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Interferons induce proteolytic degradation of TRAILR4 ☆

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Abstract

IFNγ and its transcriptional target tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) are two major effector molecules of activated CTLs and NK cells. Here, we show that IFNγ as well as the type I interferon IFNα strongly inhibit cell surface expression of the decoy receptor TRAILR4 while having only a moderate inhibitory or even an inducing effect on TRAILR2 and CD95. Interferon-induced inhibition of TRAILR4 expression was blocked by a protease inhibitor cocktail and also by MG132, suggesting that down-regulation of TRAILR4 involves the proteasome. Inhibition of TRAILR4 expression by siRNA sensitized for TRAIL-, but not CD95L-induced apoptosis. Thus, the apoptosis-inducing action of interferons may not only rely on the well-established induction of TRAIL in effector cells but also on concomitant down-regulation of its antagonizing decoy receptor TRAILR4 in target cells.

Keywords: Apoptosis; IFNα; IFNγ; Proteasome; TRAIL; TRAILR4

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), also known as Apo2L, is a typical member of the TNF ligand family and has been implicated in tumor surveillance, killing of virus-infected cells, and control of autoreactive T-cells [1,2]. TRAIL interacts with five members of the TNF receptor superfamily: The two death receptors TRAILR1 and TRAILR2, the transmembrane decoy receptor TRAILR4, the glycosyl phospholipid anchored decoy receptor TRAILR3, and the soluble decoy receptor osteoprotegerin OPG, which also interacts with receptor activator of nuclear factor κB ligand (RANK-L), a TNF family member involved in the regulation of osteoclastogenesis and T-cell activation [1]. In accordance with its biological functions, TRAIL is predominantly expressed in immune cells such as natural killer cells and cytotoxic T-cells. Moreover, TRAIL expression is induced by type I and type II interferons as well as by NFκB stimulating proinflammatory cytokines [3–7]. Notably, IFNγ-induced TRAIL expression in activated effector cells of the immune system is complemented by IFNγ-mediated sensitizing of potential target cells for death receptor-induced apoptosis [8]. Enhancement of death receptor-induced apoptosis by IFNγ requires pretreatment for 1–2 days and has been attributed to the transcriptional up-regulation of several proapoptotic proteins, including caspases, death receptor ligands, and Bcl2 family members [8]. Here, we demonstrate that interferons strongly down-regulate expression of TRAILR4 by inducing its proteolytic degradation and thus identify this decoy receptor as a novel, non-transcriptional target of IFN signaling.

Materials and methods

Materials. The fibroblast cell line SV80, the colon cell line HT-29, the melanoma cell line M2A7, and the rhabdomyosarcoma cell line Kym-1 and KB cells were all cultured in RPMI medium with 10% heat-inactivated serum. The KB cell line was originally established from an epidermal carcinoma of the mouth, but was then found to have been derived from HeLa cell contamination (http://www.lgcpromochem.com/atcc/). The phytoerytin conjugated TRAILR1 to TRAILR4-specific mAbs, the corresponding isotype control mAbs, and goat anti-TRAILR4 IgG and IFNγ were obtained from R&D Systems (Wiesbaden, Germany). IFNα was

☆ *Abbreviations:* CTL, cytotoxic T-cell; IFNα/γ, interferon-α/γ; NK cell, natural killer cell; TRAIL, TNF-related apoptosis inducing ligand; TRAILR1/2/3/4, TRAIL receptor-1/2/3/4.

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purchased from EssexPharma (Munich, Germany). Anti-vinculin mAb was bought from Santa Cruz Biotechnology (CA, USA), Flag-tagged soluble CD95L (aa 139–281) and Flag-tagged soluble TRAIL (aa 95–281) were produced as described elsewhere, and the recombinant proteins were purified by affinity chromatography using anti-Flag M2 agarose beads (Sigma, Deisenhofen, Germany). MG132 was obtained from Calbiochem (Schwalbach, Germany) and the protease inhibitor cocktail was purchased from Sigma. The expression plasmids encoding full-length TRAILR4 and a GPI-anchored variant of the extracellular domain of TRAILR4 were a kind gift of Pascal Schneider (University of Lausanne, Switzerland).

FACS analysis. To analyze the effects of interferon stimulation on cell surface expression of TRAILR1–TRAILR4, KB cells were challenged in triplicates as indicated with IFNγ or IFNα at 37 °C and afterwards cells were stained with TRAILR1-, TRAILR2-, TRAILR3- or TRAILR4-specific PE-conjugated antibodies or with the corresponding isotype control antibodies. Samples were then analyzed by FACS. Receptor positive cells were obtained using a gate excluding >97% of the control antibody stained cells.

RNAse protection assay. KBs were stimulated with IFNγ or IFNα for the indicated time and total RNAs were isolated with the peqGOLD RNAPure reagent (PeqLab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. Total RNAs were then analyzed with respect to the expression of the indicated genes using the RiboQuant Multi-Probe RNase Protection Assay System and the Multi-Probe template set hApo-3d (PharMingen, Hamburg, Germany). Probe synthesis, hybridization, and RNase treatment were performed according to the manufacturer's recommendations. Protected transcripts were separated by denaturing polyacrylamide gel electrophoresis on 5% acrylamide gels and analyzed on a PhosphorImager using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Ectopic expression of TRAILR4 and down-regulation of endogenous TRAILR4 by siRNA. To achieve TRAILR4 overexpression, KB cells (25×10^6) were electroporated (4 mm cuvette; 250 V, 1800 μF, maximum resistance) in medium (10% FCS) containing 40 μg of TRAILR4 and GPI-TRAILR4 encoding expression plasmids using an Easyject Plus (PeqLab, Germany). Electroporated cells were seeded into 6 cm tissue culture plates (1.5×10^6). Next day cells were incubated for 48 h with IFNγ (20 ng/ml) or remained untreated. TRAILR4 expression was then analyzed by FACS (see above) and by Western blotting. Cell lysates were prepared in RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) and analyzed by Western blotting using a goat anti-TRAILR4 IgG (1 μg/ml) or an anti-vinculin mAb (1 μg/ml). Immunocomplexes were detected with the corresponding horseradish peroxidase-conjugated secondary antibodies (Dako, Hamburg, Germany) and the ECL detection system (Amersham Biosciences). To down-regulate endogenous TRAILR4 expression, cells were electroporated with medium (10% FCS) supplemented with the TRAILR4-specific siRNA Hs-TNFRSF10-1-HP (200 nM; target sequence CCG GAG TGA CAT CAA GTG CAA; Qiagen, Hilden, Germany) or an irrelevant neomycin-phosphotransferase specific control siRNA (kind gift from Dr. J. Soutschek, Alnylam Europe AG, Kulmbach, Germany).

Cell death assays. KB cells (25×10^6) were electroporated with 40 μg of a GPI-TRAILR4 encoding expression plasmid or siRNA oligonucleotides. Next day cells were harvested and seeded in 96-well plates (20×10^3 cells per well). After an additional day, cells were incubated in triplicates with the indicated concentrations of Flag-TRAIL and Flag-CD95L for 18 h in the presence of cycloheximide (2.5 μg/ml). Activity of the ligands was enhanced by adding 1 μg/ml of the Flag-specific mAb M2. Finally, cell viability was determined by crystal violet staining. After several washes with water, plates were air-dried and the plate-bound dye was dissolved in methanol (1–2 h) to measure the optical density at 595 nm.

Results and discussion

To elucidate whether IFNγ regulates expression of TRAIL receptors, KB cells were stimulated for 24 h with this cytokine and then analyzed for expression of TRAILR1, TRAILR2, TRAILR3, and TRAILR4 by RNA protection assay analysis and FACS analysis (Fig. 1). The TRAIL death receptors TRAILR1 and TRAILR2 were both significantly expressed at the RNA and protein level, (Figs. 1A and B). Noteworthy, TRAILR2 expression was not changed or even up-regulated at the RNA level by IFNγ, but its cell surface expression was reduced by almost 50% (Fig. 1B). There was also a significant up-regulation of the TRAILR2 related death receptor CD95 at the mRNA level, which resulted in enhanced cell surface expression of the corresponding protein (Figs. 1A and E). TRAILR3 and TRAILR4 expression was hardly detectable at the RNA level, but TRAILR4 expression was clearly evident in non-stimulated cells by FACS analysis using two different TRAILR4-specific antibodies (Fig. 1B and data not shown). More importantly, in IFNγ-treated cells TRAILR4 cell surface expression was reduced to background levels (Fig. 1B). IFNγ-induced inhibition of TRAILR4 expression occurred within 12 h and was evident when IFNγ was applied in concentrations down 0.2–2 ng/ml (Figs. 1C and D). Interestingly, two recent studies using RT-PCR described down-regulation of TRAILR4 mRNA by IFNγ, but failed to find an effect on TRAILR4 cell surface expression [9,10]. Next, we analyzed whether ectopically expressed TRAILR4 was also down-regulated by IFNγ. For this purpose, full-length TRAILR4 or a deletion mutant of TRAILR4 consisting of the extracellular domain linked to a peptide motif, mediating GPI-anchoring in the plasma membrane, was transiently expressed in KB cells. One day after transfection, cells were split and one-half was stimulated with IFNγ for additional 2 days. While expression of ectopically expressed TRAILR4 was down-regulated by more than 80% after treatment with IFNγ, expression of the GPI-anchored TRAILR4 deletion mutant remained largely unchanged (Fig. 2). In relative terms, endogenous TRAILR4 was significantly faster down-regulated by IFNγ than ectopically expressed TRAILR4. This may be related to the fact that the absolute expression levels of ectopically expressed TRAILR4 exceeded those of endogenous TRAILR4 by many folds. As the plasmid-encoded TRAILR4 gene and the endogenous TRAILR4 gene do not share any non-translated regulatory sequences, IFNγ-induced inhibition of TRAILR4 expression may involve post-translational mechanisms. We have therefore analyzed next the effect of IFNγ on TRAILR4 expression in the presence of a protease inhibitor cocktail or after treatment with the proteasome inhibitor MG132. Both treatments almost completely abrogated the inhibitory effect of IFNγ on TRAIL-R4 expression (Fig. 2C). Thus, IFNγ down-regulates TRAILR4 expression under crucial involvement of the proteasome. Noteworthy, protease inhibition reverted IFNγ-induced inhibition of TRAILR2 expression and moreover strongly enhanced the constitutive expression of the receptor (Fig. 2D and data not shown). In contrast, expression of CD95 was barely affect-

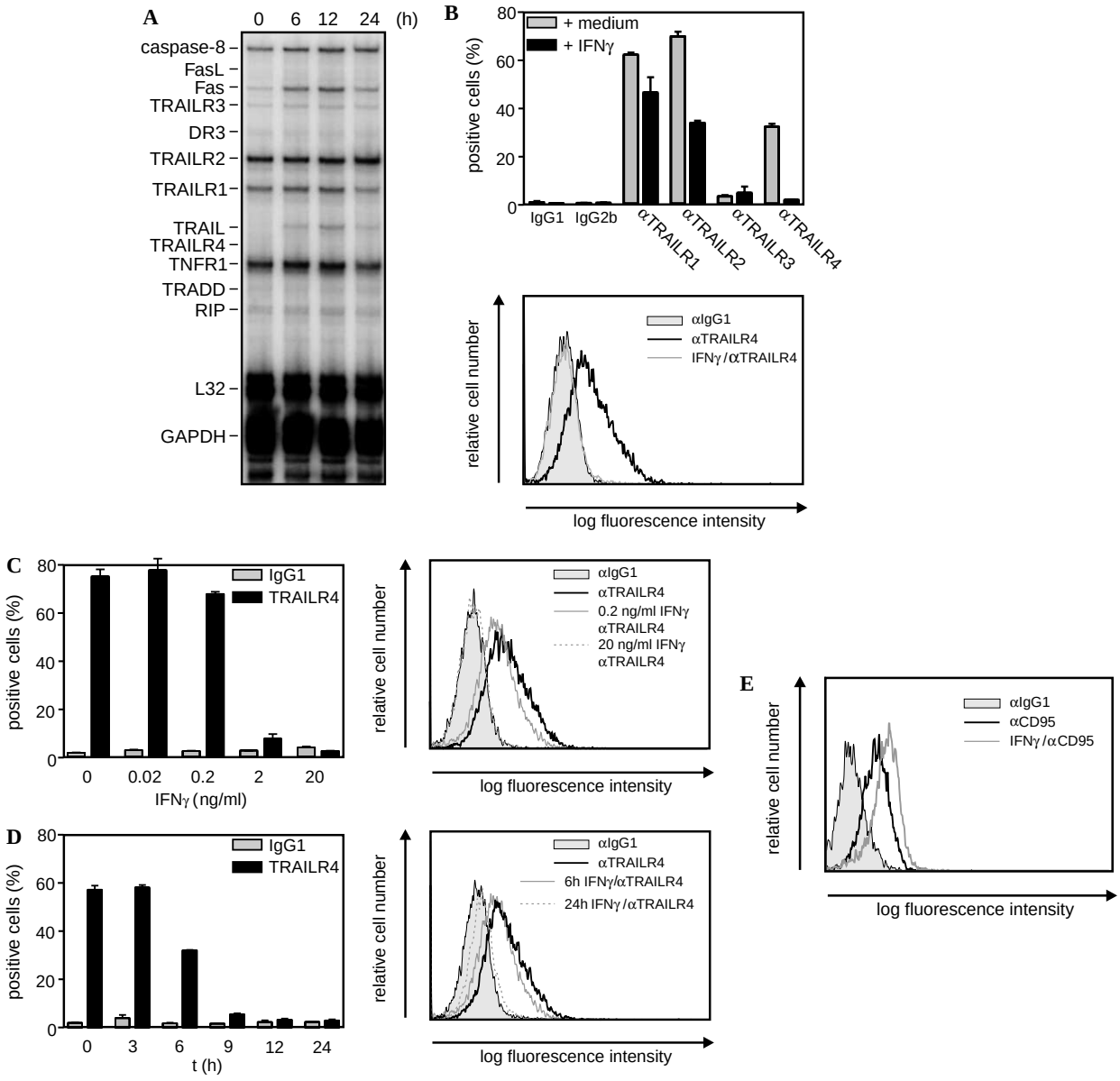

Fig. 1. Effect of IFNγ on expression of TRAILR1, TRAILR2, TRAILR3, and TRAILR4. (A) KB cells were stimulated with 20 ng/ml IFNγ for 6, 12, or 24 h, total RNAs were prepared and subsequently assayed for transcription of the indicated genes by multi-template RPA. (B–D) Triplicates of KB cells were stimulated with 20 ng/ml IFNγ for 24 h (B), for the indicated times with 20 ng/ml IFNγ (C) or with the indicated concentrations of IFNγ for 24 h (D). Cells were then stained with PE-labeled TRAILR1-, TRAILR2-, TRAILR3- or TRAILR4-specific antibodies as well as the corresponding Isotype controls. Gate was positioned to exclude >97% control antibody stained cells and TRAIL receptor positive cells were determined. Representative histograms of the most relevant groups are shown. (E) KB cells were again treated with IFNγ (20 ng/ml) for 24 h and were then analyzed by FACS with respect to CD95 cell surface expression.

ed by blocking the proteasome (Fig. 2E). Thus, proteasomal degradation seems to be of special relevance in the regulation of TRAILR2 and TRAILR4 cell surface expression (Fig. 2). In fact, it is known that IFNγ can change the substrate specificity of the proteasome and in addition stimulate its proteolytic activity, e.g., by inducing the proteasome regulators PA28α and PA28β [11]. IFNγ-induced proteasome dependent inhibition of TRAILR4 expression was also found in several other cell lines including HT29, M2A7, Kym-1, and SV80 cells (Figs. 3A–D) and was also observed with the type I interferon IFNα (Figs. 4A–C and data not shown). Thus, efficient inhibition of TRAILR4 expression seems to be a common cellular response to interferons, which base on the down-regulation of TRAILR4 mRNA described elsewhere and/or enhancement of the proteasome dependent turn-over of TRAILR4 protein.

Important functions of interferons are the inhibition of viral spread from infected cells and in course of tumor surveillance suppression of tumorigenic cells [1–3]. As both

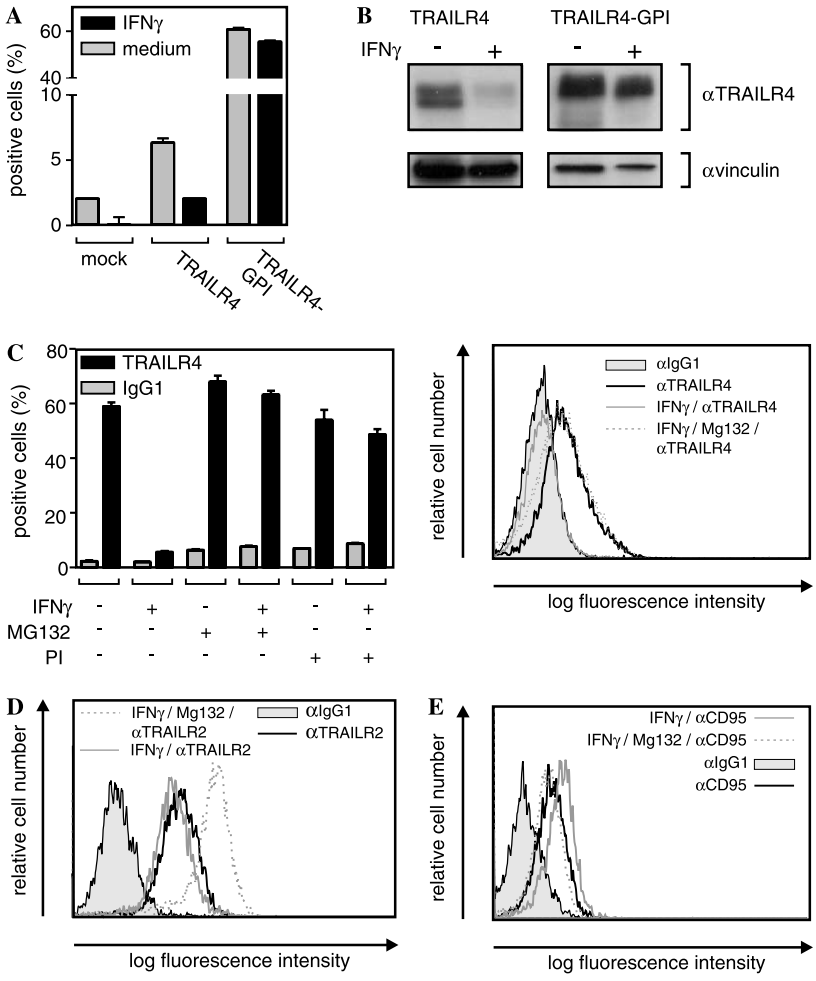

Fig. 2. TRAILR4 is down-regulated by IFNγ by proteolytic degradation. (A,B) KB cells were transiently transfected with empty vector or expression plasmids encoding full-length TRAILR4 or a GPI-anchored variant of the extracellular domain of TRAILR4. Next day, cells were split and stimulated with IFNγ (20 ng/ml). After 48 h triplicates of stimulated and non-stimulated cells were analyzed by FACS with respect to TRAILR4 expression (A). Gate was set above the expression level of endogenous TRAILR4 to select for cells with significant levels of ectopically expressed TRAILR4 molecules. An additional group was analyzed by Western blotting with anti-TRAILR4 and anti-vinculin as a load control (B). (C) KB cells were treated in triplicates with the indicated combinations of IFNγ (20 ng/ml), MG132 (20 μM), and a protease inhibitor cocktail and were analyzed after 16 h by FACS with respect to TRAILR4 cell surface expression. (D,E) KB cells were challenged with 20 ng/ml IFNγ in the presence and absence of 20 μM MG132 and were analyzed after 16 h by FACS with respect to TRAILR2 (D) and CD95 (E) cell surface expression.

functions involve NK- and CTL-induced TRAIL-mediated apoptosis, interferon stimulated degradation of the decoy receptor TRAILR4 in target cells may relieve killing of potential harmful cells by these effector cells. In fact, IFNγ significantly sensitizes KB cells for TRAIL-induced apoptosis [12]. However, the relevance of TRAILR4 degradation for apoptosis induction by TRAIL in IFNγ-primed cells can hardly be attributed to the complex effects of IFNγ on apoptosis-related genes and proteins. Indeed, we have recently found that IFNγ-priming sensitizes KB cells for CD95L- and TRAIL-induced apoptosis upstream of caspase-8 activation and correlates also with down-regulation of the death receptor inhibitory FLIP protein [12]. To verify that TRAILR4 expression is in principle able to modulate TRAIL-induced apoptosis in KB cells, we transfected these cells with an expression construct encoding GPI-anchored TRAILR4 to analyze its effect on TRAIL- and CD95L-induced apoptosis. Transfection efficiencies reached up to 80% and conferred a significant protection against TRAIL-induced apoptosis while CD95-mediated cell death remained unaffected (Figs. 5A and B). The selectivity of this protective effect is in accordance with other studies showing that expression of TRAILR4 can attenuate TRAIL-induced apoptosis [13–15]. As the ectopically expressed TRAILR4 levels exceeded the endogenous expression by many times, it is difficult to estimate the relevance of this effect for TRAIL sensitivity. We therefore inhibited endogenous TRAILR4 expression by transient transfection with TRAILR4-specific siRNA. One day post-transfection, TRAILR4 expression was almost completely blocked (Fig. 5C). In good accordance with the previously observed protective effect of overexpressed TRAILR4, siRNA treatment resulted in a significant sensitization for TRAIL- but not CD95L-induced apoptosis (Fig. 5D) again arguing for

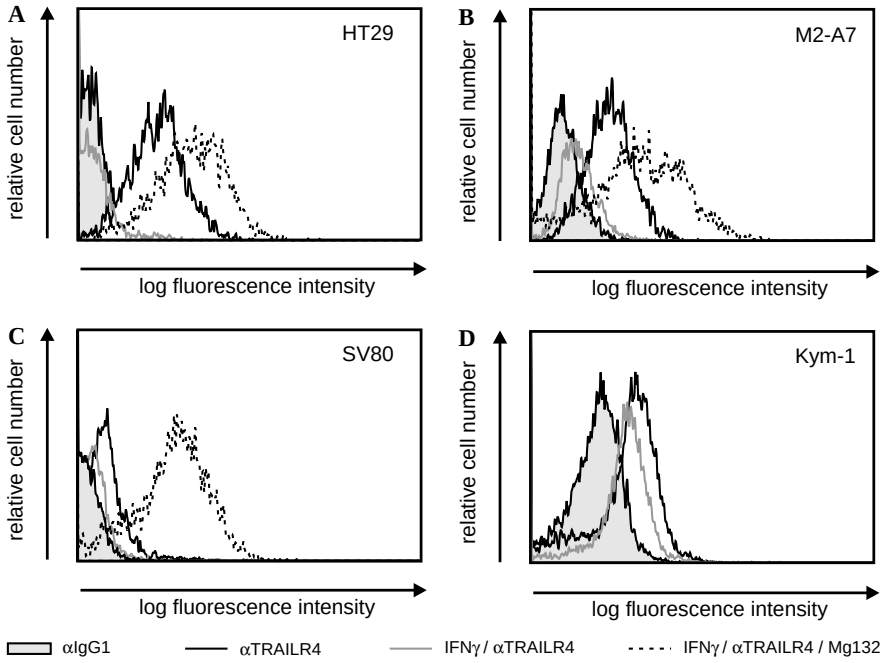

Fig. 3. Down-regulation of TRAILR4 occurs in cell line of diverse origin. (A–D) HT-29 (A), M2-A7 (B), SV80 (C), and Kym-1 (D) cells were treated with the indicated combinations of IFNγ (20 ng/ml) and MG132 (20 μM), and were analyzed after 16 h by FACS with respect to TRAILR4 cell surface expression.

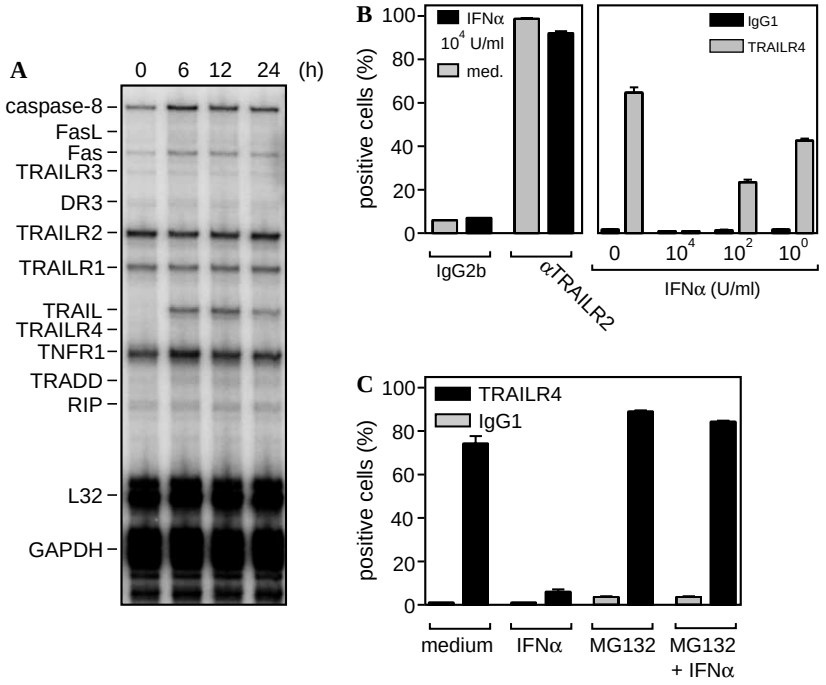

Fig. 4. TRAILR4 is down-regulated by IFNα by proteolytic degradation. (A) KB cells were stimulated with 10,000 U/ml IFNα for 6, 12 or 24 h, total RNAs were prepared and subsequently assayed for transcription of the indicated genes by multi-template RPA. (B) Triplicates of KB cells were stimulated with the indicated concentration of IFNα for 24 h and were then stained with PE-labeled antibodies for TRAILR2 and TRAILR4 expression. (C) KB cells were treated in triplicates with the indicated combinations of IFNα (10,000 U/ml) and MG132 (20 μM), and were analyzed after 16 h by FACS with respect to TRAILR4 cell surface expression.

a protective function of TRAILR4 expression. Thus, in an interferon-rich microenvironment down-regulation of the antagonizing decoy receptor TRAILR4 in target cells may act together with TRAIL induction in NK and CTL effector cells to ensure rapid and potent apoptosis induction by TRAIL.

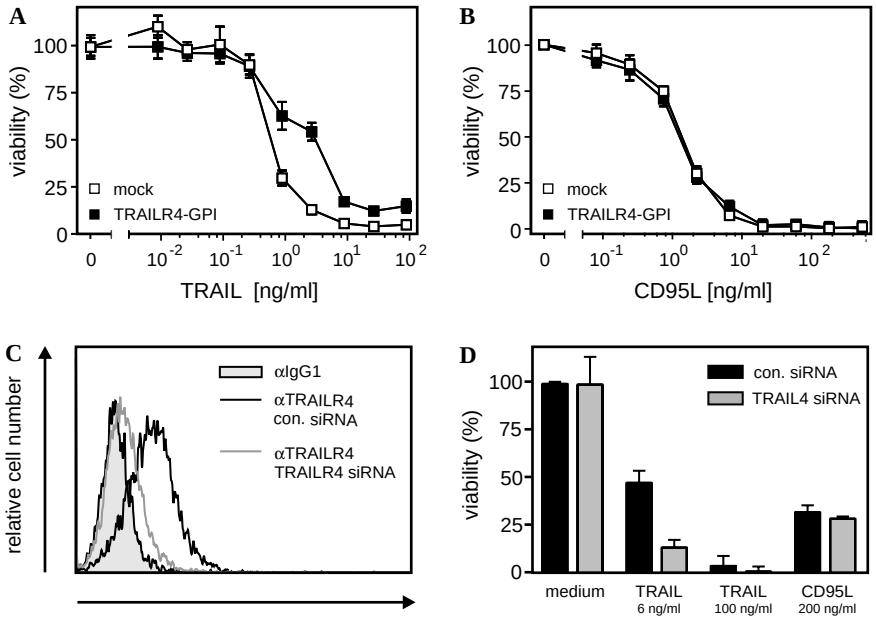

Fig. 5. TRAILR4-GPI expression attenuates TRAIL- but not CD95L-induced apoptosis. (A,B) KB cells were transiently transfected with empty vector or an expression plasmid encoding GPI-anchored TRAILR4. Next day, cells were seeded in 96-well plates (10×10^3 cells per well) and after an additional day cells were challenged in triplicates with the indicated concentrations of soluble Flag-tagged TRAIL (A) or soluble Flag-tagged CD95L (B) in the presence of CHX (2.5 μg/ml). Both reagents were crosslinked with the monoclonal anti-Flag antibody M2 (1 μg/ml) to allow formation of ligand complexes of high activity. After additional 18 h, cell viability was determined by crystal violet staining. (C,D) TRAILR4-specific and as a control neomycin-specific siRNAs were transiently introduced in KB cells by electroporation with medium containing the corresponding siRNA (200 nM). The following day TRAILR4 expression was determined by FACS (C) and an aliquot of cells were seeded in 96-well plates (10×10^3 cells per well). After an additional day, cells were stimulated in triplicates with the indicated concentrations of TRAIL or CD95L in the presence of CHX (D). After additional 18 h, cell viability was again determined by crystal violet staining.

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