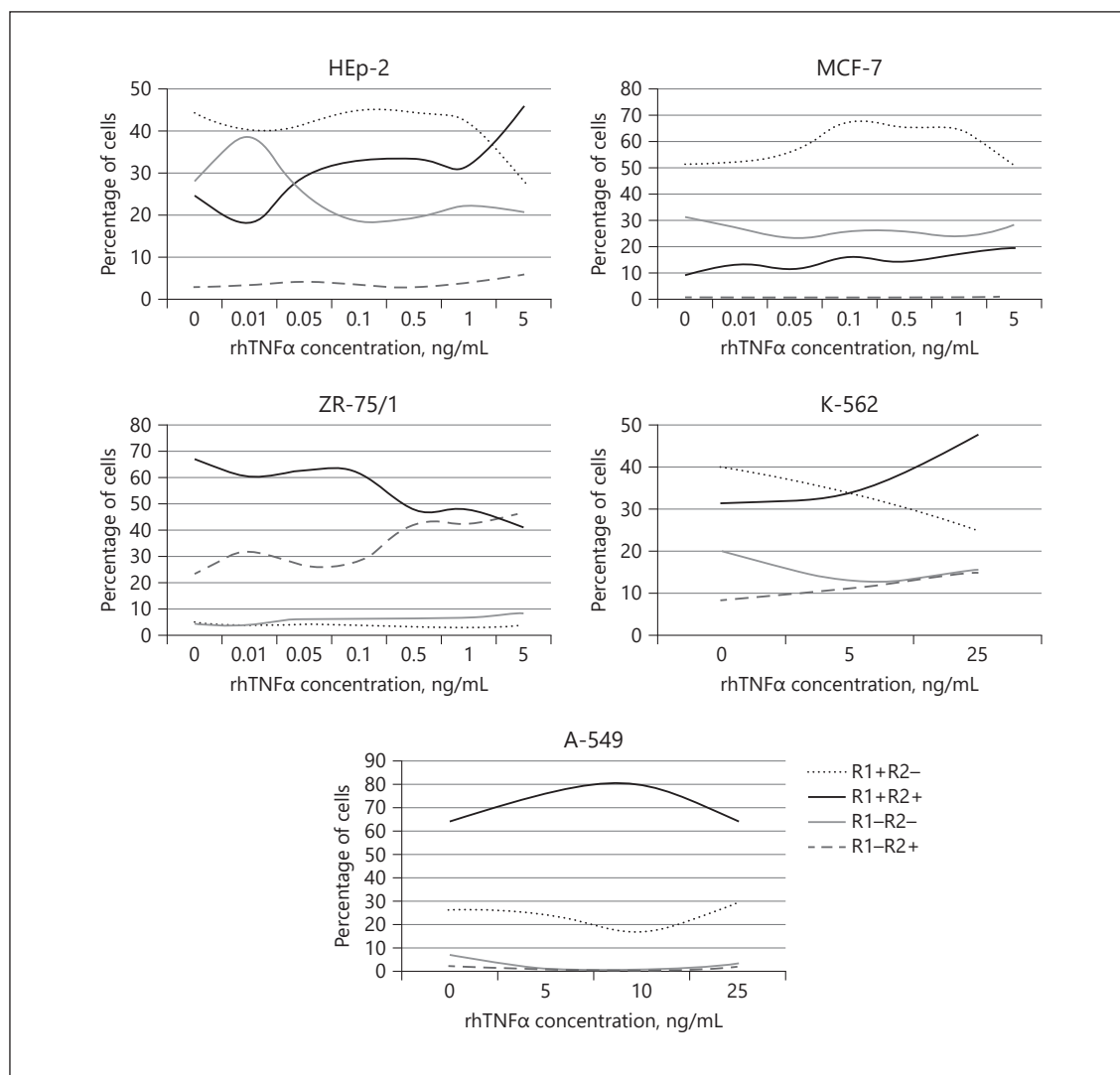


Fig. 4. Changes in the expression of type 1 and 2 receptors to TNF α on cultures of epithelial-like cell lines with different concentrations of TNF α added to the culture. Data are given as the mean value; $n = 5$ repeats for each cell line. The indicated correlation coefficients correspond to the condition $p < 0.05$.

The Density of Receptor Expression on Cells

The quantitative expression of receptors on cells is shown in Table 1. Among cell lines of different morphological groups, there were significant differences ($p < 0.05$) in: (i) the quantitative expression of TNFR1 on double-positive cells between A-549 and MOLT-4, K-562 and MCF-7, K-562 and MOLT-4, K-562 and Raji, and MOLT-4 and ZR-75/1 lines; (ii) in the quantitative expression of the type 1 receptor on TNFR1+TNFR2 $-$ cells between K-562 and MOLT-4, K-562 and Raji, MOLT-4 and ZR-75/1, and Raji and ZR-75/1 lines; (iii) in the quantitative expression of the type 2 receptor on TNFR1+TNFR2 $+$ cells between A-549 and MOLT-4, K-562 and MOLT-4,

K-562 and MCF-7, and MCF-7 and Raji lines; and (iv) in the quantitative expression of the type 2 receptor on cells expressing only TNFR2 between A-549 and MOLT-4, K-562 and MOLT-4, K-562 and MCF-7, and MCF-7 and Raji lines.

For each cell line, the mean number of type 1 and 2 receptors was determined on cells expressing an appropriate receptor (Fig. 2, 3). The K-562 cell line had the highest expression density of both type 1 and 2 receptors per cell. However, a high density of receptor expression on the cell membrane is not necessarily related to the presence of a large fraction of cells expressing this receptor. K-562 cells expressed the greatest amount of TNFR2

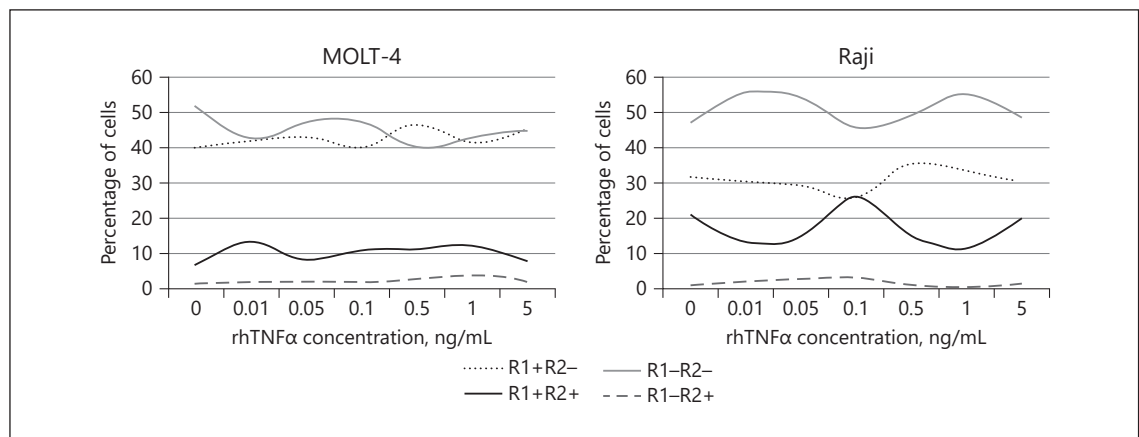


Fig. 5. Changes in the expression of type 1 and 2 receptors to TNF α on cultures of lymphoblast-like cell lines with different concentrations of TNF α added to the culture. Data are given as the mean value; $n = 5$ repeats for each cell line. The indicated correlation coefficients correspond to the condition $p < 0.05$.

on the membrane, but the relative amount of TNFR2+ cells in the culture was $<50\%$. The MOLT-4 and MCF-7 cell lines expressed the smallest amount of TNFR1 and TNFR2 on the cell membrane, respectively.

The expression of the type 1 receptor on cultured cells was characterized by a larger percentage of cells expressing TNFR1 in the culture rather than by the high quantitative expression of the receptor on the cell membrane. The exception was K-562 cells that had the highest expression density of the type 1 receptor. In contrast, the expression of TNFR2 in most cases was characterized by the high quantitative expression of TNFR2 by a relatively low percentage of cells expressing this receptor. Exceptions were the A-549 and ZR-75/1 cell lines characterized by a high percentage of TNFR2+ cells in the culture.

A positive correlation between the percentage of TNFR1+ cells and the quantitative expression of the type 1 receptor was found only for MOLT-4 cells ($r = 0.97$, $p < 0.05$). There were no statistically significant correlations between the relative number of TNFR2+ cells and quantitative expression of the type 2 receptor.

TNF-Induced Changes in Receptor Expression on Cell Lines

To evaluate the effect of TNF α on the expression of TNF receptors, cell lines were cultured in the absence or presence of recombinant TNF α at concentrations of 0.01, 0.05, 0.1, 0.5, 1, and 5 ng/mL (Fig. 4, 5). Within cell line cultures, cells differing in the type of TNFR1 and TNFR2 co-expression responded differently to the addition of TNF α . HEP-2 cells were characterized by an increased

percentage of TNFR1+TNFR2+ cells, a reduced fraction of TNFR1–TNFR2– cells, and a decrease in the relative number of cells expressing only TNFR1, as the TNF α concentration was increased. However, these effects on HEP-2 cells were not statistically significant ($p > 0.05$).

In the MCF-7 cell line, there were statistically significant correlations ($p < 0.05$) regarding the increased percentage of cells expressing both receptors ($r = 0.69$) and the relative number of TNFR1–TNFR2+ cells ($r = 0.78$). For the A-549 line, there were no statistically significant correlations between an increase in the TNF α dose and a change in the relative number of cells of different fractions.

ZR-75/1 cells were characterized by an increase in the fraction of cells expressing only type 2 receptors and a decrease in the percentage of TNFR1+TNFR2+ cells as the TNF α concentration in culture was increased. However, these patterns were also not statistically significant ($p > 0.05$). Therefore, in cultures of epithelial-like cell lines, cells with different types of receptor co-expression respond differently to an increase in the TNF α concentration in culture.

In cultures of lymphoblast-like MOLT-4 and Raji cell lines, there were no statistically significant changes in the percentage of cell fractions with different types of co-expression of type 1 and 2 TNF α receptors in response to the addition of different TNF α concentrations. For culture of the erythromyeloblastoid cell line K-562, statistically significant ($p < 0.05$) correlations were found between an increase in the TNF α concentration in culture and an increase in the relative number of TNFR1+TNFR2+ cells ($r = 0.84$), as well as between a decrease in the percentage of

Table 2. Statistically significant ($p < 0.05$) correlation coefficients between an increase in TNF α concentration and a change in the relative number of cells that differ by the type of co-expression of TNFR1 and TNFR2 as well as a change in the quantitative expression of type 1 and 2 TNF receptors

Cell line	TNFR1 on TNFR1+TNFR2- cells, n	TNFR1 on TNFR1+TNFR2+ cells, n	TNFR1+TNFR2+ cells, %	TNFR1-TNFR2+ cells, %	TNFR1+TNFR2- cells, %
MOLT-4	0.67				
ZR-75/1		-0.55			
MCF-7			0.69	0.71	
K-562			0.84	0.81	-0.85

cells expressing only the type 1 receptor ($r = -0.85$) and an increase in the fraction of TNFR1-TNFR2+ cells ($r = 0.81$).

We also evaluated changes in the quantitative expression of type 1 and 2 TNF α receptors after the addition of different cytokine concentrations to the culture. Statistically significant correlations between an increase in the cytokine dose and a change in the quantitative expression of receptors were found for the MOLT-4 line in the form of an increase in the amount of TNFR1 on TNFR1+TNFR2+ cells ($r = 0.67$, $p < 0.05$) and also for the ZR-75/1 line in the form of a decrease in the quantitative expression of TNFR1 on TNFR1+TNFR2+ cells ($r = -0.55$, $p < 0.05$). Statistically significant correlations between an increase in the TNF α dose and a change in the relative number of cells differing in the type of co-expression and quantitative expression of TNFR1 and TNFR2 are shown in Table 2.

Discussion

This study is the first to obtain data on the relationships between indicators of type 1 and 2 receptor expression in cell lines and the dose-dependent effect of TNF. The obtained data on cultures of some cell lines are consistent with previous studies in that these cell lines mainly expressed only one receptor type: lines A-549 [12] and HEp-2 [13, 14]. However, previous studies investigated the separate expression of type 1 and 2 receptors. In our study, we evaluated the co-expression of both receptors; therefore, the sum of the relative amounts of TNFR1+TNFR1+ and TNFR1+TNFR2+ cell fractions will result in the maximum total percentage of cells bearing the type 1 receptor on their membrane.

According to previous studies, Raji line cells express only the type 2 receptor [13, 14]. In our study, the relative number of Raji cells expressing TNF α receptors was small, but both TNFR1+TNFR2- and TNFR1+TNFR2+ frac-

tions were present among them. In this case, most of these cells bear the type 1 receptor on the membrane, which is consistent with the data presented earlier. The difference between our findings and previous studies might be associated with the different strains of the Raji cell line.

Thus, cell lines of different origin and morphology differ by the co-expression of type 1 and 2 TNF α receptors. These differences may be associated with the different degree of cell differentiation and the functional status of the cells. In particular, low differentiated lines (e.g., Raji) are characterized by the predominance of type 2 receptor expression, whereas highly differentiated lines (MCF-7 and Raji) are characterized by the predominance of type 1 receptors; in this study, both cell lines responded to TNF by increasing the percentage of TNFR1+TNFR2- or TNFR1+TNFR2+ cells. Therefore, low differentiated lines with a predominant expression of the type 2 receptor have a greater proliferative potential when activated, especially by TNF.

Among epithelial-like cell lines, K-562 cells had the highest surface number of type 1 and 2 TNF α receptors. Among lymphoblast-like lines, IM-9 cells had the lowest surface number of type 1 TNF α receptors; in this study, RPMI-8226 cells expressed the highest number of type 1 receptors and the lowest number of type 2 receptors.

Therefore, we analyzed the expression level of membrane-bound TNF receptors and demonstrated that lines of a similar origin may have a different number of receptor molecules on their surface and may be characterized by the different co-expression of TNF α receptors. On the basis of these study results, we selected cell lines characterized by various combinations of membrane-bound TNF receptors to further evaluate the biological effects of TNF.

TNF α affects the expression of type 1 and 2 receptors by changing the relative number of cells expressing the receptors and the quantitative expression of receptors on the cell membrane. However, TNF α only changes the quantitative

expression of receptors in some cell lines (MOLT-4 and ZR-75/1) and the percentage of cells expressing TNF α receptors in other cell lines (MCF-7 and K-562).

To evaluate the effect of recombinant TNF α on the functional activity of cells, cell lines were cultured in the presence or absence of TNF α , and the dose-dependent effect of TNF α was also assessed. Cell lines with a weak expression of both receptor types (HEp-2, MOLT-4) and the K-562 line with predominant TNFR2 expression did not respond to TNF α stimulation. The ZR-75/1 cell line expressing both receptors and the RPMI-8226 line expressing the type 1 receptor responded to the addition of TNF α by enhancing proliferative activity.

Conclusion

We demonstrated that cell lines of the same genesis differ by the type of receptor expression and by dose-dependent reaction to TNF α . The predominant expression of type 1 TNF receptors may be associated with a high degree of cell line differentiation, while the expression of type 2 TNF receptors may be associated with a low degree of cell line differentiation. Further investigation of the influence of TNF on the proliferative and pro-apoptotic potential of these cells is necessary to confirm this hypothesis.

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Statement of Ethics

Not applicable (this study did not involve humans or animals).

Disclosure Statement

The authors have no conflicts of interest to declare.

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No special finding was received for this study.

Author Contributions

All authors contributed equally to the manuscript preparation. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.