

# Resistance to tumor necrosis factor (TNF) cytotoxicity by autocrine TNF production is independent of intracellular signaling pathways

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**Abstract** We previously showed that autocrine tumor necrosis factor (TNF) production in the TNF-sensitive L929sA fibrosarcoma cell line induced TNF resistance, which is correlated with downmodulation of both TNF receptors on the cell surface. We now analyzed whether autocrine TNF production also interfered with intracellular TNF signaling pathways. The L929sA-CAT-R55i cell line, in which cell death can be induced by controlled cytoplasmic expression of a trimeric fusion protein between chloramphenicol acetyltransferase and the intracellular domain of TNF-R55 (CAT-R55i), was supertransfected with the murine TNF gene. Expression of the latter conferred resistance to cell death induced by exogenous TNF, while cytotoxicity induced by CAT-R55i was not impaired. This demonstrates that autocrine TNF did not induce intracellular mechanisms that block TNF signaling leading to cell death. Thus the induction of TNF resistance via autocrine TNF production in L929sA cells is solely due to downmodulation of TNF receptors on the cell surface.

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**Key words:** Cytotoxicity; Fusion; Signaling; Tumor necrosis factor

## 1. Introduction

The pleiotropic cytokine tumor necrosis factor (TNF) is mainly produced by macrophages, but a wide variety of other cells can also produce TNF. TNF exerts a whole spectrum of biological activities, depending on the cell type, via interaction with well-characterized transmembrane TNF receptors with  $M_r$ s of 55 kDa (TNF-R55) and 75 kDa (TNF-R75) [1]. TNF signaling is initiated by receptor clustering after interaction with trimeric TNF, followed by the recruitment of multiple factors to the TNF receptor [2]. TNF is cytotoxic to many types of tumor cells, especially in synergy with interferon- $\gamma$  (IFN- $\gamma$ ). However, several tumor cell lines are resistant to the cytotoxic effect of TNF, and some of them produce autocrine TNF [3–6]. Moreover, transfection of TNF-sensitive cell lines with an exogenous TNF gene, under control of a constitutive promoter, confers resistance to TNF-mediated cytotoxicity [7,8]. We previously demonstrated that TNF resistance caused by autocrine TNF production in the TNF-sensitive L929sA cell line is correlated with downmodulation of the TNF receptors on the plasma membrane [8]. We now investigated whether intracellular TNF resistance mechanisms are also involved in this TNF unresponsiveness of TNF-producing L929sA cells. In order to answer this question we used a cell line in which intracellular TNF effects can be mimicked without actual need for TNF itself or its transmembrane re-

ceptor. In this cell line, called L929sA-CAT-R55i, expression of a cytosolic fusion protein of chloramphenicol acetyltransferase (CAT) and the intracellular domain of TNF-R55 (CAT-R55i) is under control of the type I IFN-inducible Mx promoter. Expression of trimeric CAT enforces clustering of the intracellular domain of the TNF receptor, which leads to activation of the nuclear factor  $\kappa$ B, production of interleukin-6 and cell death [2,9]. The cell line was supertransfected with the murine TNF gene in order to determine the intracellular sensitivity caused by controlled expression of the CAT-R55i fusion protein in a cell resistant to exogenous TNF.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

The fibrosarcoma cell lines L929sA [8] and L929sA-CAT-R55i [9] were cultured as previously described. These cell lines were repeatedly screened for mycoplasma with a DNA-fluorochrome assay and were found to be negative.

### 2.2. Cytokines and antisera

Purified *Escherichia coli*-derived murine TNF was produced in our laboratory and had a specific biological activity of  $2 \times 10^8$  IU/mg. Recombinant murine IFN- $\beta$  was also produced in our laboratory and had a specific activity of  $3 \times 10^8$  U/ml as determined on murine cells in an L929/vesicular stomatitis virus assay. Murine IFN- $\gamma$  was produced in Chinese hamster ovary cells and had a concentration of  $10^5$  U/ml. Polyclonal rabbit antiserum against murine TNF-R55 and TNF-R75 was a kind gift of Dr. W.A. Buurman (University of Limburg, Maastricht, The Netherlands).

### 2.3. Plasmids and transfection

pSV23S-mTNF, encoding the murine TNF gene driven by the constitutive SV40 early promoter [10], and pPHT, encoding the hygromycin-resistant (hygro<sup>r</sup>) gene, were stably transfected in L929sA-CAT-R55i cells. The protocol involved improved DNA-calcium phosphate coprecipitation as described previously [8].

### 2.4. Flow fluorocytometry

The presence of TNF-R55 or TNF-R75 on the cell membranes was determined by incubating cells for 1 h at 4°C with polyclonal rabbit antiserum against TNF-R55 or TNF-R75 (1  $\mu$ g antiserum per  $4 \times 10^5$  cells in 200  $\mu$ l), followed by incubation for 1 h at 4°C with biotinylated donkey anti-rabbit polyclonal antiserum (Amersham Life Science, Amersham, UK), and by subsequent incubation for 1 h at 4°C with phycoerythrin-conjugated streptavidin. Soluble TNF was removed from the cell surface receptors by prior acidic treatment for 3 min at room temperature with glycine-HCl buffer (50 mM, containing 150 mM NaCl, pH 3.0). Analyses were performed by flow fluorocytometry using a Coulter Epics 753 equipped with an argon-ion laser (Coulter, Hialeah, FL).

### 2.5. Determination of sensitivity to exogenous TNF or intracellular CAT-R55i expression

TNF sensitivity was determined as previously described [6]. The sensitivity of cells to IFN- $\beta$ -induced CAT-R55i expression was analyzed as follows. Cells were seeded in 96-well plates in 100  $\mu$ l of medium at a concentration of  $2 \times 10^4$  cells per ml. 12–18 h later,

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CAT-R55i was induced by adding 1000 U/ml IFN- $\beta$  in 100  $\mu$ l for an incubation period of up to 24 h. Surviving cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and assayed colorimetrically [11].

### 2.6. Determination of expression of the CAT-R55i fusion product

Confluent monolayers of L929sA-CAT-R55i transfectants were treated with 1000 U/ml IFN- $\beta$  or were left untreated. After 4.5 h, cells were washed with phosphate-buffered saline, pelleted, and lysed on ice with phosphate-buffered saline containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 1 mM ethylenediaminetetraacetate and 10 mM Pefabloc (Pentapharm, Basel, Switzerland). Cleared lysates were assayed for CAT enzymatic activity. The assay mixture, a total volume of 90  $\mu$ l, contained 1  $\mu$ Ci of [ $^{14}$ C]deoxychloramphenicol, 20  $\mu$ g of protein extract. These reagents were pre-incubated for 5 min at 37°C and the reaction was started by addition of 10  $\mu$ l of 9 mM acetyl coenzyme A. The reaction lasted 2.5 h and was stopped by addition of 1 ml ice-cold ethyl acetate which was used to extract the chloramphenicol. The organic layer was dried and taken up in 20  $\mu$ l ethyl acetate; 5  $\mu$ l of this solution was spotted on silica gel thin-layer plates and resolved with chloroform/methanol (95:5; ascending). Visualization of the separated acetylated forms was obtained by Phosphor Image Quantification (Molecular Dynamics, Sunnyvale, CA).

### 3. Results and discussion

Previously, we reported that transfection of L929sA cells with the murine TNF gene led to autocrine TNF production and TNF resistance, which was correlated with downmodulation of the TNF receptors on the plasma membrane [8]. Now we analyzed whether autocrine TNF also affects intracellular TNF signaling pathways leading to cell death. Hence we determined the effect of autocrine TNF production on the cytotoxicity induced by intracellular expression of a chimeric CAT-R55i protein, which clusters the intracellular domains of TNF-R55. We supertransfected the TNF gene in the L929sA-CAT-R55i cell line, previously transfected with the CAT-TNFR55i fusion gene under control of the Mx promoter inducible by IFN type I (IFN- $\alpha$  or IFN- $\beta$ ). IFN- $\alpha$ -induced intracellular expression of CAT-R55i protein complex leads to cell death in L929sA-CAT-R55i cells [2,9]. After stable transfection of L929sA-CAT-R55i with the *hygro*<sup>r</sup> gene alone or in combination with the TNF gene, *hygro*<sup>r</sup> colonies were obtained and tested for their TNF production, TNF sensitivity and finally for their sensitivity to IFN- $\beta$  treatment which activates the expression of CAT-R55i leading to cell death.

The production of TNF in the culture supernatant of 10 L929sA-CAT-R55i TNF transfectants tested varied between 0.7 and 70 IU TNF/ml. No secreted TNF was detected in the supernatant of control cells transfected with the *hygro*<sup>r</sup> gene

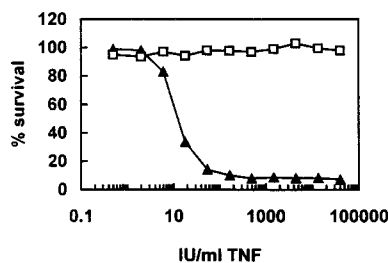


Fig. 1. TNF sensitivity of L929sA-CAT-R55i cells. Cells were transfected with the *hygro*<sup>r</sup> gene alone ( $\blacktriangle$ ) or cotransfected with the TNF gene ( $\square$ ). Sensitivity was determined after 24 h in the presence of TNF.

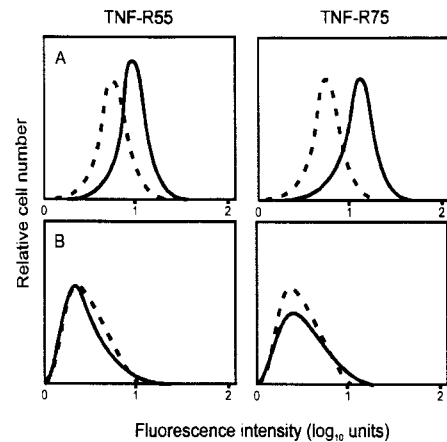


Fig. 2. Flow cytometric analysis of TNF-R55 and TNF-R75 expression on L929sA-CAT-R55i cells. Cells were transfected with the *hygro*<sup>r</sup> gene alone (A) or cotransfected with the TNF gene (B). Cells were then treated with antiserum directed against TNF-R55 or TNF-R75 and secondary antibody (full line) or with secondary antibody alone (broken line).

alone. Autocrine TNF production, as low as 0.7 IU TNF/ml, protected L929sA-CAT-R55i TNF transfectants completely against the cytotoxic effect of TNF, while L929sA-CAT-R55i cells transfected with the *hygro*<sup>r</sup> gene alone remained TNF-sensitive (Fig. 1). The representative transfectant used in all further experiments produced 15 IU TNF/ml. In order to determine whether the induction of TNF resistance was correlated with downmodulation of the TNF receptors on the cell surface, as is the case for L929sA TNF transfectants, the expression level of both TNF receptor types in L929sA-CAT-R55i TNF transfectants was measured by flow fluorocytometric analysis. As shown in Fig. 2, in contrast to *hygro*<sup>r</sup> control transfectants, no TNF-R55 and TNF-R75 molecules could be detected on TNF-producing L929sA-CAT-R55i transfectants. Even after pretreatment with glycine-HCl buffer, meant to remove receptor-bound TNF from the TNF receptors [8], no TNF receptor molecules could be detected on TNF-producing transfectants. Taken together, these results demonstrate that L929sA-CAT-R55i cells supertransfected with the TNF gene behave like parental L929sA cells transfected with the TNF gene as described previously [8]. But transfection of the TNF gene in L929sA-CAT-R55i cells did not affect inducible CAT-R55i expression (Fig. 3).

In order to investigate whether autocrine TNF production could also protect against TNF cytotoxicity independently of transmembrane TNF receptors, we determined whether IFN- $\beta$ -induced expression of a trimeric CAT-R55i fusion protein could still induce cell killing. As shown in Fig. 4, cell death was evident 6 h after IFN- $\beta$  administration and followed very comparable time kinetics both in non-TNF-producing and in TNF-producing L929sA-CAT-R55i transfectants. To rule out the possibility that the sensitivity of these transfectants to IFN- $\beta$  was independent of CAT-R55i expression and was mediated by IFN- $\beta$  itself, the effect of IFN- $\beta$  treatment was investigated on parental L929sA and TNF-producing L929sA cells, both transfected with the *hygro*<sup>r</sup> gene. No cell death was induced by IFN- $\beta$  administration in these transfectants, supporting the conclusion that the observed sensitivity of L929sA-CAT-R55i transfectants to IFN- $\beta$  was due to CAT-R55i expression (Fig. 5). To further establish that IFN- $\beta$ -in-

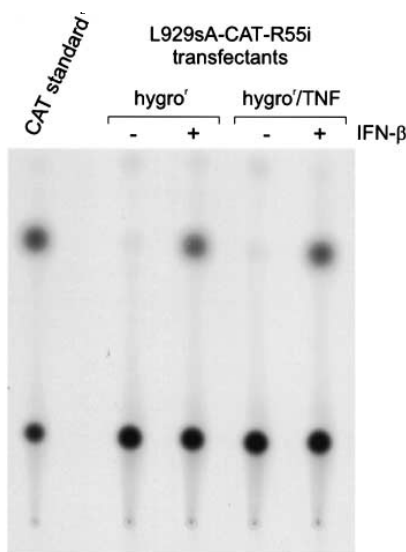


Fig. 3. Determination of IFN- $\beta$ -inducible CAT-R55i expression in L929sA-CAT-R55i cells. Cells were transfected with the *hygro*<sup>r</sup> gene alone or cotransfected with TNF as described in Section 2.

duced cell killing in L929-CAT-R55i transfectants could be ascribed to Mx promoter-driven expression of the CAT-R55i cytotoxic fusion product, the effect on cell survival of IFN- $\gamma$ , which induces many effects similar to those observed with type I IFNs, but which cannot activate the Mx promoter, was investigated. Moreover, considering that most IFN effects are protein synthesis-dependent, we also tested, as a control, treatment with IFN- $\beta$  combined with actinomycin D. IFN- $\gamma$  treatment did not induce cell death in any of the cell lines tested (Fig. 5), while reduced cytotoxicity was observed in L929sA-CAT-R55i transfectants after a combined treatment with IFN- $\beta$  and actinomycin D compared with an IFN- $\beta$  treatment alone (Fig. 5). These results and the fact that CAT-R55i expression could only be detected after IFN- $\beta$  treatment indicate that IFN- $\beta$ -induced cytotoxicity in L929sA-CAT-R55i transfectants was indeed dependent on Mx promoter-induced expression of the CAT-R55i fusion product. It may also be mentioned that IFN- $\beta$ -induced expression of CAT alone does not lead to cell killing in L929sA cells (data not shown). This observation confirms that the occurrence of cell death after CAT-R55i expression is due to signalization via the clustered intracellular domains of TNF-R55.

Taken together, our results clearly demonstrate that auto-

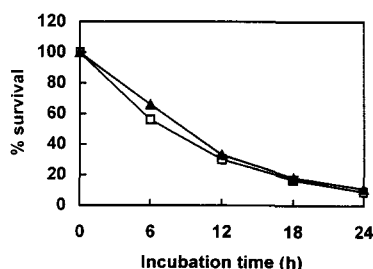


Fig. 4. Time kinetics of cell death obtained by IFN- $\beta$ -induced CAT-R55i expression. Cytotoxicity on L929sA-CAT-R55i cells transfected with the *hygro*<sup>r</sup> gene alone ( $\blacktriangle$ ) or cotransfected with TNF ( $\square$ ) was measured at different time points after treatment with 1000 U/ml of IFN- $\beta$ .

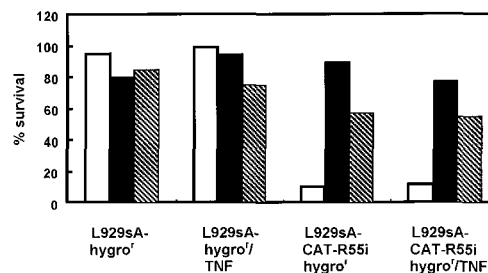


Fig. 5. Determination of sensitivity to IFN treatment of L929sA or L929sA-CAT-R55i cells. Cells were transfected with the *hygro*<sup>r</sup> gene alone or cotransfected with TNF, and were subjected to a 24-h treatment with 1000 U/ml of IFN- $\beta$  (open bars), IFN- $\gamma$  (filled bars), or IFN- $\beta$  in combination with actinomycin D (hatched bars).

crine TNF production confers resistance against the cytotoxic effect of exogenous TNF via downmodulation of the TNF receptor on the cell surface. Receptor downmodulation presumably is an intracellular process occurring at the level of de novo synthesis. Considering that receptor downregulation occurs only with an active TNF molecule, and not with an inactive TNF mutant having highly reduced TNF receptor-binding capacity [8], an accurate interaction between the TNF receptor and TNF may lead to intracellular retention of the TNF receptor. If TNF receptor clustering occurs intracellularly, triggering of the signaling pathway may be expected, resulting in cell killing. This is, however, not the case, since TNF-producing L929 cells do not die. The phenomenon might be explained by a mechanism involving autocrine TNF which induces TNF resistance proteins or other protection mechanisms blocking the TNF signaling pathway leading to cell death. We here show, however, that cell killing can still occur in TNF-producing L929sA cells by TNF signalization induced via expression of clustered intracellular TNF receptor domains (CAT-R55i). This observation implies that autocrine TNF does not induce or select for intracellular TNF protection mechanisms. In conclusion, the data presented in this report demonstrate in a direct way that the acquisition of unresponsiveness to TNF cytotoxicity via autocrine TNF production is solely a consequence of the disappearance of TNF receptor molecules on the cell surface, by which TNF-producing L929sA cells become blind to the effects of this cytokine.

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