TNF-α induced changes in cell membrane antigen expression on K-562 cells associated with increased lactate dehydrogenase (LDH) release*

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TNF-α is a pleiotropic cytokine, which induces death of sensitive cells, whose effect depend on cell membrane receptor expression, cell cycle phases, as well as on intracellular ratio of pro-apoptotic and anti-apoptotic molecule expression. Since determination of LDH release from cultured cells *in vitro,* reflects early membrane alterations, we estimated and compared LDH release from cultured cells with changes in cell membrane antigen expression on K-562 cells after TNF-α treatment by flow cytometry. The significant increase in LDH release activity and cytotoxicity values was associated with decrease in membrane molecule expression for CD45 and CD30 as well as for low expressed CD45RA and CD38 after TNF-α treatment. However, percentage of decrease of all examined molecules is not uniform, and appears to depend on the respective level of pre treatment values expression and molecule type. These results indicated the complexity of events on cell membrane, including association between increasing LDH release and decrease of antigen expression of membrane molecules following TNF-α mediated processes.

Key words: antigen expression, apoptosis, CD30, CD38, CD45, CD45RA, flow cytometry, Lactate dehydrohenase (LDH) release assay, necrosis, TNF-α

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic cytokine produced by macrophages, cytotoxic T lymphocytes and Natural killer (NK) cells as an effector molecule in immune response which transduce diverse signals after engagement appropriated receptors [10, 12, 31]. TNF-α mostly triggers cell death in different cells, normal or tumor [30, 33, 42] with effects dependent on cell membrane receptor expression [10, 34], cell cycle [5] and ratio of intracellular expression of pro-apoptotic and anti-apoptotic molecules [19, 21, 35]. TNF-α stimulated growth of some lymphoid cells and may even participate in development of tumors.

We reported previously that lactate dehydrogenase (LDH) release determined in cultured cells *in vitro* [15] reflects cell membrane alteration [16]. The release of intracellular molecules such as LDH trough cell membrane appears after damage, as well as following alterations in transport channels or pore forming after drug treatments or activation process [20, 24, 40]. The erytroleukemic K-562 cells were originally described by Lozzio and Lozzio from patients in blast transformation and characterized as NK sensitive and usually it is a target for evaluation of cell cytotoxicity. We reported previously [15] that TNF-α, as a single cytotoxic agent *in vitro*, induced significantly LDH release, indicating cell death process in K-562 cells in comparison with effects of human NK cells against some targets.

For better understanding of the very complex events on cell membrane K-562 cells, during triggering of cell death process (apoptosis and necrosis) or cell activation, we analyzed and compared LDH release in relation to changes in CD45, CD30, CD45RA, CD38 and glycophorin A membrane molecule expression on gated cell population, K-562 cells before and after TNF-α treatment, using flow cytometry.

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Material and methods

Cell culture. The human erythroleukemia cell line K562 was grown in RPMI 1640 medium without phenol red (Sigma, USA) supplemented with 2 mM glutamine, 10% bovine serum albumine (Sigma, USA) in presence of antibiotics: 100 mg/ml of penicillin and 100 μg/ml of streptomycin (ICN, Yugoslavia). The cells were sub-cultured twice a week at the concentration of 50.000-100.000 cells/ml. Cells were cultured in flasks (Costar, USA) at 37 ˚C in 100% of humid atmosphere containing 5% CO_2.

Cell treatment. TNF-α (Pro-moll, Germany) was dissolved in RPMI medium without phenol red (Sigma, USA) and used in final concentration of 500 and 1000 pg/ml of culture medium for *in vitro* treatment of K-562 cell cultures at 37 ˚C in humide atmosphere containing 5% CO_2 in micro-well plates (Costar, USA) for 2, 4, and 6 h for LDH release determination and for flow cytometry analyses.

LDH release assay. Modified lactate dehydrogenase (LDH) micro-assay, as previously described [15, 16], was used for determination of TNF-α effects on K-562 cells line. Control or treated K-562 cells after centrifugation and medium replacement were transferred to micro-well plates. The LDH release assay was done in 96 micro-well plates (Costar, USA) incubated for the next 2 h at 37 ˚C in humid atmosphere containing 5% CO_2. After that, the plates were centrifuged for 5 min at 200 g and supernatants from each well (100 μl) were transferred into new 96 flat bottom micro-well plates to which 100 μl of the LDH substrate mixture composed of $5.4x10^{-2}$ M of L(+)-lactate, $6.6x10^{-4}$ M of 2-p-iodophenyl-3-p-nitropheyl tetrazolium chloride, $2.8x10^{-4}$ M of phenazyne metosulphate and $1.3x10^{-3}$ of NAD (all from Sigma, USA) in 0.2 M Tris-buffer at pH 8.2, was added for determination of LDH release activity. The micro-titer plate reader (Behringer EL-311, Germany) was used for evaluation of the changes in the absorbance using dual filters at 492–630 nm/min.

The cytotoxicity of TNF-α was calculated using a formula:

$$\frac{\text{LDH after treatments} - \text{LDH culture medium}}{\text{LDH after sonification} - \text{LDH culture medium}} \times 100$$

In this formula LDH after sonication represents maximal LDH activity from identical concentration of K-652 cells, obtained after lysis by ultrasound (Soniprep, Germany), 3 times for 15 sec. at 35 kHz on ice. Each analysis was done in triplicate.

Immunophenotype of K-562 cells. Cell surface antigens on control and treated K-562 cells were determined using the following directly or indirectly labeled monoclonal antibodies: Anti-CD45, Anti-CD30, Anti-CD38 and glycophorin A (Becton Dickinson, USA). The samples were prepared as previously described [34]. Briefly, $1.0x10^6$ of K-562 cells in 100 μl of culture medium supplemented with 5% bovine serum albumin were incubated for 30 min at 4 ˚C with 20 μl of appropriate combination of monoclonal antibodies in each tube, washed twice with ice medium and fixed with 1% paraformaldehyde prior to FACS analysis. Surface marker expression was quantified by measurement of color fluorescence on FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA). A total number of 10 000 gated event, was analyzed using Cell QUEST software. Exclusion of non-specific fluorescence staining was based on matched isotype mAb combinations conjugated with FITC. Results were expressed as percentage positive cells.

Determination of TNF-α effect on the decrease of antigens expression. Estimation of TNF-α effect on changes in antigen expression of K-562 cells was calculated using formula and expressed as percentage decreases from basal values, for better statistical analyses:

$$\frac{\text{The values before treatment} - \text{The values after treatment}}{\text{The values before treatment}} \times 100$$

Statistical analysis. The data were analyzed by using ANOVA, Mann-Whitney U-test, and comparison of slope curve by Multivariate analyses using EXCEL Microsoft software.

Results

LDH release from cultures of K-562 cells ($1.0x10^6$/ml of culture medium) with or without TNF-α were shown in Figure 1A, while total intracellular LDH activity of K-562 cells in Figure 1B. The results show that TNF-α induced significant increase of LDH release activity expressed in absorbance (Mann-Whitney U-test, $p<0.05$), after 4 and 6 h when compared to first 2 h. The total intracellular LDH activity from identical concentration of ultrasound lyses K-562 cells was without changes with or without presence of 1000 pg/ml of TNF-α in cultures. No difference was found (Mann-Whitney U-test, $p>0.05$) in total intracellular LDH activity in K-562 determined after 2, 4, or 6 h (Fig. 1B). The percentage of LDH release after treatment with TNF-α, calculated in terms of spontaneous to total LDH activity and expressed as percentage of cytotoxicity was shown in Figure 1C. The results show significant increase cytotoxicity percentage of TNF-α depending on times (Mann-Whitney-U test, $p<0.05$).

The immuno-phenotype characteristics for selected antigens on K-562 cells before and after treatment with TNF-α are shown in Figure 2. Results showed different percentage expression of CD45, CD30, CD38, CD45RA, and glycophorin A antigens on the cell surface membrane before and after TNF-α treatment, determined after 6 h. How-

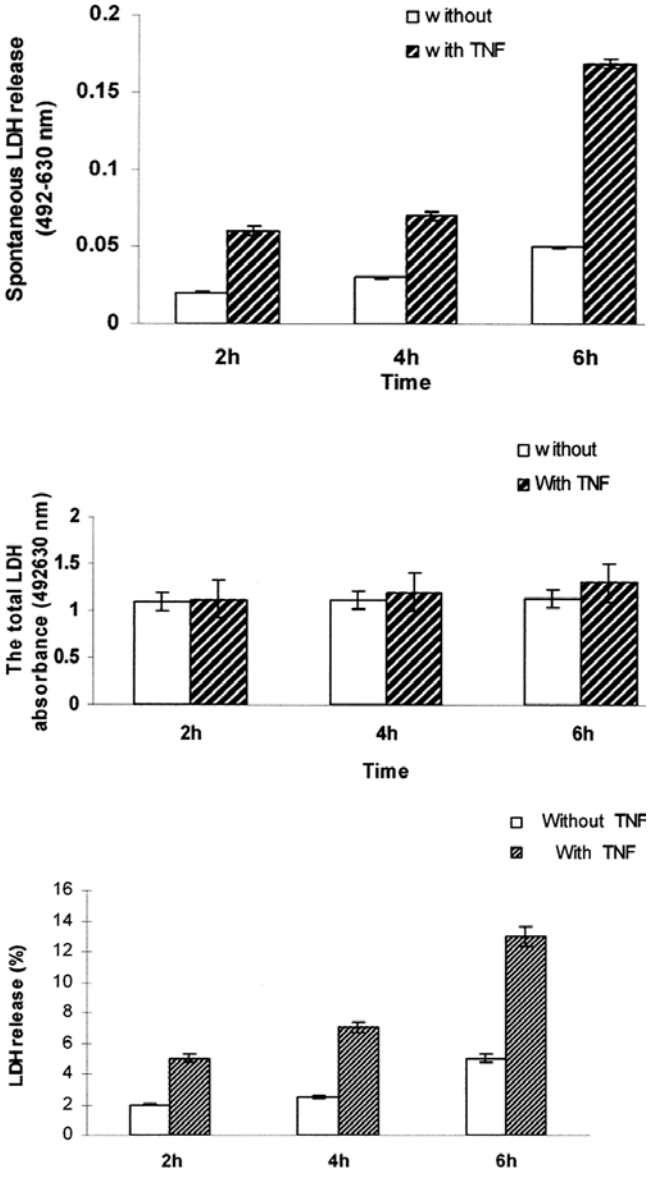

Figure 1. The significant increase (Mann-Whitney U-test, $p<0.05$) of LDH release activity in supernates from cultures K-562 cells, expressed in absorbance determined after 2, 4, and 6 h induced with 1000 pg/ml of TNF-α in comparison to spontaneous LDH (A). The total intracellular LDH activity in sonified K-562 cells with or without TNF-α was without significance (Mann-Whitney U-test) (B). The percentage of the LDH release through cell membrane of K-562 cells (C) calculated from spontaneous LDH release in respect to total intracellular LDH showed significance in presence of TNF-α (Mann-Whitney U-test).

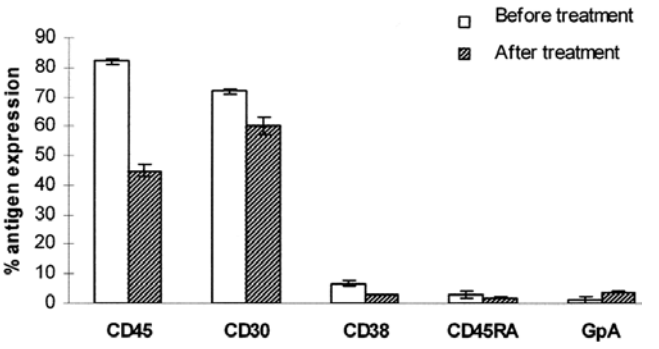

Figure 2. Percentage expression of CD45, CD30, CD45RA, CD38 and Glycophorin A, the membrane molecules on K-562 cells before and after TNF-α treatment estimated by flow cytometry.

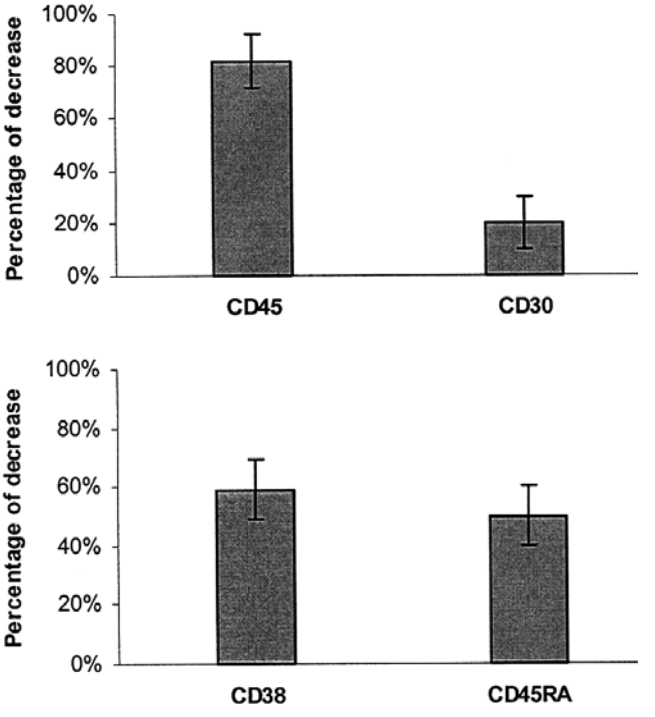

Figure 3. The rate of decrease CD45 or CD30 membrane molecules was shown on Figure 3A (highly expressed) and CD45RA or CD38 (low expressed) on Figure 3B, calculated from values after treatment in respect to pre-treated values.

ever, we analyzed percentage decrease of selected membrane molecules, which were divided into two groups; high membrane molecule expression (CD45 and CD30) and low molecule expression bellow 10% (CD45RA and CD38). Results are shown in Figure 3A and 3B, respectively. The rate of decrease was calculated from values obtained after treatment in respect to pre-treatment value (by formula in material and methods) and results show different percentage of decrease. The percentage of decrease of CD45 membrane molecules with median values of 82% (for several independent experiments) was significantly different (Mann-Whitney U-test, $p<0.05$) in comparison to 20% (median values) for CD30 molecules (Fig. 3A). However, decrease of antigens which shows very low pre-treatment values of membrane K-562 cells (CD38 and CD45 RA) show similar percentage of decrease (Fig. 3B), without significance ($p>0.05$, Mann Whitney U-test) between them. Contrary to this, glycophorin A showed significantly in-

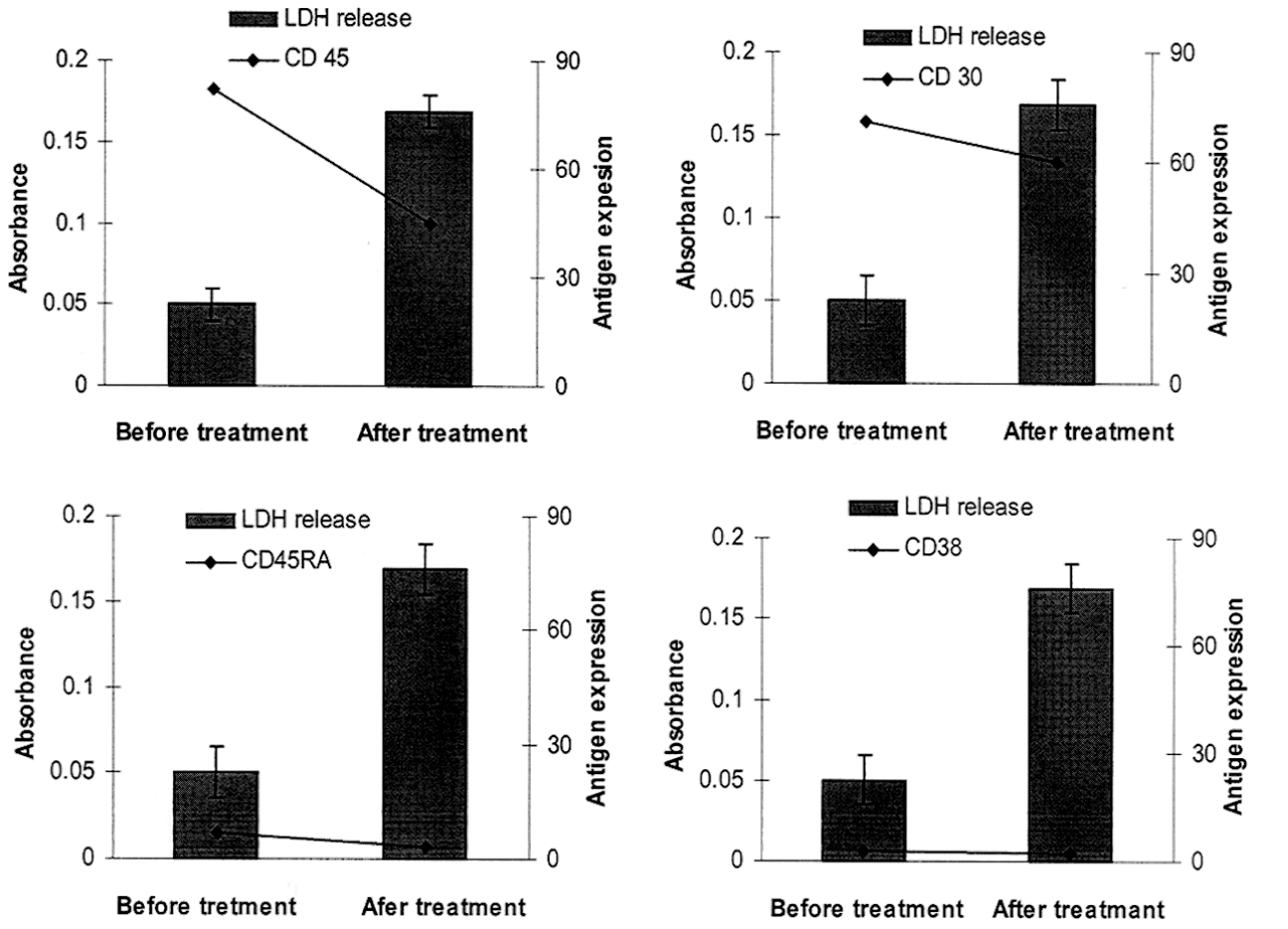

Figure 4. The association between increase of LDH release activity (expressed in absorbance at 492-630 nm) with rate of decrease membrane expression for CD45, CD30, CD45 and CD38 antigens on gated K-562 cells before and after treatment.

creased values after TNF-α treatment, although, this data were estimated on very low pre-treatment values, which may be in-appropriate.

The association between the increase of LDH release with changes in CD45, CD30, CD45RA or CD38 membrane molecule expression, before and after TNF-α treatment of K-562 cells, is presented in Figure 4. In figure a significant greater decrease in CD45 molecules and lower decrease in CD30 molecule, are shown to be accompanied with a significant increase in LDH release, but without changes in expression of CD45RA or CD38. Furthermore, different curve slope indicated difference in decrease of selected antigen associated with significant differences in LDH release ($p<0.05$, multivariate analyses) before and after TNF-α treatment.

Discussion

Based on the possibility of TNF-α to induce opposite and diverse effects, including triggering of apoptosis [4, 7, 10] or induction of cell activation [1, 9, 31] is an area of great interest for research.

Recently, different methods for estimation of cell death processes and cell membrane alterations were described [4, 20, 28, 41]. The recommendations from some studies when analyzing cell death process by single method indicated that more assays are necessary for better data interpretation and understanding complex events on cell membrane [4]. This is an actual problem, apart from the consideration that the techniques of flow cytometry better explanted changes in the outer or inner cell membrane during signal transduction [41]. The problem in interpretation of cellular changes following dynamic processes was additionally complicated because any events following apoptosis have a very short-time duration, molecules involved in this process only translate second signals [11] or induce protein transport from one to the other intracellular compartment [26].

In this study, we compared results obtained by two different methods for better explanation of the dynamic changes on the cell membrane after TNF-α treatment. The first assay was based on determination of released in-

tracellular molecules, LDH through the cell membrane. This assay, as very sensitive [3, 15, 41], shows significant LDH release from cultured cells [14] depending on cell type (tumor or normal), cell number [17] or cell separation process. Cultures of peripheral blood lymphocytes from healthy volunteers after TNF-α treatment showed significant increase in LDH release activity [14]. The determination of spontaneous LDH release, reflecting membrane alterations on molecular level is a very appropriated for the estimation of the safety of vaccine application and virus toxicity also [8].

We detected that TNF-α induced significant and dose dependent increase of LDH release activity in supernatants of cultured cells, indicating different mechanisms in LDH transport through cell membrane, and its appearance in extra-cellular space, analysed by the LDH release assay [26, 27].

TNF-α induced lower LDH release in comparison to the effect of human healthy NK cells against K-562 [16] which promptly and efficiently destroyed cell membranes mostly using a perforin following the necrosis process. Regardless of obtained low values of LDH released activity after TNF-α treatment as a single mediator, this finding clearly indicated early and detectable events confirming influence of external mediators to LDH release trough the cell membrane, reflecting changes in transport trough the membrane [26]. Effect of TNF-α on total intracellular LDH activity on tumor K-562 cells is without any significance in short term cultures (Fig. 1B) contrary to effects on long-term cultures.

For better explanation TNF-α effects on cell membrane disintegration or transport alteration in membrane of K-562 cells second assay was applied. Using flow cytometry techniques changes in the expression of membrane molecules were analyzed. The evaluation of cell membrane molecules is common method for cell characterization, confirmation of the hematological malignancy type, cell differentiation or cell activation status [13, 29, 32, 37]. TNF-α after *in vitro* culture induced decrease of antigen expression on K-562 cells, dependent on pre-treatment values, and are showing decrease in highly expressed antigens such as CD45 and CD30.

CD45 is a type I trans-membrane molecule found on leukocytes and exclusively on nucleated hematopoietic cells and their precursors. CD45 has a pivotal role during B or T cell antigen receptor stimulation and cell proliferation [25]. CD45 is molecular substrate in controls of cytokine receptor signaling. Contrary to the study that describe ultra structural characteristics of this important molecule in cell activation or cell death processes [14] we prooved functional alteration (80% decrease in CD45 cell membrane molecule expression) after TNF-α treatment, associated with increase of LDH release, suggesting of the cell death process. This is supported by some previously reported data based on consideration that loss of CD45 was accompanied by hyperactivation of the Jak-STAT pathway, cytokine hyperactivation and increase of toxicity although examined on CD45-null mice [25]. Decrease in CD45 membrane molecule expression during the treatment of K-562 cells postulate the possibility of interaction between these cell molecules via receptor cooperation or probably its participation in intracellular signal transduction [7, 34].

If TNF-α induce direct and prompt effect on the cell membrane disintegration following necrosis, we would expect identical loss and uniform decrease of all examined cell surface molecules. However, there was no uniform decrease of all examined molecules as we obtained smaller decrease of CD30 molecules (only 20%) in comparison to higher decrease (82%) in CD45 molecules after treatment. The persistence of high values in the expression of CD30 molecules after treatment (mean values about 60% in repeated experiments) postulated that TNF-α, don't destroy the cell membrane directly and fully.

This finding indicates other mechanism by which TNF-α induce LDH release, probably by activation of some intracellular enzymes (serine kinase, protein kinase C, sphingomyelinase, or phospholipase C) associated [2, 23, 39] with TNF-α mediated signaling and induction changes in pore forming channels [27]. The understanding of diversity in decrease of antigen expression was based on consideration that CD30 molecule, as member of TNF receptor superfamily, is involved and participates in signal transduction from the membrane. Its cytoplasmic tail played more significant role in NF-kappa B activation and interaction with TNF-receptor associated factors than in apoptosis network pathways [29]. These data are showing association of low TNF-α effects on activation molecules and higher effects on cell death inducing molecules and suggest participation of additional signals which induced LDH release [11, 23, 36].

The analyses in expression of other membrane molecules [13] confirms the complexity in regulation of TNF-α dependent on the events in cell membrane, such as moderate decrease in CD45RA and CD38 contrary to the increase of glycophorin A expression. However, this increase is in connection with very low pre-treatment values. Although expression of CD38 molecule in leukemia cells accompanies cell irregularity and is a negative prognostic marker [14] we found moderate decrease in its expression after TNF-α treatment.

Similarly to the presented data, showing association of increased LDH release in cultured cells during membrane molecule cooperation, other studies presented similar results. Expression of the thymocyte immature antigen on the cell membrane [6] was accompanied by LDH release. This is confirmed by determination of mRNA synthesis [42]. These data indicate novel traffic function during thymocyte development [6, 43] and suggest necessary energy for the described processes [40, 42].

We conclude that following TNF-α mediated signaling a very complex and dynamic processes on cell membrane appears [16], including changes in cell membrane expression and that these events were accompanied by LDH release through membrane.

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