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ORIGINAL ARTICLES

# TNF-α Induced Apoptosis is Accompanied with Rapid CD30 and Slower CD45 Shedding from K-562 Cells

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**Abstract** TNF-α can induce cell death (apoptosis and necrosis), and these effects mostly depend on expression of TNF-receptor superfamily molecules. As determination of certain intracellular enzymes like LDH, released from cultured tumor cells, reflects early membrane alterations, we compared LDH release with changes in cell surface membrane molecule expression during culture of K-562 cells in the presence of TNF-α. TNF-α-mediated CD45 and CD30 shedding is shown to be to be time- and dose-dependent and associated with significant increase in LDH release, with maximal effects after 24 h of treatment. The percentage of decrease of all examined cell surface molecules on K-562 cells after TNF-α treatment was not uniform and appeared to depend on the respective constitutive level of expression and molecule type. The presence of these molecules was confirmed in supernatants using Western blot analyses. These results indicated the complexity of events on the cell membrane, including early LDH release that is associated with a difference in shedding of CD30 and CD45. Shedding of CD30 occurs before apoptosis induction, while shedding of CD45 is associated with apoptosis.

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## Introduction

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 the immune response. This cytokine is involved in multiple immunological responses and in the pathogenesis of a variety of infections, autoimmune inflammation including septic shock, rheumatoid arthritis and Crohn disease after engagement of appropriate receptors (Haranaka and Satomi 1991; Grell et al. 1994; Smith et al. 1994; Andreakos et al. 2002). Its effects mostly depend on cell membrane receptor expression, cell cycle phases in target cells as well as balance of intracellular pro- and antiapoptotic molecules (Kumamura et al. 1996; Jurisic et al. 1999, 2000; Meilhac et al. 1999).

Two TNF receptors have been identified with molecular masses of 55 kDa (TNF-R1) and 75 kDa (TNF-R2), and some experiments have demonstrated that expression of these receptors on the cell surface is independently regulated (Grell et al. 1994; Pollock et al. 2000; MacEwan 2002; Jupp et al. 2003). Binding of TNF to its death receptor recruits procaspase to an oligomeric activation complex using adaptor protein FADD. The activated upstream and downstream caspases cleave various cellular proteins leading to apoptotic cell death. Among its biological activities, TNF-induced apoptosis and activation of nuclear factor-kappa B (NF-κB) have gained particular attention in recent years (Hu 2003). Caspases play an essential role during TNF-induced apoptosis, although

many molecular events may be involved in cell death (Chen et al. 1999). On the other hand, TNF-α can stimulate growth of some lymphoid cells and may participate in development of tumors. The diverse stimuli that upregulate TNF-α expression are known activators of several distinct MAP kinase signaling pathways also (Tibbles and Woodgett 1999).

We previously described that during TNF-α-induced cell death in tumor cells or normal lymphocytes lactate dehydrogenase (LDH) release in cell cultured medium can reflect cell membrane disintegration (Weidmann et al. 1995; Decker and Lohmann-Matther 1998; Jurisic et al. 2000; Konjevic et al. 2001; Jurisic 2003). The release of intracellular molecules such as LDH through the cell membrane occurs after cell membrane damage, as well as following alterations in transport channels or pore formation after drug treatment or activation processes (Nehar et al. 1997; Haggins 1999; Porter 1999). The erythroleukemic cell line K-562, used in our study, is common target for the evaluation of NK cell cytotoxicity, different drugs or cytotoxic molecules. We previously reported that TNF-α, as a single cytotoxic agent in vitro, induced significant LDH release, indicating necrotic cell death in K-562 cells (Jurisic et al. 1999).

For a better understanding of the very complex events on the cell membrane of K-562 cells, we analyzed and compared LDH release in relation to changes in CD45, CD30, CD45RA, CD38 and glycophorin A, membrane molecule expression on K-562 cells with different concentrations of TNF-α and time of treatment. Additional analyses were used to confirm the presence of these molecules in cell supernatants and cell lysate.

## Materials and Methods

### Cell Culture and Treatment

The erythroleukemic K-562 cell line developed by Lozzio and Lozzio was maintained in RPMI 1640 without phenol red (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (GIBCO, Paisley, UK), 3.0 mmol/l L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C. Exponentially growing cells in 35-mm Petri dishes were used in our study, and viability was determined by the trypan blue dye exclusion test.

### LDH Release Assay

Modified LDH assay, as previously described (Jurisic et al. 2000), was used for determination of cell membrane damage and for cell death. Control or treated K-562 cells after centrifugation and medium replacement were transferred to microwell plates. The LDH release assay was done in 96-microwell plates (Costar, Cambridge, MA), which were incubated for the next 2 h at 37°C in a humid atmosphere containing 5% $CO_2$. After that, plates were centrifuged for 5 min at 200×*g* and supernatants from each well (100 µl) were transferred into new flat-bottomed 96-microwell plates, to which 100 µl of the LDH substrate mixture (composed of $5.4 \times 10^{-2}$ M of $L^+$-lactate, $6.6 \times 10^{-4}$ M of 2-*p*-iodophenyl-3-*p*-nitropheyl tetrazolium chloride, $2.8 \times 10^{-4}$ M of phenazine methosulfate and $1.3 \times 10^{-3}$ of NAD [all from Sigma] in 0.2 M Tris buffer at pH 8.2) was added for determination of LDH release activity. A microtiter plate reader (EL-311; Boehringer Ingelheim, Ingelheim, Germany) was used for evaluation of the changes in absorbance using dual filters at 492–630 nm/min.

### Flow Cytometry

Cell surface antigens on control and treated K-562 cells were determined using the following monoclonal antibodies: anti-CD45, anti-CD30, anti-CD38 and glycophorin A (Becton Dickinson, San Jose, CA) on gated cells. Briefly, $1.0 \times 10^6$ K-562 cells in 100 µl of culture medium supplemented with 5% BSA were incubated for 30 min at 4°C with 20 µl of the 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 a FACSCalibur flow cytometer (Becton Dickinson). A total of 10,000 gated events were analyzed using CellQuest software (Becton Dickinson). Exclusion of nonspecific fluorescence staining was based on matched isotype mAb combinations conjugated with FITC. Results were expressed as percentage positive cells.

The decrease in antigen expression was calculated by the following formula:

$$\frac{(\text{Values before treatment} - \text{Values after treatment})}{\text{Values before treatment}} \times 100$$

### Cell Lysis

Culture supernatants before and after treatment of K562 cells were collected by centrifugation (500×*g* for 10 min). Cells were washed twice in cold PBS and lysed at a density of $2 \times 10^7$ ml in lysis buffer (1% Nonidet P-40, 50 mM Tris [pH 7.6], 300 mM NaCl, with 1 mM EDTA and 1 mM sodium orthovanadate) for 30 min on ice, passed through a 21 G needle and incubated for another 30 min on ice. Whole-cell lysates were clarified by centrifugation at 12,000×*g* for 10 min.

### Western Blotting for CD30 and CD45

For Western blot analysis, K-562 cells ($3 \times 10^6$ cells/ml) were stimulated with different concentrations of TNF (125, 250, 500 and 1,000 pg/ml) for 4 and 24 h. After activation, the supernatant was collected and the K562 cells were lysed in 1× lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM $Na_2EDTA$, 1% Triton, 1 mM $Na_3VO_4$) at 4°C for 1 h and then centrifuged at 14,000×*g* for 15 min. The supernatant was collected, and total protein was quantified. The total amount of the proteins of interest was studied by Western immunoblotting. After electrophoresis, proteins were electroblotted onto nitrocellulose membranes (Hybond ECL; GE Healthcare, Waukesha, WI). After transfer, filters were blocked in Tris-buffered saline containing 5% w/v nonfat dry milk and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO). Membranes were then incubated with primary antibody overnight at 4°C and with HRP-conjugated secondary antibody for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL).

### Apoptosis Investigation

The apoptosis and necrosis of K-562 cells ($1.0 \times 10^6$ ml of culture medium) were analyzed by flow cytometry at 2, 6, 8 and 24 h of treatment with TNF-α (0, 125, 250, 500 and 1,000 pg/ml of medium). According to the manufacturer's instructions, cells were further incubated in the dark with staining buffer (20 µl of propidium iodide [PI] and 20 µl of Annexin V in HEPES buffer) (Annexin V-FITC kit; Pharmingen, Hamburg, Germany) for 30 min on ice. After the incubation period, stained cells were resuspended in HEPES buffer and analyzed by the standard procedure with the FACS Calibur 440 E flow cytometer (Becton Dickinson). A total of 10,000 gated events were analyzed, using CellQuest software. Results were expressed as a percentage of necrotic and apoptotic cells, calculated as mean values from three to four independent experiments.

### Statistical Analysis

The data were analyzed by ANOVA and Mann–Whitney *U*-test, and comparison of slope curves by multivariate analyses used Excel (Microsoft, Redmond, WA).

## Results

TNF-α induced significant increases of LDH release activity from cultures of K-562 cells ($1.0 \times 10^6$ ml of culture medium) expressed as absorbance (Mann–Whitney *U*-test, $P < 0.05$) after 6 h compared to the first 2 h (Fig. 1).

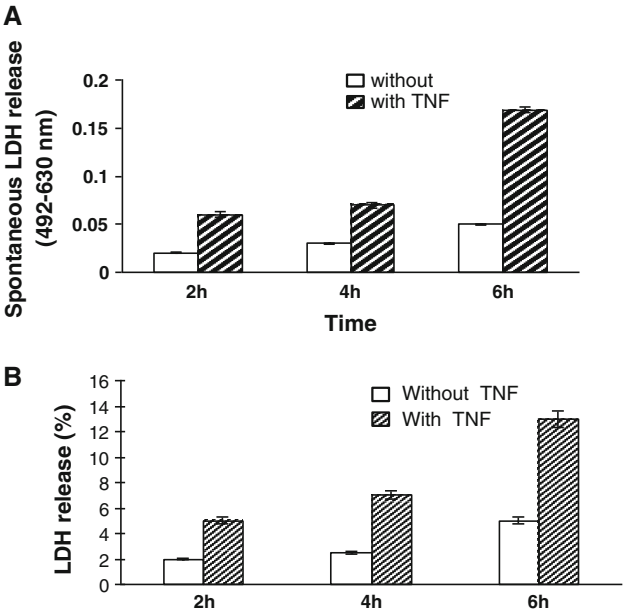


**Fig. 1** Significant increase (Mann–Whitney *U*-test, $P < 0.05$) of LDH release activity in supernatants from cultured K-562 cells ($1.0 \times 10^6$ ml of culture medium), expressed as absorbance determined after 2, 4 and 6 h induced with 1,000 pg/ml of TNF-α in comparison to spontaneous LDH (**a**). Percentage of LDH release through the cell membrane of K-562 cells (**b**) calculated from spontaneous LDH release with respect to total intracellular LDH showed significance in the presence of TNF-α (Mann-Whitney *U*-test)

When we analyzed spontaneous LDH release activity in terms of total intracellular LDH activity, results were expressed as a percentage of TNF-α toxicity. TNF-α after 6 h induced a significant increase in the percentage of LDH release (Fig. 1b) from K-562 cells (Mann–Whitney *U*-test, $P < 0.05$).

The immunophenotypic characteristics for selected antigens on K-562 cells before and after treatment with TNF-α are shown in Fig. 2a. The results showed diverse levels of expression of CD45, CD30, CD38, CD45RA and glycophorin A antigens on K-562 cells before and after TNF-α treatment when evaluated after 24 h. However, we analyzed the percentage decrease of selected membrane molecules, which were divided into two groups: high membrane molecule expression (CD45 and CD30) and low molecule expression, below 10% (CD45RA and CD38). Results are shown in Fig. 2b and c, respectively.

To better explain the change in the decrease of some antigens, we additionally calculated the rate of decrease for selected CD30 and CD45 molecules. This parameter is calculated from values obtained after treatments with respect to pretreatment values (formula is given in

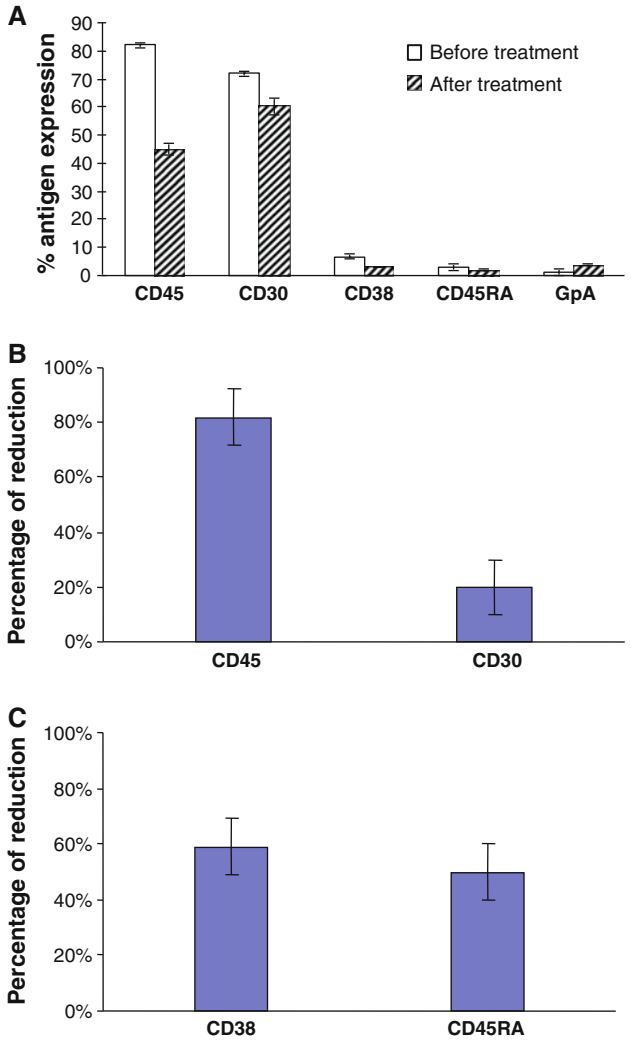


**Fig. 2** Cell surface expression of CD45, CD30, CD45RA, CD38 and glycophorin A (*GpA*) on K-562 cells before and after TNF-α treatment analyzed by flow cytometry and expressed as mean values for several repeated experiments with SEM (**a**). Rates of reduction of CD45 and CD30 expression (highly expressed) (**b**) and of CD45RA and CD38 (low expressed) were calculated from values after treatment with respect to pretreated values

Materials and Methods), and the results showed different degrees of reduction. The percentage of reduction of CD45 membrane molecules with median values of 82% (for several independent experiments) significantly differed (Mann-Whitney *U*-test, $P < 0.05$) compared to median values of 30% for CD30 molecule (Fig. 2b). However, the decrease of antigens on K-562 cells, which show very low pretreatment values (CD38 and CD45 RA), was similar (Fig. 2c) and without a statistically significant difference between them ($P > 0.05$, Mann–Whitney *U*-test). Contrary to this, glycophorin A showed significantly increased values after TNF-α treatment, although it cannot be used as a referent (Fig. 2a).

Kinetic study of shedding of CD45 and CD30 shows dose- and time-dependent decreases, with maximal effects after 24 h. Moreover, investigation of the dose-dependent effects of TNF-α on shedding of CD45 and CD30 indicates that 1,000 pg/ml of TNF-α induced a statistically significant decrease in comparison to 125 pg/ml (Mann–Whitney *U*-test, $P < 0.05$) (Fig. 3a, b, respectively). Representative dot plots for CD30 and CD45 shedding from K-562 cells without and with 1000 pg/ml of TNF-α after 4 and 24 h are given in Fig. 3c.

Analyses of apoptosis by flow cytometry indicate a significant increase of degree of apoptosis induction (Mann–Whiteny *U*-test, $P < 0.05$) after 24 h with 1,000 pg/ml of TNF-α compared to the same concentration after 4 h of treatment (Fig. 4a). We give representative dot plots of TNF-α-induced apoptosis in K-562 cells (Fig. 4b) after 4 and 24 h with 1,000 pg/ml of TNF-α.

Furthermore, we performed Western blot analyses with the aim to better explain the finding of a TNF-α-induced decrease of membrane molecules from K-562 tumor cells. We confirmed the appearance of CD45 and CD30 molecules in cell culture supernatant after K-562 cell treatment with TNF-α (Fig. 5).

The analyses of antigen shedding from treated K-562 cells indicated early appereance of the investigated molecules, after 4 h. Shed CD30 molecules were detected at 35 kDa, which corresponds with the known soluble CD30 molecular form (sCD30).

In addition, the quantity of shed molecules was much greater after 24-h treatment (Fig. 4b) in comparison to 4 h (Fig. 5a). Moreveor, after 24 h, shedding of these molecules appears to be dose-dependent. On the contrary, analyses of CD30 and CD45 in K-562 cell lysate show greatly reduced concentrations of these molecules after 24-h treatment with TNF-α (Fig. 5b) compared to 4 h (Fig. 5a).

## Discussion

Based on the possibility of TNF-α inducing opposite and diverse effects (Nehar et al. 1997; Okazaki et al. 1998; Arnott et al. 2002), this is an area of great interest for research. Recently, we used three different methods for estimating cell death processes and for analyzing the degree of cell membrane alterations for better data evaluation and understanding of complex events on cell membrane associated with exogenous TNF-α (Marjanovic et al. 1991; Jurisic et al. 2000; Jupp et al. 2003). This is an actual problem, and for its consideration several techniques for analyses of cell death can be used (Okazaki et al. 1998; Pollock et al. 2000).

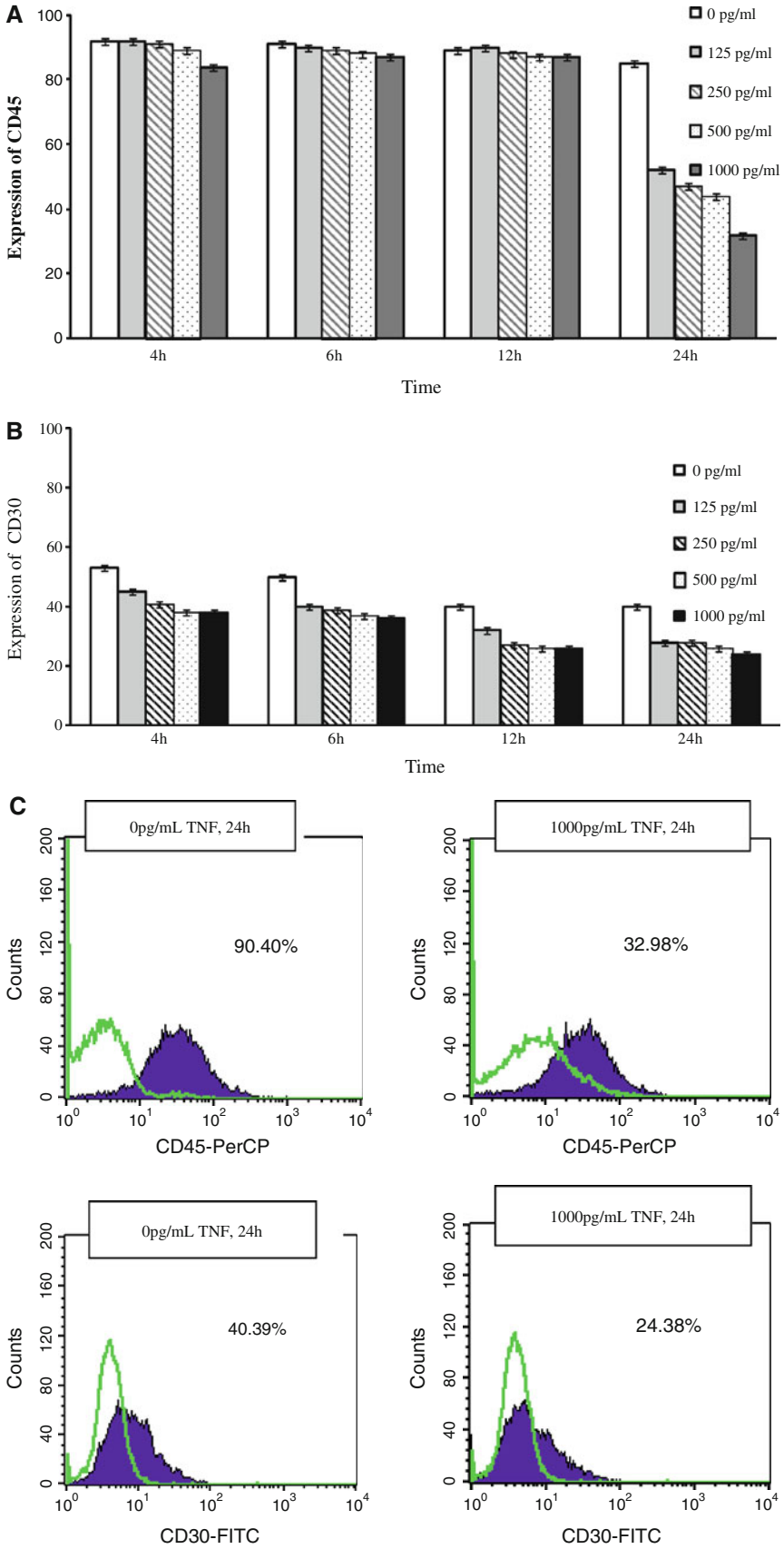


**Fig. 3** Expression of CD45 (**a**) and CD30 (**b**) molecules on K-562 cells before and after TNF-α treatment in a dose- and time-dependent manner. Cells were treated with TNF-α for 4, 6, 12 and 24 h at concentrations of 125, 250, 500 and 1,000 pg/ml. Untreated cells were used as negative control.
**c** Representative histogram of CD30 and CD45 expression on K-562 cells after 24-h treatment with and without TNF-α (1,000 pg/ml)

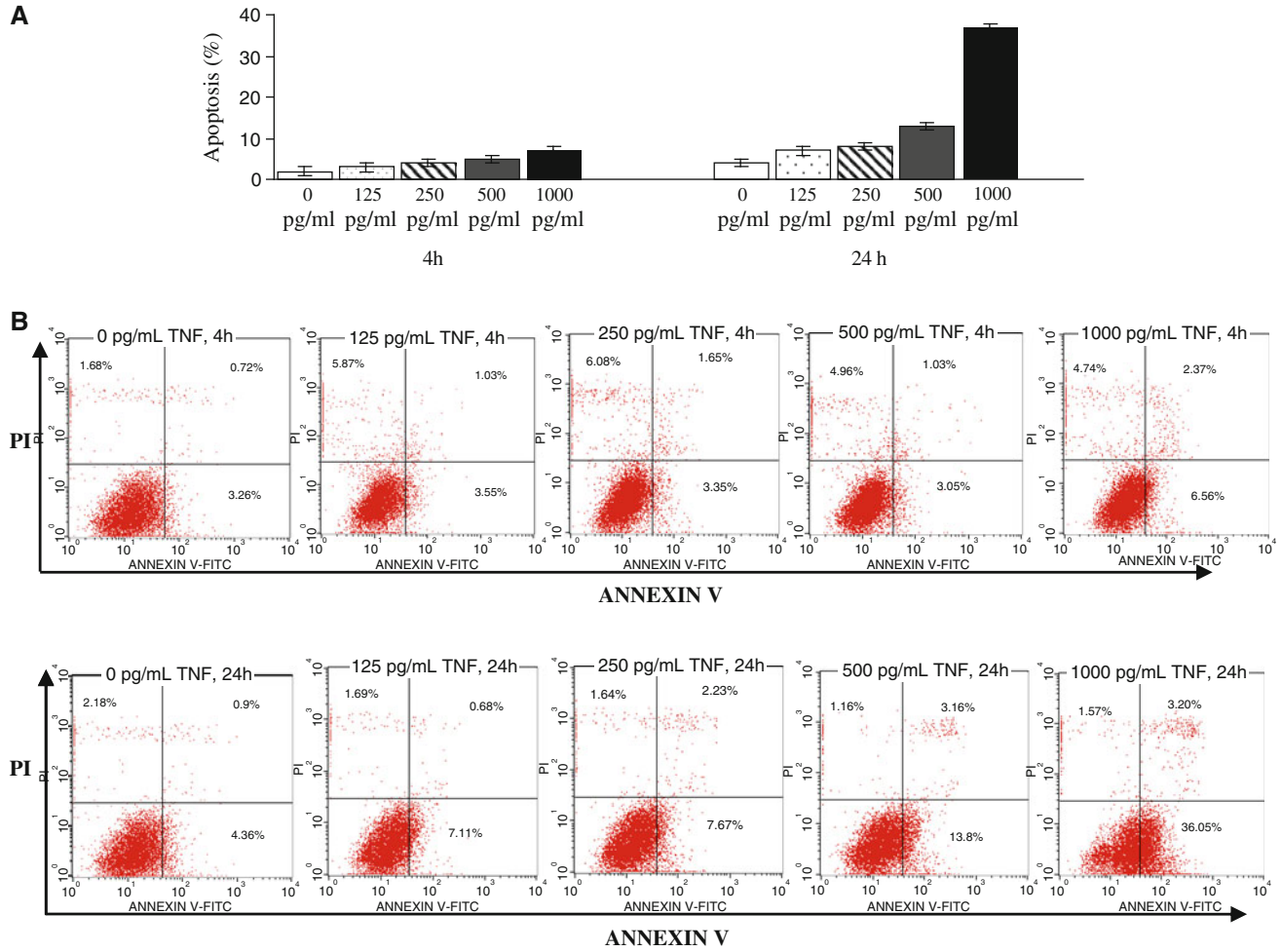


**Fig. 4** **a** Dose-dependent effects of TNF-α on apoptosis induction. K-562 cells were incubated in the presence of TNF-α (125, 250, 500, 1,000 pg/ml) or its absence (control) for 4 and 24 h. Cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. **b** Representatives dot plots showing significant percentage of early apoptotic cells (*lower right quadrant*) after 24-h incubation with TNF-α

We previously reported that TNF-α can induce an increase of LDH release activity in supernatants of cultured K-562 cells, indicating an early mechanism in LDH transport through the cell membrane (Konjevic et al. 1997; Decker and Lohmann-Matther 1998; Jurisic 2003; Goto et al. 2000). Based on this, we further focused on the molecular mechanisms of LDH release in comparison to changes in cell membrane expression of CD45 and CD30 following TNF-α-mediated cell membrane disintegration.

CD45 is a type I transmembrane molecule found on leukocytes and exclusively on nucleated hematopoietic cells and their precursors, including K562, erythromyeloid cells in blast transformation (Penninger et al. 2001; Vajant and Scheurich 2001; Barbui et al. 2006). CD30 is expressed on lymphoma cells, in Hodkin disease, on large-cell anaplastic lymphoma and on normal PBLs. We here report a dose- and time-dependent decrease in its expression after TNF-α treatment that is associated with increase of LDH release, suggesting cell death processes. In case TNF-α induces direct and prompt effects on the cell membrane disintegration following induction of necrotic cell death, we would expect an identical loss and a uniform decrease of all examined cell surface molecules.

However, there was no uniform decrease of the examined molecules as we obtained a smaller decrease of CD30 molecule in comparison to a higher decrease of CD45 molecule after treatment. The persistence of CD30 molecules on K-562 cells after treatment indicates that TNF-α does not destroy the cell membrane directly and fully, which follows from flow-cytometric data indicating only 30% apoptosis after 24-h cell cultures.

This finding indicates another mechanism by which TNF-α induces LDH release, as well as decreasing membrane molecules that is not solely dependent on apoptosis. This can occur by activation of some intracellular enzymes

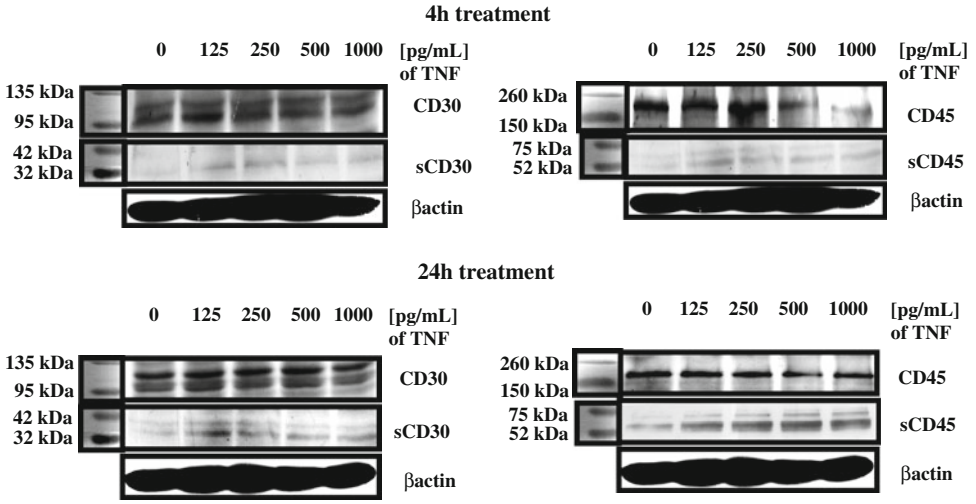


**Fig. 5** TNF-α stimulates shedding of CD45 and CD30 molecules from K-562 cells. CD45 and CD30 were detected in cell lysate and cell-free supernatant as soluble molecules (sCD45 and sCD30) by Western blot. Cells were treated for 4 and 24 h with TNF-α (125, 250, 500 and 1,000 pg/ml). Untreated cells were used as controls. *β*-actin was used as loading control

(serine kinase, protein kinase C, sphingomyelinase, phospholipase C) associated with TNF-α-mediated signaling (Porter 1999; Wissing et al. 1997; Okazaki et al. 1998; Chen et al. 1999; Voelkel-Jonson et al. 1995; Jupp et al. 2003), which supports our finding that TNF-α mediated early release not only of LDH but also of CD30 and CD45 in cell supernatants within 2–4 h.

Two membrane metalloproteinases, ADAMS 10 and 17, have emerged as key molecules in most of the shedding events characterized to date (Eichenauer et al. 2007; Vahdat et al. 2010). TNF-α converting enzyme (TACE) was the first discovered mammalian enzyme to play a critical role in ectodomain shedding. TACE proteolytically cleaved not only TNF but also TGF-α, TNF receptors and L selectin and can participate in the shedding of CD30 and CD45 that we describe here.

Our studies of the time- and dose-dependent kinetics of the TNF-α-mediated effect on K562 cells show that shedding of CD30 and CD45 is associated with LDH release. Moreover, we here for the first time report that this shedding, similar to LDH release, is an early event on the cell membrane. This shedding is not identical for CD45 and CD30 regarding kinetics. The shedding of CD30 preceded apoptosis, which was evident at a later time point, i.e., after 24 h of treatment. It has been shown that various cell surface receptors and molecules can be shed after various in vitro activations, such as PHA, anti-CD3 and bortezomib, or even constitutively, which supports our findings that CD30 is shed during cultures of K562 cells not only without TNF-α treatment but also much more under different time and dose conditions during TNF-α treatment.

Furthermore, we performed Western blot analyses with the aim of explaining the finding of TNF-α-induced decreases of the cell membrane molecules from K-562 tumor cells. We confirmed the appearance of the CD45 and CD30 molecules in cell culture medium after treatment. This indicates that TNF-α induces shedding of these molecules since their appearance in the cell cultured medium correlated with a decrease of these molecules on the cell membrane analyzed by flow cytometry and, of the cell lysate, by Western blotting.

Based on the consideration that cell membranes transduced many signals for apoptosis, necrosis or activation after TNF-α treatment, we conclude that interaction of TNF-α with cell membrane molecules, partly of the TNF-α receptor superfamily, including CD30, indicates the presence of a very complex and dynamic process.

In this sense, our study confirms a contribution of TNF-α to cell surface molecule release that sets in more rapidly than its contribution to cell killing. Therefore, we conclude that CD30 and CD45 shedding is not a consequence of apoptosis but occurs earlier or in parallel.

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