

# A Mathematical Vision of TNF Receptor Interaction

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## ABSTRACT

Tumor necrosis factor (TNF) induces a broad spectrum of cellular responses like differentiation, immune response or programmed cell death (apoptosis) via a highly complex signaling network. For example, TNF induced signal transduction is controlled by two distinct membrane receptors, TNFR1 and TNFR2, and a variety of intracellular signaling molecules. TNF induced apoptosis is largely attributed to TNFR1. The role of TNFR2 in TNF mediated apoptosis remains less well understood, although a positive cooperation has been demonstrated. Using in this work an integrated approach of mathematical modeling in combination with experimental data, we have investigated the mechanisms by which TNFR2 might cooperate with TNFR1 induced apoptosis in HeLa cells.

In the case of the antiapoptotic pathway the intracellular adapter molecule TRAF2 is believed to play a key role in receptor crosstalk. In order to gain a better understanding of the dynamics of TNF signaling and the TNFR1/TNFR2 crosstalk we have developed several mathematical models of the signaling pathways which enabled us to test hypotheses. We find that the apoptotic crosstalk of TNFR1 and TNFR2 does in fact depend on TRAF2 depletion, but must also rely on additional TNFR2 dependent, TRAF2 independent, mechanisms. With the help of mathematical modeling we propose a new regulatory principle of TNF receptor crosstalk based on the adaptor molecule RIP. RIP might play a key role in the TNFR1/TNFR2 crosstalk by regulating the balance between the apoptotic and gene inductive pathway. RIP concentration therefore influences NF- $\kappa$ B activation and thus NF- $\kappa$ B induced antiapoptotic gene products. Among these, c-FLIP and cIAP are of special interest, as these are major regulators of caspase activation.

As it has been shown that mitochondria are involved in the apoptotic pathway in HeLa cells [Scaffidi et al., 1999], we have included this pathway into our model. With the help of computational simulation we investigate the possible role of the mitochondrial pathway for caspase activation. Our computational simulations show that the mitochondrial pathway delays caspase activation although the maximum level of activated caspase molecules is increased.

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## 1. INTRODUCTION

TNF<sup>1</sup> is mainly produced by activated macrophages and T cells in response to infection and might either stimulate immune defense mechanisms or, alternatively, induce apoptosis. Apoptosis (programmed cell death) is an important regulatory mechanism to eliminate unwanted cells in development or irreversibly damaged cells in infection. TNF exerts its diverse biological functions by binding to and activating two distinct cell surface receptors, TNFR1 and TNFR2 [Ashkenazi and Dixit, 1998]. Activation of TNFRs occurs by oligomerization through the trimeric ligand TNF. In the recent years a large number of proteins has been identified that associate directly or indirectly with the cytoplasmic domains of the two receptors. Among these two groups of signal transduction molecules have been identified, the so called death domain (DD) proteins like TRADD and FADD and the TNF receptor associated factor (TRAF) family. Overexpression of death domain containing molecules leads to the induction of apoptosis. The cytoplasmic part of TNFR1, but not TNFR2, carries a death domain and is thus capable to bind other death domain proteins.

Following ligand binding, the DD containing adapter molecule TRADD binds through its death domain to TNFR1, thereby forming a platform for the initiation of both the apoptotic pathway or, alternatively, the antiapoptotic pathway. Regarding the apoptotic pathway, FADD binds to TRADD and induces the caspase cascade via caspase 8 binding and proteolytic activation. Thus, caspase 8 is the initiating caspase whereas caspases 3 and 7 represent major executional caspases which cleave protein targets leading to apoptosis. But besides this direct caspase activation pathway, there is an alternative pathway leading to the mitochondria which can be activated also by many stimuli besides death receptors. To initiate this pathway, activated caspase 8 cleaves Bid into tBid which induces the oligomerization of Bax [Wei et al., 2001], [Perez and White, 2000] and results in a mito-

<sup>1</sup>The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; NF- $\kappa$ B, nuclear factor  $\kappa$ B; I- $\kappa$ B, Inhibitor of NF- $\kappa$ B; IKK, I- $\kappa$ B Kinase; TRAF2, TNFR associated factor 2; RIP, receptor interacting protein; TRADD, TNFR1 associated death domain protein; FADD, Fas associated death domain protein; CHX, cycloheximide; XIAP, X-linked inhibitor of apoptosis; cIAP, caspase inhibiting IAP, c-FLIP, FLICE inhibitory protein

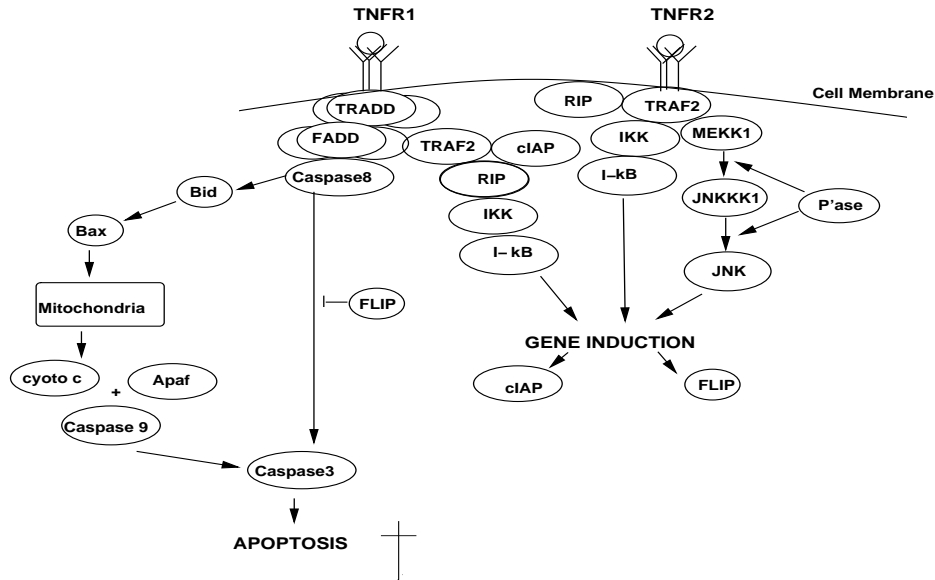


Figure 1: Schematic representation of TNFR1 and TNFR2 signaling pathways

chondrial dysfunction leading to the release of cytochrome c. Cytoplasmic cytochrome c then binds to a docking protein called Apaf-1 that facilitates binding and activation of caspase 9 [Scaffidi et al., 1999], [Zou et al., 1999], [Bratton et al., 2001]. Activated caspase 9 in turn cleaves procaspase 3 which leads to DNA fragmentation. In death receptor signaling the mitochondrial pathway has been proposed to function as an amplifier [Scaffidi et al., 1999] of caspase activation.

Regarding the antiapoptotic pathway, binding of TRAF2 and RIP to the TNFR1-TRADD complex is essential to induce strong NF- $\kappa$ B activation [Tran et al., 2001], [Hohmann et al., 1990], [Hsu et al., 1996] via IKK [Delhase et al., 1999]. In unstimulated cells, the transcription factor NF- $\kappa$ B is kept in the cytoplasm by interaction with I- $\kappa$ B inhibitory proteins [Baldwin, 1996]. Cellular TNF stimulation results in phosphorylation, ubiquitination and degradation of I- $\kappa$ B. Likewise liberated NF- $\kappa$ B molecules are translocated into the nucleus in order to activate transcription of gene targets. Figure 1 shows schematically the apoptotic and antiapoptotic pathways triggered by TNF stimulation.

Much less is known about the signal transduction pathways of TNFR2. TRAF2 can directly associate with TNFR2 and activate NF- $\kappa$ B and N-terminal c-Jun kinase (JNK). However, TNFR2 has been shown to enhance TNFR1 induced apoptosis in many cellular systems and as a likely mechanism TRAF2 depletion has been proposed by several groups [Weiss et al., 1997], [Weiss et al., 1998], [Chan and Lenardo, 2000]. Another adapter molecule which was shown to interact with both receptors is RIP, that mediates caspase activation by TNFR2 [Pimentel-Muinos and Seed, 1999] via TRADD. Until now this could only be shown for one cell type.

The TNF signaling system is a challenging example to demonstrate the complexity of signaling networks. This complexity

arises from the large number of interacting signaling compounds governed by positive and negative regulatory feedback loops. As such complex networks can not be anticipated by intuition, mathematical modeling provides a useful framework to gain a better understanding of the dynamical behavior of such systems and its regulatory mechanisms.

In the following we present a compartment model of nonlinear Differential Equations (ODEs) based on a formal kinetic approach. We consider each compartment as homogenous and well stirred. In our previous work we could already show at the example of the EGF receptor that this approach can result in a mathematical model with a highly predictive character [Schoeberl et al., 2001].

## 2. MATERIALS AND METHODS

As a cellular system we used in our work transfected HeLa cells expressing TNFR2. HeLa cells from the American Type Culture Collection were maintained in RPMI 1640 medium supplemented with 5 % heat inactivated fetal calf serum (Biochrom, Berlin, Germany). HeLa transfectants stably expressing TNFR2 have been already described elsewhere [Weiss et al., 1997]. Typically, these cells express about 3000 TNFR1 and 50000 TNFR2 at the cell surface. TNFR2 was stimulated with an agonistic TNFR2-specific rabbit IgG fraction ( $2\mu\text{g/ml}$ ) and TNFR1 with the agonistic TNFR1 specific mAb htr-1 ( $3\mu\text{g/ml}$ ).

Effects of different stimulation protocols on TNFR1- and TNFR2-mediated caspase activation:

HeLa-TNFR2 cells were pretreated with CHX ( $2.5\mu\text{g/ml}$ ) for 3 hours. Cells were then stimulated for the indicated times with TNF ( $10\text{ ng/ml}$ ) alone or in combination with anti TNFR2-IgG ( $2\mu\text{g/ml}$ ). In an additional group cells were stimulated with anti TNFR2-IgG for 4 hours before TNF treatment. Cell lysates were prepared to analyze processed caspase -8 and -3 by immunoblotting.

### 3. MODEL DERIVATION

The investigation of fluorescently conjugated proteins by different microscopy techniques has lead to new insights into the dynamic mechanisms of signaling processes. In nearly all cases studied a significant fraction of cytosolic, membrane bound or transmembrane proteins diffuse relatively freely within the cytosol and the membrane. From this knowledge the application of nonlinear ODEs seems to be sufficient in a first approach to describe the dynamics of signal transduction pathways. This has the advantage that the systems can be analyzed more easily with the help of nonlinear systems theory. The components of the mathematical model are state variables, indicating the state of a system at a certain time (e.g. the number of molecules of a certain compound) and control variables. The control variables are the basis for the chemical kinetic equations, which are retrieved from the literature and comprise Michaelis-Menten constants, turnover numbers, rate constants of association and dissociation.

In the mathematical models presented in the following, we consider receptor internalization and degradation. We considered direct receptor internalization as well as the so called coated pit path in order to model receptor internalization of all receptor-ligand complexes [Starbuck, 1992]. We assume that the receptors in the endosome continue to transduce signals and use the same signal transduction pathways as receptors remaining on the cell surface until they are degraded in the lysosome. This assumption leads to a significant reduction of the number of kinetic parameters.

Furthermore we assumed in the mathematical model that the receptors and the adapter molecule TRAF2 are constitutively trimerized [Pullen et al., 1999]. Due to this trimeric structure of the receptors and the monomeric structure of all the adapter molecules besides TRAF2 the number of combinations of possible interactions and thus the number of activated molecules by one receptor increases significantly as can be seen in Figure 2. We assumed that the binding order of the different adapter molecules does not have any influence which means the protein complex A-B is identical with B-A.

According to the induced proximity model procaspase 8 is activated at the DISC in an autocatalytic manner [Muzio et al., 1998]. Therefore we assume that at least two caspase molecules have to bind in proximity at the receptor in order to activate caspase 8 as can be seen in Figure 2.

Figure 2 represents the biochemical reaction scheme of TNFR1 on which the mathematical model is based on. All compounds are indicated with numbers  $N=1..j$  and all the reaction rates with  $r=v_{1..i}$ . Regarding the compounds three numbers have been used in the biochemical reaction scheme: the first indicates the signaling complex remaining on the cell membrane, the second the signaling-complex-coated-pit-protein complex and the third number the internalized signaling complex. In case of the reaction rates, the regular and bracketed expression represent the reaction rates of the external and internalized compounds respectively. This nomenclature was utilized in the Matlab program. In the case of TNFR2 the signal transduction pathways are mostly unknown. Nevertheless we included the binding of TRAF2

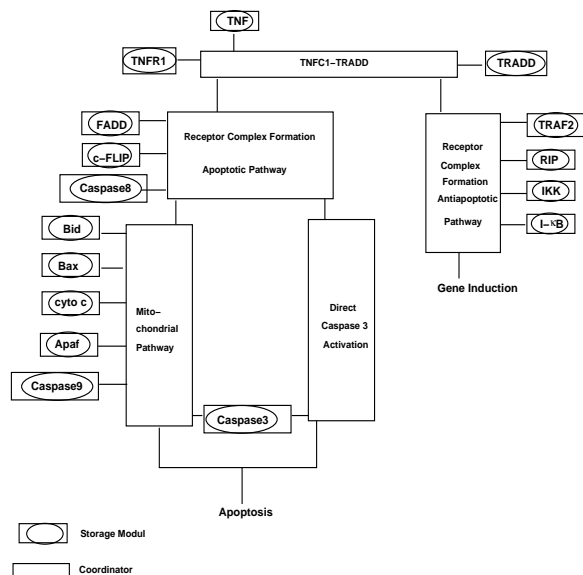
to TNFR2 and JNK activation. The mathematical model describing the TNFR1/TNFR2 interaction consists of about 280 ODEs and about 110 kinetic parameters. The number of kinetic parameters is relatively small in comparison to the number of ODEs. One reason are the different combinations of receptor complexes which can be seen in Figure 2 because we assume that the affinity stays the same even if the binding order of the different adapter molecules differs. Another reason is the inclusion of receptor internalization and degradation in our model and the assumption that external as well as internalized receptors use the same signal transduction pathways. The equation for each compound was derived by adding up the product forming reactions and subtracting the product consuming reactions.

$$\frac{d[C_j]}{dt} = \sum r_{Production} - \sum r_{Consumption} \quad (1)$$

In order to estimate the kinetic parameters of the reaction network represented in Figure 2, not yet accessible by published kinetic data, we fitted the model to time dependent quantitative observations of different laboratories and own experimental data. In order to carry out a parameter estimation, a sensitivity analysis was performed in DIVA. DIVA is a simulation tool which was developed at the Institute of System Dynamics and Control at the University of Stuttgart. It has the advantage of powerful numerics. For the parameter estimation we employed a least square algorithm in combination with an evolutionary strategy based on the theory of Rechenberg [Rechenberg, 1994]. The evolutionary strategy was implemented into a Pascal routine at the Institute of System Dynamics and Control of the University of Stuttgart and converted into Matlab at the Max-Planck-Institute for Dynamics of Complex Technical Systems. As ODE solver the ODE15s routine of Matlab 6.2 was utilized.

In order to represent several interacting signal transduction pathways or even a whole cell in a well structured manner new modeling concepts are needed. Therefore we apply the Modular Modeling Concept, which is currently under development at the Max-Planck-Institute for Dynamics of Complex Technical Systems [Kremling, 2000], [Stelling et al., 2001]. The basic idea comes from the engineering sciences, where this way of modular thinking has been successfully applied. [Lauffenburger, 2000]. Therefore, a promising way to achieve a system-level understanding of cells and organisms is to extend these theoretical concepts which are already successfully applied for the analysis and synthesis of complex technical systems. The elementary reactions described above can be combined into functional units and be represented according to the Modular Modeling Concept as aggregated modeling objects as shown in Figure 3. Each aggregated modeling object (= functional unit) comprises a disjunct set of elementary modeling objects representing the explicit biochemical reactions, which are shown in Figure 2. The proteins being part of the signal transduction pathway can be found in the storage modules. The coordinator modeling object contains the equations, describing a certain functional unit. These functional units can be very easily reused by the drag and drop principle for other receptors of the TNF family once they have been implemented into the modeling tool PROMOT [Ginkel et al., 2000], [Stelling et al.,





**Figure 3: Representation of the TNFR1 signal transduction pathways according to the Modular Modeling Concept [Kremling, 2000]**

2001]. As a consequence modeling of related receptors or signal transduction pathways consisting of similar functional units can be accelerated by this software enormously.

A compartment model was chosen in order to model the presented signal transduction pathways. Therefore three cellular compartments were taken into account: cell membrane, cytoplasm and endosomes. An idealized cell may be considered as a sphere with a diameter of  $15 \mu\text{m}$  resulting in a cell volume of  $1 \times 10^{-12} \text{ l}$ . The estimated radius of an average endosome is  $100 \text{ nm}$ , resulting in a volume of  $4.2 \times 10^{-18} \text{ l}$  [Haugh and Lauffenburger, 1998].

Usually, the kinetic equations of chemical reactions and the concentrations are written in terms of molar concentrations. If the same compound participates in reactions taking place in different compartments with different volumes, the effective concentration of this compound is different in each compartment. As the number of TNF receptor complexes formed at a certain concentration is usually calculated, the use of molecules per cell seemed more convenient to evaluate the number of activated molecules per cell and receptor in the signaling cascade. Therefore we converted the concentrations and the association rates occurring for the different compounds in molecules per cell using the proper volume for the particular compartment and Avogadro's Number.

## 3.1 Results

### 3.1.1 Role of TRAF2 in caspase activation

TRAFs mediate signal transduction from many members of the TNF receptor superfamily. In contrast to TNFR2, TNFR1 binds TRAF2 indirectly through the adapter protein TRADD [Hsu et al., 1996]. The indirect TRAF2 recruitment by TRADD triggers the antiapoptotic pathway. Alternatively the adapter molecule FADD initiates the apoptotic pathway by binding to TRADD. As TRAF2 and FADD

compete for TRADD, TNFR1 might alternatively form pro- or antiapoptotic complexes using the available adapter molecules. It is therefore probable that recruitment of TRAF2 to TNFR2 affects the pro- and antiapoptotic balance of TNFR1 signaling.

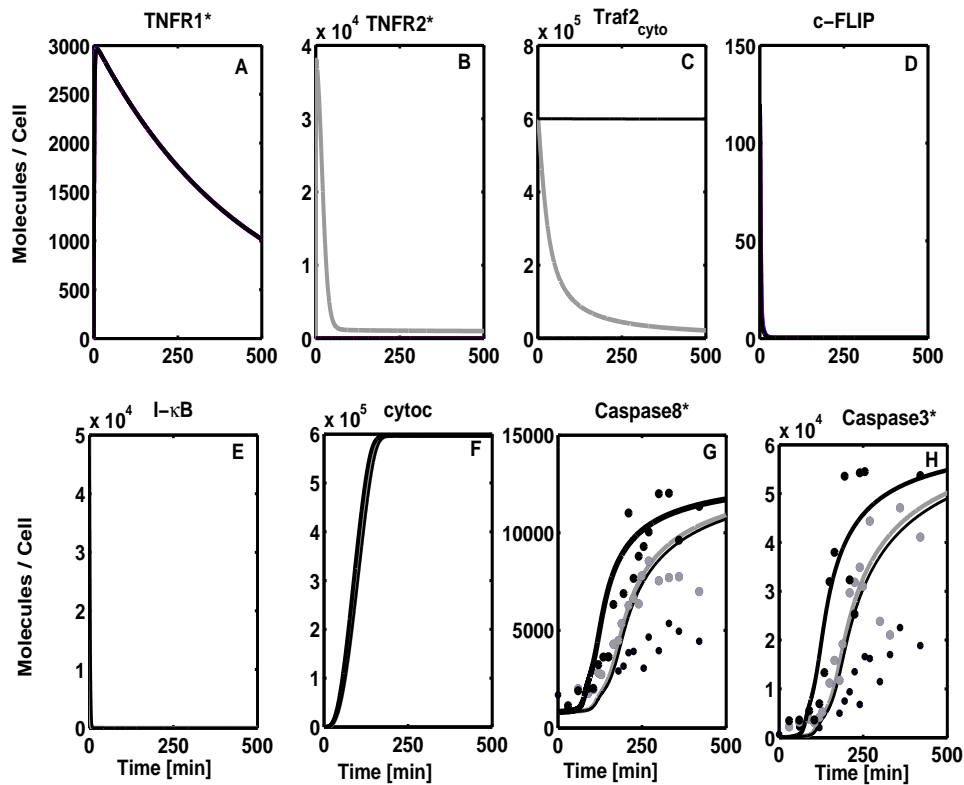
It was shown that the interaction between TRADD and TRAF2 is significantly stronger ( $K_D=7.8 \mu\text{M}$ ) [Park et al., 2000] than the direct interaction of TNFR2 and TRAF2 ( $K_D=40 \mu\text{M} - 1 \text{ mM}$ ) [Yeh and Wu, 2000], which is rather weak compared to other protein interactions. NF- $\kappa\text{B}$  activation triggered by TNFR2 is relatively small in comparison to that induced by TNFR1. We therefore neglected NF- $\kappa\text{B}$  activation by TNFR2. As we could observe TNFR2 induced caspase activation in HeLa cells, we included this caspase activating reaction pathway into our model which was observed in T cells [Pimentel-Muinos and Seed, 1999].

It could be shown that costimulation of TNFR2 enhances TNFR1 induced apoptosis. Depletion of endogenous TRAF2 from the cytoplasmic fraction could be observed, when TNFR2 was stimulated for prolonged times [Hostager et al., 2000], [Duckett and Thompson, 1997], [Weiss et al., 1998]. Recent findings indicated that TRAF2 is recruited into endosomes after TNFR2 stimulation [Fotin-Mleczek et al., 2001] and finally becomes degraded. In order to analyze the role of TRAF2 depletion in TNF receptor crosstalk in apoptosis we applied mathematical modeling.

Biochemical analyses of caspase activity were performed in TNF stimulated transfected HeLa cells under three distinct conditions: First, we costimulated TNFR1 and TNFR2. Second, we prestimulated TNFR2 for 6 hours and third, we stimulated TNFR1 on its own. The pre- and costimulation protocols resulted in an almost maximal caspase activation whereas selective stimulation of TNFR1 only lead to a partial caspase activation.

We further determined the total cellular TRAF2 concentration and found that after 6 hours of TNFR2 prestimulation TRAF2 was almost completely depleted [Fotin-Mleczek et al., 2001].

These experimental data of pre- and costimulation were taken as a starting point of our mathematical model. In the case of prestimulation we set the initial TRAF2 concentration in the model to zero. In order to fit the kinetics of caspase activation to the experimental data of pre- and costimulation, the mathematical model predicted a rather high initial concentration of TRAF2 which is in good agreement with experimental data [Zapata et al., 2000] and own unpublished data. In Figure 4 some selected time courses are shown in the graphs A-H. The thick black line represents the simulation results of TNFR2 prestimulation, the gray line costimulation of TNFR1 and TNFR2 and the thin black line the selective stimulation of TNFR1. In the graphs A and B the time courses of total receptor complexes, including both external and internalized receptors, are shown. The decrease in the number of complexes is due to receptor degradation following receptor internalization which corresponds well with literature data [Bajzer and Vuk-Pavlovic, 1992], [Pennica et al., 1992]. In graph C TRAF2 depletion is depicted with a half life time of about 30 min. Binding of TRAF2 to TNFR2



**Figure 4: Selective time courses of TNFR1/TNFR2 crosstalk under three distinct conditions. (Solid lines: simulation, dots: experimental data - Prestimulation, - Costimulation, - TNFR1 stimulation only )**

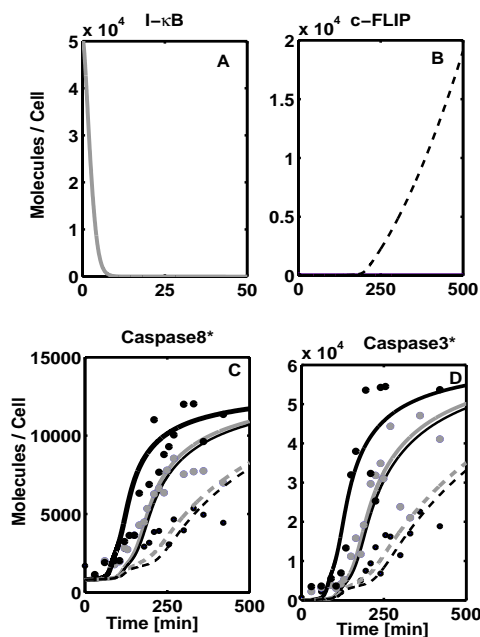
and the degradation of TNFR2 itself does not lead to the observed strong TRAF2 depletion. In the model we assume that TRAF2 interacts with a protein which is only active in the presence of TNFR2 and which leads to a TRAF2 depletion which is in good agreement with the experimental data (data not shown). Graph D shows the time course of c-FLIP, a potent inhibitory molecule acting as a competitor for caspase 8. The initial concentration of c-FLIP in CHX pretreated cells is very low and the caspase 8/c-FLIP ratio was found to be 100:1 in T cells and other apoptosis sensitive cell lines [Scaffidi et al., 1999], making it ineffective for caspase inhibition. However, raising c-FLIP concentration in our model and simulating caspase activation, indicated the high potency of the molecule in caspase inhibition. Graph E depicts the TNFR1 induced I- $\kappa$ B degradation which then leads to NF- $\kappa$ B activation. The cytochrome c release of the mitochondria is shown in graph F. The time courses of caspase 8 and caspase 3 activation are represented in the graphs G and H.

Comparing the experimental data (shown in dots) with the simulation results (shown in lines) one can state that the experimental data of pre- and costimulation can be well described by the model. The simulation of TNFR1 stimulation on its own (thin black line) however, does not fit to the experimental results (small black dots) and is virtually identical with the simulation results of costimulation. This finding indicates that depletion of TRAF2 does not affect caspase activation as much as expected [Weiss et al., 1997]. The reason according to the mathematical model is the high

number of TRAF2 molecules in comparison to the number of receptors.

In order to better describe the experimental data of selective TNFR1 stimulation, we introduced the capability of TNFR1 to induce the antiapoptotic proteins c-FLIP and cIAP at the transcriptional level (see Figure 5 graph dashed line). These changes resulted in a satisfying description of the experimental data of TNFR1 selective stimulation. In fact, subsequent experimental data confirmed the time kinetics of TNFR1 induced c-FLIP expression in HeLa cells (data not shown).

When we investigated the time course of I- $\kappa$ B processing, this was identical for experiments with receptor costimulation and TNFR1 selective stimulation as shown in Figure 5 graph A. Accordingly, in both cases c-FLIP and cIAP expression should be equally and strongly induced leading to a major inhibition of caspase activation. However, the simulated data from the costimulation experiment, when c-FLIP and cIAP induction was included (dashed gray line in Figure 5 graph C and D) were not in accordance with the experimental results. We therefore experimentally determined NF- $\kappa$ B activation in all three protocols of receptor stimulation and surprisingly found that NF- $\kappa$ B activation after costimulation was significantly lower than NF- $\kappa$ B activation after selective TNFR1 stimulation. [Fotin-Mleczek et al., 2001]. These data urged us to look for additional crosstalk mechanism influencing the antiapoptotic pathway. As it has been shown by one group that RIP can bind via



**Figure 5: Selective time courses of TNFR1/TNFR2 crosstalk under three distinct conditions under consideration of gene induction of antiapoptotic proteins. (- Prestimulation of TNFR2, - Costimulation of TNFR1 and TNFR2, - selective stimulation of TNFR1 and - - TNF stimulation with gene induction of antiapoptotic proteins )**

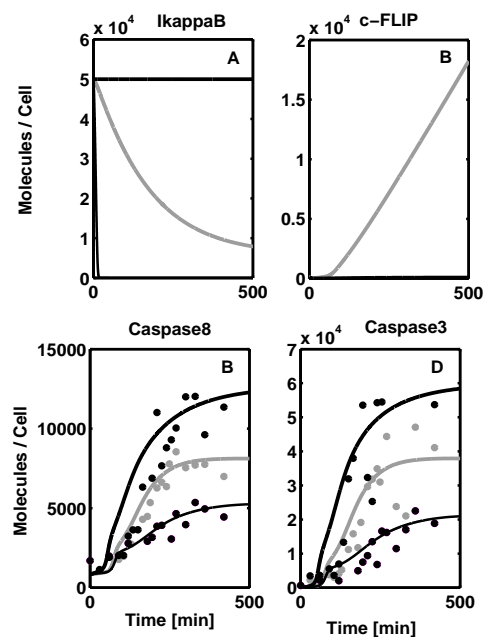
TRAF2 to to TNF-R2 [Pimentel-Muinos and Seed, 1999], we included TNFR2 and RIP interaction into the model.

### 3.1.2 RIP as potential key regulator protein

If we assume that the RIP concentration in the cells is much smaller than the TRAF2 concentration e.g. in the range of 20 nM (own unpublished data) this molecule becomes an important player in TNFR1/TNFR2 crosstalk. The competition of TNFR1 and TNFR2 for RIP depletes TNFR1 in RIP containing antiapoptotic complexes and therefore shifts TNFR1 signaling towards apoptosis via a reduction in NF- $\kappa$ B activation. According to the findings of Pimentel-Muinos [Pimentel-Muinos and Seed, 1999] and own experimental findings we included caspase activation of TNFR2 via binding of the adapter molecules TRADD and FADD to RIP.

The inclusion of the TNFR2/RIP interaction into the model revealed results as depicted in Figure 6. In Graph B the number of induced c-FLIP molecules is shown. I- $\kappa$ B degradation is given in graph A and in graph C and D caspase activation.

The slower I- $\kappa$ B degradation which is shown in graph A in Figure 6 might lead to a decrease in NF- $\kappa$ B activation but this point has not been further investigated. In any case the observed increase in caspase activation in the costimulation protocol compared to selective TNFR1 stimulation is caused by a reduction in RIP molecules available for TNFR1 rather than gene inductive effects. Thus, the



**Figure 6: TNF receptor crosstalk regulated by RIP. (- Prestimulation, - Costimulation, - TNFR1 stimulation only )**

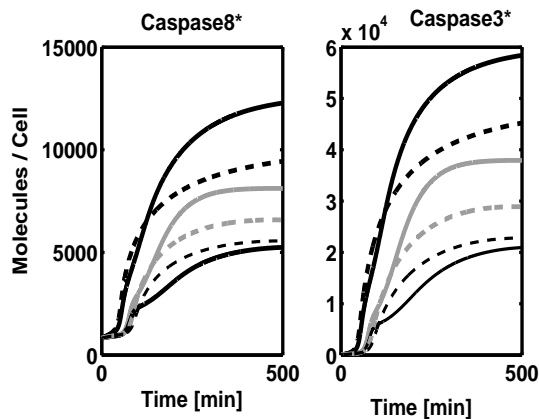
inclusion of RIP competition by the two receptors makes it now possible to satisfyingly describe all three experimental protocols with the computational simulation.

To further evaluate the predictive potential of the model, we simulated the dependence of NF- $\kappa$ B and caspase activation on TNF concentration. Activation of NF- $\kappa$ B in HeLa cells is a rapid process and can be detected as early as 2 min after stimulation and is maximal after about 15 min [Hohmann et al., 1990] for 1 nM TNF. The respective simulation resulted in a maximal activation at concentrations above 0.01 nM TNF after 30 min. This is in good accordance with literature data [Hohmann et al., 1990]. In contrast to NF- $\kappa$ B activation, efficient caspase processing needs much higher TNF concentrations [Weiss et al., 1997], results which are in good accordance with published data.

### 3.1.3 Role of the mitochondrial pathway in caspase activation

Scaffidi et. al. showed that there exist at least two different ways of caspase activation [Scaffidi et al., 1999]. So called type I cells induce apoptosis directly by large amounts of activated caspase 8 activated by the receptor complex. In contrast, in type II cells very little initial caspase 8 activation can be observed but this is amplified by the mitochondrial pathway described above [Scaffidi et al., 1999].

Our cellular system (HeLa cells) is well known to consist of typical type II cells [Goldstein et al., 2000]. In a next step we therefore included the mitochondrial pathway in our model. Blocking the mitochondrial pathway and testing the three experimental protocols of receptor stimulation revealed some interesting results which are depicted in Figure 7. As shown by the dashed lines the model predicts



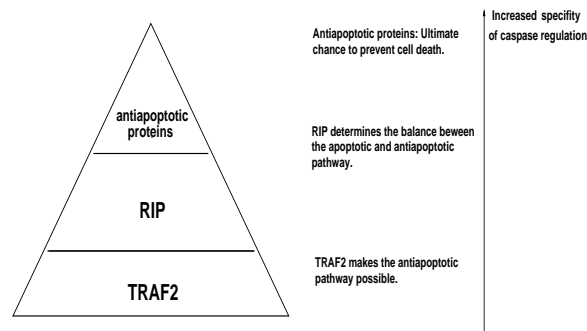
**Figure 7: Influence of the mitochondrial pathway in caspase activation. (Solid lines: - Prestimulation, - Costimulation, - TNFR1 stimulation only; Dashed lines: Caspase activation without the mitochondrial pathway )**

that the mitochondrial pathway leads to an amplification of caspase cleavage when TNFR2 has been prestimulated or costimulated. In contrast, the mitochondrial pathway hardly affects caspase activation when TNFR1 has been selectively stimulated. In the latter case the mitochondrial pathway even seems to attenuate caspase activation. These model predictions are hard to evaluate experimentally, but might give an idea of the functional role of the mitochondrial pathway.

#### 4. DISCUSSION

TNF is a potent immunoregulatory cytokine that mediates its effects through two distinct receptors, TNFR1 and TNFR2. While it is known that TNFR1 on its own is capable to trigger apoptosis, the role of TNFR2 in TNF induced cell death is still a matter of debate. Nevertheless, there exist several studies indicating that TNFR2 overexpression enhances TNF induced apoptosis [Chan and Lenardo, 2000], [Fotin-Mleczek et al., 2001], [Weiss et al., 1997], [Haridas et al., 1998]. The underlying mechanisms, however, remain largely undefined.

In order to gain a better understanding of TNFR1/TNFR2 crosstalk, we applied mathematical modeling under various situations of TNF receptor stimulation. These included the selective stimulation of TNFR1, costimulation of TNFR1 and TNFR2 as well as costimulation of TNFR1 and TNFR2 after a selective stimulation of TNFR2 for 6 hours (prestimulation). The modeling approach was performed focused on two typical cellular TNF responses, activation of NF- $\kappa$ B and induction of apoptosis. Experimental data for caspase 8 and 3 activation under all these conditions were available. In addition, the kinetics and dose responses of NF- $\kappa$ B activation have been determined experimentally. The results derived from an iterative process between biochemical experiments and mathematical modeling finally revealed a good accordance with the experimental data pointing out some signaling molecules which are likely to be highly involved in TNF receptor crosstalk.



**Figure 8: Hierarchical regulation of caspase activation**

The molecule TRAF2 is capable to bind indirectly to TNFR1 and directly to TNFR2. Due to these properties and the fact that it has been shown that TRAF2 is committed to degradation by prolonged TNFR2 stimulation, TRAF2 was assumed to be a good candidate to regulate TNF receptor crosstalk. In fact, under condition of TNFR2 prestimulation, resulting in a near to total depletion of the cells of cytoplasmic TRAF2, the model nicely fits the experimental data showing an enhanced caspase activity. However, in the two additional experimental setups where TNFR1 was selectively stimulated and TNFR1 and TNFR2 were costimulated, respectively, no significant differences were obtained in caspase activation in the simulation results, although the experiments showed a much weaker caspase activation after TNFR1 stimulation on its own. The obvious reason given by the model, later confirmed experimentally, was the high expression level of TRAF2, making any competition for TRAF2 neglectable after TNFR1/TNFR2 costimulation.

In a next step we included TNF mediated gene induction of antiapoptotic proteins, especially of the powerful proteins c-FLIP and cIAP, into our model. This resulted in a reduction of caspase activation in those experimental setups, where gene induction can occur, i.e. the costimulation protocol and the selective TNFR1 stimulation. However, the predicted kinetics of caspase activation in these two experiments were still identical in contrast to the experimental data. In line with experimental data demonstrating that under the conditions of costimulation NF- $\kappa$ B activation is reduced compared to TNFR1 stimulation alone, we looked for an additional molecule which might be essential for TNFR1 induced NF- $\kappa$ B activation and is also part of the TNFR2 signaling complex. The best candidate was the adapter molecule RIP which is, together with TRAF2, essential for TNFR1 mediated NF- $\kappa$ B activation but can also be recruited indirectly via TRAF2 to TNFR2. This molecule is considerably lower expressed in comparison to TRAF2 and might be therefore limiting under certain conditions. The inclusion of RIP binding to TNFR2 into the model resulted in two major effects, first, the delayed I- $\kappa$ B degradation and second, an enhanced caspase activation in the receptor costimulation protocol.

Analyzing the role of the adapter molecules proteins in the signal transduction pathway, it is possible to identify a certain role of each of the adapter molecules. Binding of TRADD serves as platform for the apoptotic and antiapoptotic path-

way, probably due to sterical reasons. The high amount of TRAF2 favours the antiapoptotic response, whereas FADD enables the apoptotic pathway. By means of mathematical modeling we have identified RIP to play a key role in the regulation of the apoptotic or antiapoptotic cellular response. Thus, we found a hierarchical regulation of caspase activation. TRAF2 regulates caspase activation at the beginning of the signal transduction cascade and delivers the platform for a second step of regulation by RIP. The competition of TNFR1 and TNFR2 for RIP regulates very efficiently the amount of activated caspase molecules. At the third level of caspase activation we find the gene induction of antiapoptotic proteins. This might be the last level where cells have the possibility to avoid apoptosis. This hierarchical structure is schematically shown in Figure 8.

## 5. CONCLUSION

Taken together, this work shows that mathematical modeling in connection with experimental data can give new insights into potential mechanisms of intracellular signal transduction and regulation. We found that the model is very sensitive to its structure, which means with regard to the actual signal transduction pathway, and less sensitive to its kinetic parameters. Without the inclusion of RIP binding to TNFR2, we were not able to describe all three experimental protocols.

In addition, even hypotheses which can hardly be tested experimentally like the contribution of the mitochondrial pathway, can be examined with the help of a mathematical model. The obtained results might then lead to a new understanding. The current model of TNF signaling, which is the result of an alternating process, may therefore serve as a platform for further experimental and theoretical investigations.

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