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Alternative functions for TRAIL receptors in eosinophils and neutrophils[1]

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Summary

Background: Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in many tumour cells but only rarely in normal cells. The receptors of TRAIL belong to the superfamily of tumour necrosis factor (TNF)/nerve growth factor (NGF) receptors. Here we investigate TRAIL receptor expression and function in eosinophils and neutrophils.

Methods: Granulocytes were isolated from human blood and purified using standard protocols. Receptor expression was analysed by reverse transcription (RT)-PCR and flow cytometry. Cell death was analysed by the ethidium bromide exclusion test. Apoptosis was determined by analysing phosphatidyl serine (PS) surface exposure and morphological evaluation.

Results: Freshly purified eosinophils and neutrophils expressed TRAIL-R1, TRAIL-R3, and TRAIL-R4, but not TRAIL-R2 surface proteins. Stimulation of eosinophils with TRAIL resulted in either inhibition of apoptosis or no effect, depending on the individual from whom the cells were isolated. In neutrophils, TRAIL stimulation did not influence apoptosis. In eosinophils and neutrophils which showed no effect on TRAIL stimulation alone, TRAIL partially blocked cytokine-mediated antiapoptosis.

Conclusions: Both eosinophils and neutrophils express functional TRAIL receptors on their surface. In contrast to tumour cells, TRAIL does not induce apoptosis in granulocytes but rather induces survival in eosinophils from approximately 50% of the donors. Alternatively, TRAIL may limit cytokine-mediated antiapoptosis under certain inflammatory conditions.

Keywords: apoptosis; eosinophils; inflammation; neutrophils; TRAIL receptors

Introduction

Receptors of the TNF/NGF family have been shown to mediate a number of physiological functions including induction of apoptosis [1]. A subset of this family contains, in addition to the cysteine-rich motifs found in the extracellular domain of all members, a common intracellular sequence able to induce apoptosis in some systems and termed death domain. Six of these "death receptors" have been described so far. These include the TNF receptor (R)1 [2], Fas/Apo-1/CD95 [3], TRAMP/DR3/Apo-3/WSL-1/LARD [4, 5], TRAIL-R1/DR4 [6], TRAIL-R2/DR5/KILLER [7, 8], and DR6 [9]. Some of these death receptors, however, have been shown to be capable of transducing extracellular signals via alternative pathways. For instance, engagement of the TNF receptor can lead to either survival or apoptosis [1]. In addition to its involvement in apoptosis, the TNF receptor has also been shown to induce necrosis in some systems [10]. Accordingly, signalling through death receptors is not restricted to apoptosis induction but may also make use of alternative pathways.

TNF-related apoptosis-inducing ligand or TRAIL (Apo-2L) has widely distributed expression and has been shown to induce apoptosis in a number of transformed cells [11, 12]. While transformed cell lines and tumour cells are sensitive to TRAIL-induced apoptosis, most normal cells are resistant to it [11–14]. However, activated T cells have also been shown in some cases to be sensitive to TRAIL-induced apoptosis [15, 16]. TRAIL has recently been reported to induce apoptosis in normal hepatocytes [17] and to block cell cycle progression [18].

TRAIL has been shown to bind to five receptors of the TNF/NGF receptor family; two signalling receptors and three modulatory or "decoy" receptors. TRAIL-R1 (DR4) and TRAIL-R2 (DR5/KILLER) contain a death domain and can induce apoptosis in some systems [6–8]. However, TRAIL-R3 (Trid/DcR1) and TRAIL-R4 (DcR2)

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have no, or only partial, death domains necessary to induce apoptosis, and have been shown instead to protect against TRAIL-induced apoptosis [7, 8, 19, 20]. In addition, a soluble secreted TRAIL receptor called osteoprotegerin (OPG) has also been shown to inhibit TRAIL signalling [21]. The presence of several functional and decoy receptors allows for major modulation of the cellular response to TRAIL. Engagement of the functional TRAIL receptors TRAIL-R1 and TRAIL-R2 has been shown to activate the same signalling pathways as reported for other death receptors. For instance, they have the ability to use the adapter molecules FADD and TRADD and to induce caspase, NF-κB, and JNK activation in different cell types [16, 22–25].

In this paper we report on TRAIL receptor expression and function in granulocytes. Both neutrophils and eosinophils express mRNA for all four membrane-bound TRAIL receptors as well as three TRAIL receptor proteins (TRAIL-R1, TRAIL-3, and TRAIL-R4) on their surface. Functionally, these TRAIL receptors are unable to induce granulocyte apoptosis upon ligand binding. However, we have observed alternative responses to TRAIL.

Material and methods

Media and reagents

The complete culture medium was RPMI 1640 (Life Technologies, Inc., Basel, Switzerland) supplemented with 2 mM L-glutamine, 200 IU/ml penicillin, 100 μg/ml streptomycin, and 10% foetal calf serum. FITC-labelled Annexin V was manufactured by Pharmingen (Hamburg, Germany). Recombinant soluble TRAIL was manufactured by Alexis (Läufelfingen, Switzerland). GM-CSF was a kind gift from Dr. T. Hartung (University of Konstanz, Konstanz, Germany). G-CSF, IL-5, IFN-γ, and goat anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3, and anti-TRAIL-R4 were manufactured by R&D Systems (Abingdon, UK). Normal goat IgG was manufactured by Santa Cruz Biotechnologies (Santa Cruz, CA). Normal mouse IgG1 and rabbit anti-goat Ig FITC-conjugated antibodies were manufactured by Dako (Zug, Switzerland). Goat anti-mouse IgG PE-conjugated antibody was manufactured by BioSource International (Camarillo, CA). Sheep anti-mouse Ig horseradish peroxidase (HRP)-conjugated was manufactured by Amersham (Zürich, Switzerland). Unless stated otherwise, all other reagents were manufactured by Sigma.

Cell isolations

Neutrophils were isolated from peripheral blood of healthy donors and eosinophils from atopic patients routinely presenting mild blood eosinophilia (6–10% blood eosinophils). Granulocytes were isolated as previously described [26, 27] by Ficoll-Hypaque (Seromed-Fakola AG, Basel, Switzerland) centrifugation. After removal of the PBMC, erythrocytes were lysed (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.3) and the remaining population chiefly contained neutrophils. Eosinophils were further isolated by negative selection using anti-CD16 mAb-labelled microbeads and a magnetic cell separation system (MACS, Miltenyi Biotec) employing a type C column attached to a 21-gauge needle in a permanent magnetic field [28, 29]. Cell purity was assessed by staining with Diff-Quik (Baxter, Düdingen, Switzerland) and light microscopy analysis. The resultant populations contained at least 95% neutrophils or 98% eosinophils.

Cell cultures, viability measurements, and apoptosis assays

Cells were cultured in RPMI 1640 containing 10% FCS. When indicated, GM-CSF, G-CSF, IL-5, and IFN-γ were added at a concentration of 50 ng/ml, soluble recombinant TRAIL at 100 ng/ml and its enhancer at 2 μg/ml. Cell viability was measured by ethidium bromide (1 μM) exclusion and flow cytometric analysis (EPICS XL, Coulter) after 40 hours in culture as previously described [26–29]. Exposure of phosphatidylserine (PS) on the surface of apoptotic cells was performed after 24 hours in culture using FITC-labelled Annexin V according to the manufacturer's instructions (Pharmingen). Cells were also examined morphologically to determine apoptosis [30]. Cytospin preparations were made and stained with Diff-Quik, and apoptotic and non-apoptotic cells were counted.

mRNA isolation and RT-PCR

Total RNA isolation from 10 million cells using an RNA isolation kit (Stratagene, Basel, Switzerland) was followed by first-strand cDNA synthesis (Pharmacia Biotech, Uppsala, Sweden) on the entire RNA preparation, according to the manufacturers' instructions. PCR amplification was carried out using primers synthesised (Microsynth BmgH, Balgach, Switzerland) as follows. TRAIL-R1: 5'-CAA TTC TGC TGA GAT GTG CCG-3' and 5'-CAT CAG CAT TGC ATA CAA GGC-3'. TRAIL-R2: 5'-GTA CAC CCT GGA GTG ACA TCG-3' and 5'-CCA GAG CTC AAC AAG TGG TCC-3'. TRAIL-R3: 5'-CCA AGA CCC TAA AGT TCG TCG-3' and 5'-CAA CAC ACT GGA TAT CAT CCC-3'. TRAIL-R4: 5'-CAC TAC CTT ATC ATC ATA GTG GTT TT-3' and 5'-GAA GGA CAT GAA CGC CGC CGG AAA AG-3'. Primers for the glyceraldehyde 3-phosphate dehydrogenase gene were used for quality control of the cDNA preparations. One μl of cDNA was amplified in 25 μl of a reaction mixture containing amplification buffer (Life Technologies), $MgCl_2$ (2.5 mM), W-1 detergent (0.05%), dNTPs (250 μM each), primers (0.8 μM each), and Taq polymerase (1 U). The cycling parameters were as follows: 30 cycles of 94 °C for 30 seconds, 55 °C for 60 seconds, and 72 °C for 90 seconds, followed by 7 minutes at 72 °C. The amplification products (TRAIL-R1: 645 bp, TRAIL-R2: 680 bp, TRAIL-R3: 448 bp, TRAIL-R4: 175 bp) were separated on 1% agarose gels and transferred to reinforced nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The blots were hybridised to FITC-labelled probes (random primer fluorescein labelling kit, Dupont NEN Life Science Products, Regensdorf, Switzerland) from which the sequence had been determined to confirm their identity.

Immunostaining

Freshly isolated eosinophils and neutrophils (2.5×10^5) were stained for the presence of total TRAIL receptors by resuspension in 50 μl PBS containing 0.2% BSA (PBS-BSA) and incubation for 30 minutes with 100 ng/ml recombinant soluble TRAIL and 0.6 μg/ml enhancer (mouse IgG). After one wash with the PBS-BSA solution,

the cells were incubated again in 50 µl PBS-BSA containing the secondary antibody anti-mouse IgG PE-conjugated. Normal mouse IgG antibody control was also used at 0.6 µg/ml. After a final PBS wash, cells were fixed in 200 µl of 2% paraformaldehyde in PBS, and analysed by flow cytometry (EPICS XL). Staining of freshly isolated or cultured eosinophils or neutrophils for specific TRAIL receptors was done similarly, using 0.5 µg of specific or control antibodies and anti-goat Ig-FITC-conjugated secondary antibody, with 15 minutes' incubation each. After a final PBS wash, cells were suspended in PBS-BSA and immediately analysed by flow cytometry (EPICS XL).

Statistical analysis

Results are expressed either as single data or as means ± standard errors of the means (SEM) for the number of experiments indicated. Student's *t*-test for paired values was used to calculate statistical significance. Statistically significant differences with a probability value of $p < 0.05$ are shown by an asterisk.

Results

TRAIL receptors are expressed by both eosinophils and neutrophils

We first measured TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 mRNA levels by RT-PCR in freshly isolated eosinophils and neutrophils. As shown in figure 1, both eosinophils and neutrophils expressed mRNA for all four receptors. To determine whether the expression of TRAIL receptor mRNAs correlates with the expression of their surface proteins, we performed flow cytometric studies. As seen in figure 2, freshly isolated eosinophils and neutrophils expressed TRAIL-R1, TRAIL-R3, and TRAIL-R4. In contrast, no detectable levels of TRAIL-R2 were observed on eosinophil and most neutrophil preparations (in some neutrophil preparations up to 20% of the cells expressed TRAIL-R2; not presented). These observations suggest that TRAIL-R1 may be the major TRAIL receptor able to transduce signals in both eosinophils and neutrophils. TRAIL-R1, TRAIL-R3, and TRAIL-R4 expression was not influenced by the presence or absence of the survival cytokines GM-CSF and IL-5 in 20-hour eosinophil and neutrophil cultures (not presented).

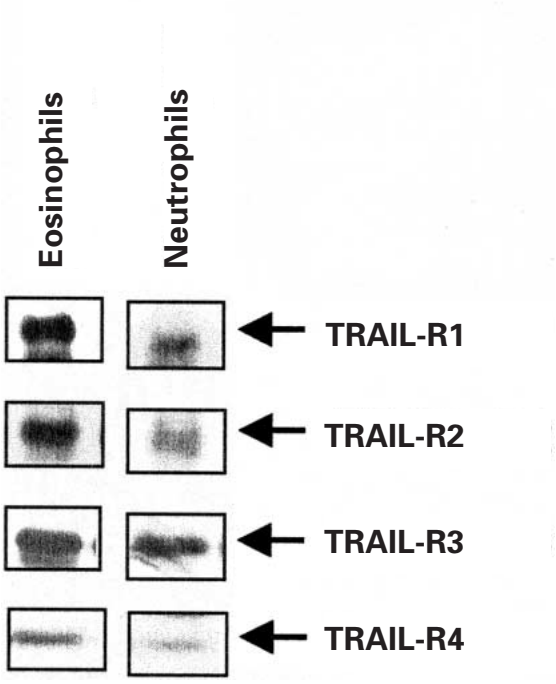


Figure 1

TRAIL receptor mRNAs are expressed by granulocytes. mRNAs for TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 were detected in both eosinophils and neutrophils using RT-PCR. These results are representative of three independent experiments.

TRAIL frequently acts as a survival factor for eosinophils but not for neutrophils

To determine the effect of TRAIL on eosinophil death, we cultured eosinophil populations from different donors either in medium alone or in the presence of recombinant soluble TRAIL. Cell viability was measured after 40 hours in culture. The results of these preparations could be subdivided into two groups. As shown in figure 3 (upper panels), TRAIL either induced longer survival (n = 6) or had no detectable effect (n = 5). In contrast, TRAIL-induced survival was not observed in neutrophils cultured under the same conditions (fig. 3, lower panel). In eosinophils the two possibilities were of almost equal frequency. In no case did TRAIL induce granulocyte death.

In the absence of survival factor activity, TRAIL blocks cytokine-mediated survival in both eosinophils and neutrophils

We were also interested in analysing the effect of combined cytokine plus TRAIL stimulations. Interestingly, the presence of TRAIL in combination with survival factors resulted in partial inhibition of the survival signal provided by GM-CSF, G-CSF, IL-5, and IFN-γ in those granulocyte populations which did not show TRAIL-induced longer survival (fig. 3, right upper panel and lower panel). This inhibitory effect was not observed in the eosinophil preparations sensitive to TRAIL-induced survival (fig. 3, upper left panel).

Inhibition of cytokine-mediated survival by TRAIL allows rapid granulocyte apoptosis

To investigate whether TRAIL-induced changes in granulocyte death were apoptosis, we determined phosphatidylserine (PS) exposure in 24-hour cultures. Dying cells lose membrane asymmetry and expose PS on their surface, and this expression constitutes an early marker of cell death. PS exposure can be measured by binding to labelled Annexin V; PS negative cells represent viable cells. As seen in figure 4A, TRAIL had similar effects on granulocyte PS exposure to those measured by ethidium bromide exclusion (fig. 3), namely one group of eosinophils is induced to survive longer (more PS negative cells) by TRAIL, whereas other eosinophils as well as most neutrophils are not. In these cells, however, TRAIL

can partially inhibit the survival signal provided by GM-CSF, as seen by partial blocking of inhibition of PS exposure in both neutrophils and eosinophils.

Furthermore, morphological analysis of granulocytes (fig. 4B) to visualise the degree of apoptosis in cells treated with TRAIL confirmed the PS exposure data. Cells undergoing apoptosis show, amongst other features, reduced size and a condensed nucleus, easily distinguishable in the case of polymorphonuclear cells such as granulocytes [30]. Cells were stained after 24-h in vitro stimulation and counted under light microscopy. As shown in figure 4B, the first group of eosinophils responded to TRAIL with decreased apoptosis. In the other group of eosinophils as well as in neutrophils, TRAIL had no influence on apoptosis but partially blocked GM-CSF-mediated delayed apoptosis.

Figure 2

TRAIL receptor surface proteins are expressed by granulocytes. TRAIL receptors were detected by flow cytometry using specific monoclonal antibodies. TRAIL-R1, TRAIL-R3, and TRAIL-R4, but not TRAIL-R2, were detected in both eosinophils and neutrophils. Specific staining is shown by filled curves and control antibody staining by open curves. These results are representative of three independent experiments.

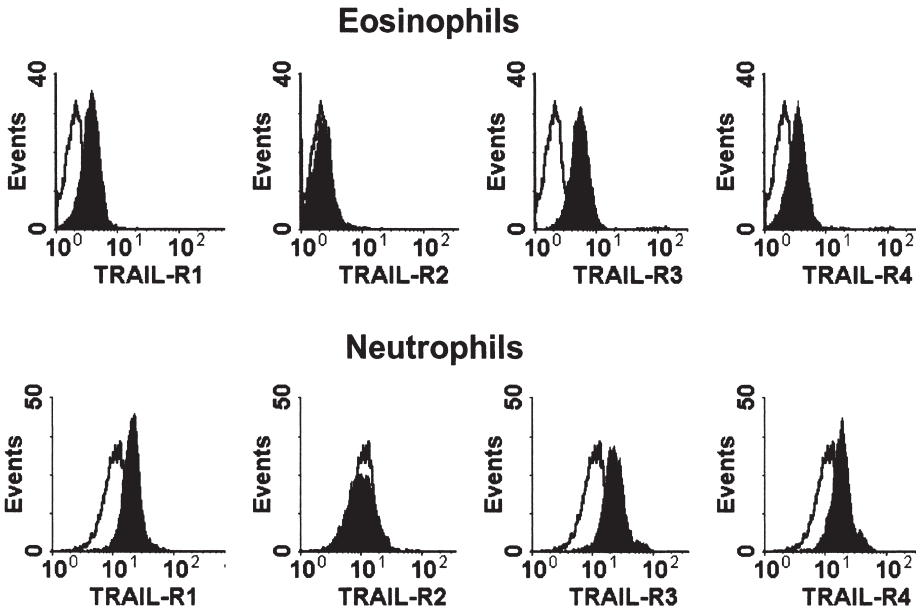
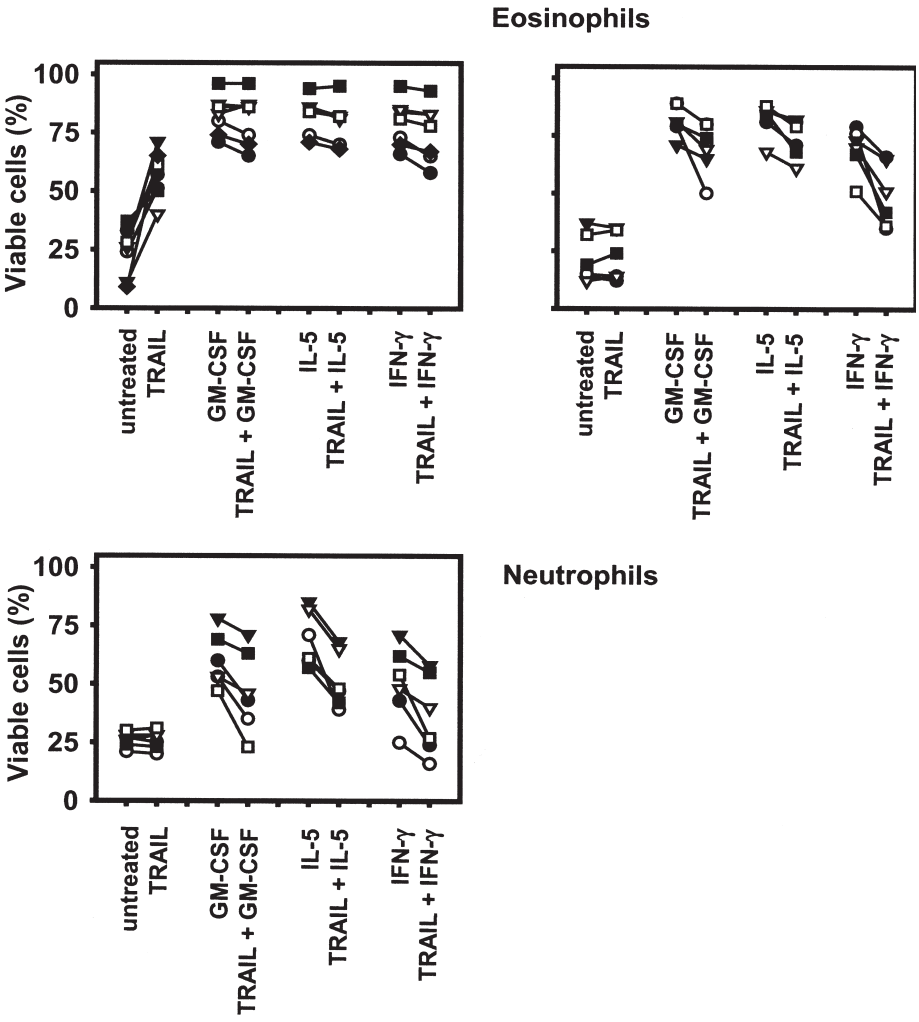


Figure 3

Cell death regulation by TRAIL in vitro. Granulocytes were cultured alone or in the presence of TRAIL with enhancer for 40 hours. When indicated, cytokines were added at a concentration of 50 ng/ml. Cell viability was measured by ethidium bromide exclusion and flow cytometric analysis.
Upper left panel: TRAIL can induce survival in a proportion (6 out of 11) of eosinophil populations (untreated: 27 ± 4%; TRAIL: 55 ± 4%; GM-CSF: 85 ± 3%). In these eosinophil populations, TRAIL did not inhibit survival induced by GM-CSF, IL-5, or IFN-γ. Six independent experiments are presented.
Upper right panel: In other eosinophil populations (5 out of 11), TRAIL treatment had no direct effect on survival (untreated 27 ± 4%; TRAIL: 25 ± 3%; GM-CSF: 78 ± 4%). TRAIL partially inhibited survival signals provided by GM-CSF, IL-5, and IFN-γ in these eosinophil preparations.
Lower panel: TRAIL did not induce neutrophil survival or death (untreated: 30 ± 3%; TRAIL: 33 ± 4%; GM-CSF; 54 ± 4%). However, TRAIL partially inhibited the survival signals provided by GM-CSF, G-CSF, and IFN-γ. Six independent experiments are presented. Results using a concentration of TRAIL of 100 ng/ml are presented, but similar results were obtained using TRAIL in a concentration range of 10 to 200 ng/ml (not presented).



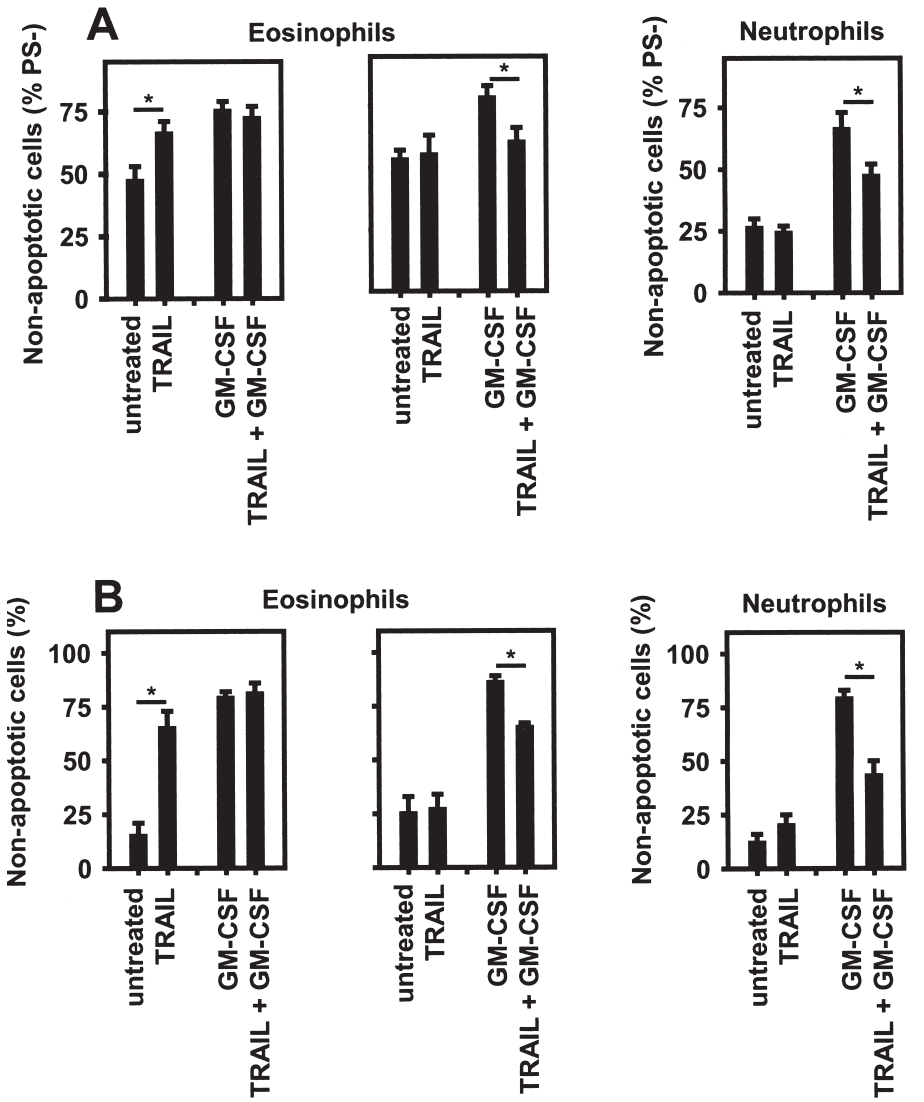


Figure 4

Apoptosis regulation by TRAIL in granulocytes in vitro. Phosphatidylserine (PS) surface exposure (A) and morphological examination (B) were used to detect apoptosis. Granulocytes were cultured for 24 h. Values are means ± SEM of four independent experiments in each case. * $p < 0.05$. The results correlate with the data presented in figure 3. TRAIL inhibits apoptosis in a proportion of eosinophil populations but not in others and not in neutrophils. In the latter, however, survival induced by GM-CSF is partially inhibited by TRAIL.

Discussion

TRAIL and TRAIL receptors are widely expressed, but have been shown to be chiefly active in inducing apoptosis in transformed cells [6–8, 11–14, 22]. In this article we provide evidence of functional TRAIL receptors in granulocytes. Freshly purified eosinophils and neutrophils express TRAIL-R1, TRAIL-R3, and TRAIL-R4, but not TRAIL-R2. Since TRAIL-R3 and TRAIL-R4 are decoy receptors [7, 8, 19, 20], our observations suggest that TRAIL-R1 is the relevant signalling receptor for TRAIL in both eosinophils and neutrophils. However, the possibility exists that undetectable amounts of TRAIL-R2 are present on the cell surface which may transduce signals. It has been noted that TRAIL-R2 has a higher affinity to its ligand than does TRAIL-R1 [31].

Despite the observation that granulocyte populations from different donors express a similar complement of the three membrane-bound TRAIL receptors, we partially observed different responses to TRAIL. In the eosinophil populations we observed either no effect or increased survival as a consequence of single TRAIL treatment. The anti-death effect is significant, even though it is not as substantial as survival induced by cytokines such as GM-CSF or IL-5. The mechanism of TRAIL-mediated delayed apoptosis may involve activation of NF-κB as previously observed in the case of TNF-α [32]. The exact mechanisms of TRAIL signalling remain to be elucidated.

In a search for a factor influencing the differential eosinophil responses to TRAIL, we carefully investigated eosinophil numbers, IgE levels, and the allergic phenotype of the eosinophil donors. These parameters did not appear to determine the response of eosinophils to TRAIL (not presented). Therefore, other factors may be present within cells which regulate responses to TRAIL. Although such factors have not yet been identified in granulocytes, in the light of previously reported observations it may be postulated that a possible factor regulating TRAIL responses is cellular FLIP [33, 34].

In eosinophils and neutrophils insensitive to TRAIL-induced survival, TRAIL receptor engagement did not induce apoptosis but was capable of partially blocking GM-CSF, G-CSF, IL-5, and IFN-γ mediated inhibition of apoptosis. This suggests that at least one TRAIL receptor present on granulocytes is capable of delivering inhibitory signals on cytokine signal transduction, despite the lack of direct apoptosis induction in this system. Both induction of survival and blocking of survival signals in granulocytes represent two newly described alternative pathways for TRAIL receptors other than apoptosis induction. It is interesting to note that they do not overlap.

We observed that eosinophils express large quantities of TRAIL-R3 and TRAIL-R4. These receptors have been shown to act as decoy receptors in some systems and to block TRAIL-induced apoptosis [7, 8, 19, 20]. The use of such a decoy receptor system to control receptor signalling has recently been reported in the case of Fas as well [35]. It is likely that TRAIL-R3 and TRAIL-R4 prevent induction of apoptosis through TRAIL-R1 and TRAIL-R2 in granulocytes, by reducing the effective amount of ligand available to bind to these receptors. However, TRAIL can induce granulocyte survival and inhibition of survival factors, despite the expression of large amounts of TRAIL-R3 and TRAIL-R4. It is possible that the decoy receptors are inactive in controlling these TRAIL responses in granulocytes. This assumption is based on observations made in melanoma cells, where TRAIL decoy receptors do not seem to regulate TRAIL receptor-induced death, which, rather, is regulated by expression of the inhibitor FLIP [34].

The results presented in this study indicate that TRAIL receptors are able to transduce a signal not only in transformed cells but also in primary cells such as granulocytes. Signalling through TRAIL receptors in granulocytes does not result in induction of apoptosis as observed in transformed cells [11, 12], activated T cells [15, 16], or hepatocytes [17], but is also involved in regulating cell numbers either by inducing survival of eosinophils or by limiting survival of granulocytes exposed to cytokines [27, 36]. Similarly, TRAIL has recently been shown to inhibit autoimmune inflammation by blocking cell cycle progression of lymphocytes, in the absence of apoptosis induction [18]. Furthermore, in the immune system, several cells can express TRAIL. For instance, monocytes, T cells, and dendritic cells have been shown to express TRAIL upon activation with different factors, including interferons [11, 37, 38]. Taken together, it appears that regulation of signalling by TRAIL, and perhaps by other "death factors", is even more complex than initially anticipated; the identification of factors regulating such responses promises to be of interest in furthering our understanding of inflammatory processes.

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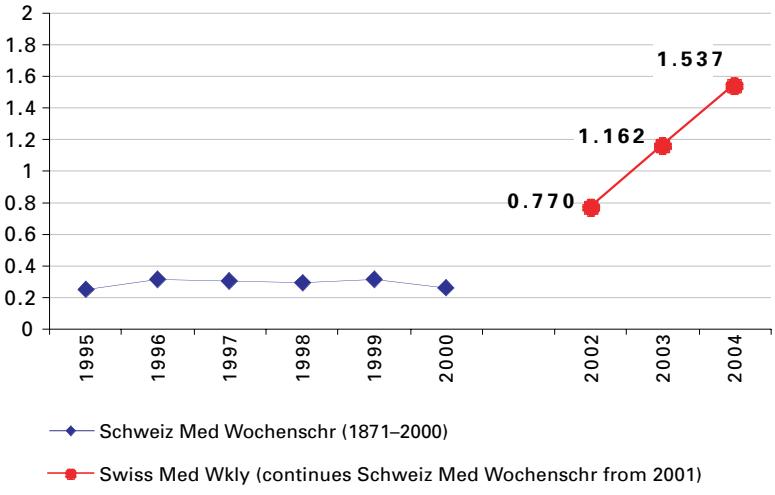

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