MATHEMATICAL MODELLING OF TNF-α INDUCED APOPTOTIC AND ANTI-APOPTOTIC SIGNALLING PATHWAYS IN MAMMALIAN CELLS BASED ON DYNAMIC AND QUANTITATIVE EXPERIMENTS

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Abstract: This work describes pro- and anti-apoptotic signal transduction pathways in a linked mathematical model. It emphasises the modelling and influence of the receptor from which those signal transduction pathways originate as well as the interaction of specific molecules within those signal cascades.
The main focus of this work is the development and verification of a model as well as a computer-assisted simulation of the processes in Kym-1 cells responsible for the NF-κB- and apoptotic pathways.
The proposed model is based on own experimental data that demonstrate that TNF-α signalling is not only dependent on the strength of the stimulus, but also on its duration. The proposed mathematical model is able to reproduce this and allows a detailed quantitative analysis of the crosstalk. This is performed with the help of dynamic sensitivity analysis.

Keywords: signal transduction, mathematical modelling, sensitivity analysis, apoptosis, tumour necrosis factor, caspases, NF-κB.

1. INTRODUCTION

The cytokine Tumour Necrosis Factor-α (TNF-α) is an important molecule crucially involved in the coordination of the immune response. In mammalian cells it induces many different responses, among them two seemingly contradictory ones as it stimulates pathways for both cell proliferation and the induction of programmed cell death, also called apoptosis. Several molecules couple these two pathways and only a systems approach can help unravel the factors deciding the cell fate. A necessary tool for understanding such complex systems is mathematical modelling, which allows one to analyse quantitative and dynamical influences of the different pathway components as well as perform predictive simulations.

This paper proposes a mathematical model of the TNF-α induced pro- and anti-apoptotic signalling pathways. The model corresponds to the signalling within a single cell and therefore exhibits fast activation of caspases (Rehm *et al.*, 2002). An important experimental finding is that a medium strength TNF-α stimulus leads to cell death for a continuous stimulus, but not for a pulse stimulus, see Section 3. The proposed model is able to replicate this. This model is then analysed to uncover the most important components of the crosstalk between TNF-α induced pro- and anti-apoptotic pathways.

This paper is structured as follows. Section 2 describes the biology of TNF-α signalling. Section 3 presents the experimental findings and Section 4 the proposed mathematical model. Simulation results are shown in Section 5 and a parameter sensitivity analysis is performed in Section 6. Finally, the paper concludes with a summary and outlook in Section 7.

2. BIOLOGICAL QUESTION

TNF-α is a classical, very well studied stimulus causing inflammatory responses, regulating immune responses and leading to apoptosis in certain tumour cells. In 1975, a single TNF-α injection was shown to cause necrosis of tumour cell in mice (Carswell *et al.*, 1975). In humans, a direct treatment with TNF-α is however not possible due to strong side effects. Nevertheless, an understanding of the TNF-α interaction is essential as TNF-α is also important for the activation and coordination of the innate immune system. Inadequate or continuous TNF-α production affects the pathogenesis of human diseases, in particular for diabetes, cancer, rheumatoid arthritis and osteoporosis. Therefore, the knowledge of the TNF-α signalling pathways is very important for the development of novel drugs.

In mammalian cells, TNF-α induces several signal transduction pathways. Some of them are shown in Figure 1.

In this study, the focus lies on the crosstalk between two pathways stimulated by the Tumour Necrosis Factor Receptor type I (p55; here called TNFR), which TNF-α binds to. This binding induces the formation of a multi-protein signalling complex at the cell membrane (Complex1). This complex activates the transcription factor Nuclear Factor-κB (NF-κB), which induces and up-regulates of anti-apoptotic proteins (FLIP, IAP) as well as of various inflammatory proteins, stimulates cell proliferation and inhibits programmed cell death (Li and Verma, 2002; Stehlik *et al.*, 1998).

Complex1 is internalised, resulting in Complex2 that activates pro-apoptotic signalling by activating caspases, a subfamily of the proteases (Micheau and Tschopp, 2003). In particular, caspase 8 (Casp8) initiates via the direct and the mitochondrial pathway the activation of caspase 3 (Casp3) that induces apoptosis. However, this paper studies type 1 cells where the mitochondrial pathway is not the primary route signalling (Barnhart *et al.*, 2003).

Intracellularly, a pronounced cross-talk between the different signal transduction pathways is decisive for the cell fate (Tucker *et al.*, 2004). Figure 1 shows the signalling pathways considered here.

3. EXPERIMENTAL DATA

Essential for mathematical modelling is quantitative data. Here, we show that for intermediate TNF-α concentrations, the activation of the pro- or anti-apoptotic pathway depends on the length of the stimulus.

3.1 Methods

Cell Culture and Reagents Kym-1 is a type 1 human rhabdomyosarcoma cell line, TNFR sensitive for TNF-α. Cells were maintained in RPMI medium (GIBCO®, invitrogen) supplemented with 10% fetal calf serum.

Cytotoxicity Assay Kym-1 cells (10^4/well) were grown overnight in 100 μl of culture medium in 96-well plates. The next day, the cells were treated with the indicated concentrations of the TNFR selective CysHisR32W/S86T-TNF. After 30 min, TNF-α was removed by three washes with medium. Afterwards, fresh medium was added with (continuous case) or without (pulse case) new CysHisR32W/S86T-TNF. ¿From the treated Kym-1 cells replenished with medium only, a part of the supernatant was taken and transferred to unstimulated growing Kym-1 cells to control the efficiency of the wash procedure. After an incubation of 12 hours, cell viability was determined using crystal violet staining (Weiss *et al.*, 1997).

3.2 Experimental Result

For low concentrations of TNF-α, neither a continuous nor a pulse treatment of TNF-α leads to the activation of the pro-apoptotic pathway (below 0.1 ng/ml), while for very high concentration (100 ng/ml) the cells undergo apoptosis, independently of the length of the stimulus, see Figure 2(a). For intermediate concentrations, a
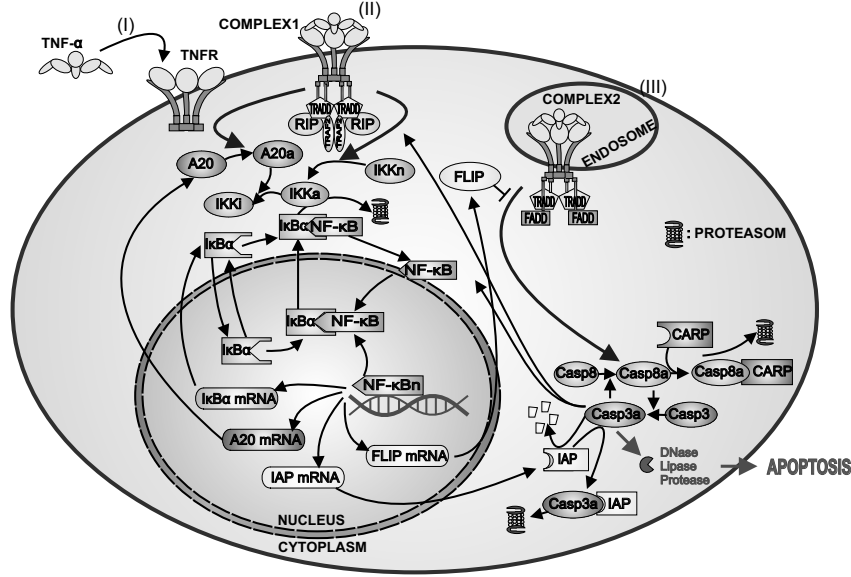
Fig. 1. TNF-α signal transduction. After binding to its receptor TNFR, TNF-α induces both the NF-κB and the apoptotic pathway, which are furthermore interconnected at multiple points.

pulse stimulation does not lead to apoptosis while a continuous stimulation does.

Another important factor is the length of the continuous stimulus. Figure 2(b) shows that TNF-α stimuli of 9 hours or longer lead to a similar apoptotic outcome. Shorter continuous stimuli approach the pulse stimulus shown in Figure 2(a).

4. MATHEMATICAL MODEL

To gain a better understanding of the TNF-α induced signalling, we developed a quantitative and dynamical mathematical model. The model consists of three modules, the receptor, the NF-κB pathway and the caspase activation pathway, described in Section 4.2. The proposed model includes mRNA synthesis, translation, transport between extracellular space, cytoplasm and nucleus and formation and dissociation of complexes.

4.1 Model structure

The model consists of a set of (mostly reversible) chemical reactions with at most two substrates, modelled according to the law of mass action. Thus, each reaction is of the form

$$A + B \rightleftharpoons C + D$$
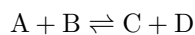
and the resulting reaction rate is

$$v = k_{on} \cdot [A] \cdot [B] - k_{off} \cdot [C] \cdot [D].$$

This results in a system of nonlinear differential equations for the concentrations over time t:

$$\frac{dc_r}{dt} = \sum_{i,j}^{n} k_{i,j} c_i c_j + \sum_{l}^{n} k_l c_k + \tilde{k}_r \qquad (1)$$

$$= \boldsymbol{c}^T \mathfrak{K}_r \boldsymbol{c} + \boldsymbol{k}^{(r)^T} \boldsymbol{c} + x\tilde{k}_r, \quad r = 1, \dots, n$$

with $\mathfrak{K}_r \in \mathbb{R}^{n \times n}$, the vector of the n concentrations $\boldsymbol{c} = \begin{pmatrix} c_1 & \cdots & c_n \end{pmatrix}^T$ and $k_{i,j}$, k_l, $\tilde{k}_r$ as reaction velocities ($\pm k_{on}$, $\pm k_{off}$ or zero). A more compact notation of (1) is

$$\frac{d\boldsymbol{c}(t)}{dt} = \boldsymbol{f}(\boldsymbol{c}(t), \boldsymbol{p}) \qquad (2)$$

where $\boldsymbol{p}$ is the vector containing all velocities.
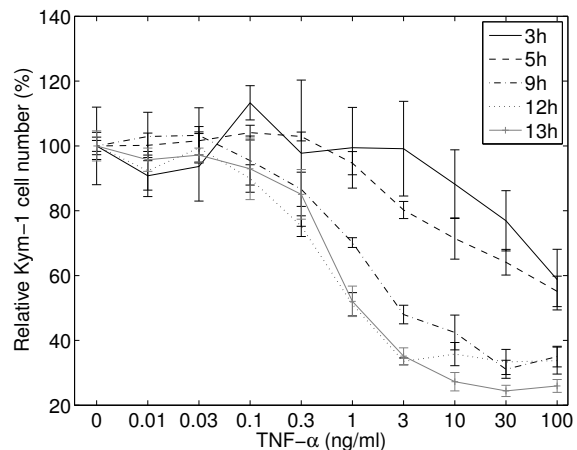
4.2 Modules of the model

Upon stimulation of TNFR, a first NF-κB activating signalling complex is formed within minutes by binding of RIP and TRAF2 to the receptor. After internalisation a second death inducing signalling complex (DISC) is formed, which activates initiator caspases. We propose for this module a model consisting of 19 compounds, 24 reactions and 48 parameters.

NF-κB contributes mainly to the expression of pro-inflammatory and anti-apoptotic genes like FLIP and IAP. For this pathway, we use the mathematical model by Lipniacki *et al.* (2004).

As Kym-1 are type 1 cells, the effector caspases (here lumped into Casp3) are activated directly by initiator caspases (here lumped into Casp8) (Barnhart *et al.*, 2003). The activation is switch-like within single-cells. This module has been modelled in Eißing *et al.* (2004).
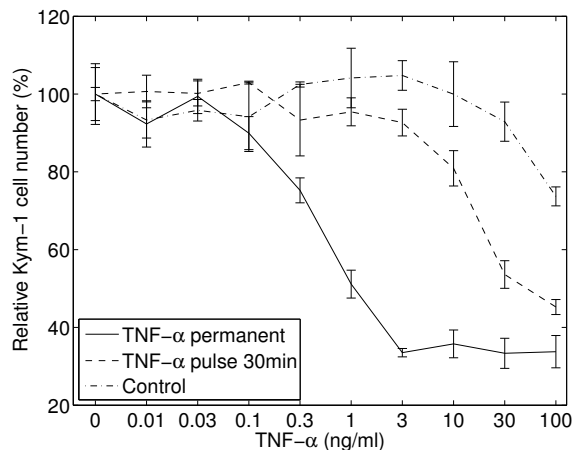
(a) Viability after 12h.

(b) Effect of stimulus duration.

Fig. 2. Cytotoxicity assays considering Kym-1 cells treated with serial dilutions of TNF-α.

4.3 Coupling of the modules and overall model

The proposed model couples the three modules, namely the receptor signalling block, the NF-κB pathway and the caspase 8-3 pathway by including NF-κB-dependent expression of regulatory and anti-apoptotic proteins (FLIP and IAP). The complete model consists of 73 reactions with 146 parameters and 41 compounds. Most parameters (k_{on} and k_{off}) are taken from (Eißing et al., 2004; Hoffmann et al., 2002; Lipniacki et al., 2004). In the overall model, 68 parameters, in particular the kinetic rate constants of the receptor pathway and those of the reactions coupling the NF-κB and the caspase pathways, were not available and therefore qualitatively fitted to own and published experimental data.

5. SIMULATION RESULTS

The proposed mathematical model fits the overall qualitative data very well and gives a good account for the characteristics of key molecules, see Figure 3, which illustrates the case of an intermediate TNF-α stimulus. The first step of the TNF-α induced signalling is the binding of TNF-α to TNFR (Grell et al., 1998). For both continuous and 30 min pulse stimulation, TNFR saturates. The main difference is the persistent presence of Complex2 for continuous stimulation, which leads to activation of Casp8 and then of Casp3, see arrows in Figure 3. The initial receptor complex (Complex1) is rapidly formed and activates the NF-κB pathway (Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004). The degradation of IκBα releases NF-κB, which translocates into the nucleus (NF-κBn) where it functions as a transcription factor. For a continuous stimulation, IκBα and NF-κBn oscillate, but

in an anti-cyclic manner, see Figure 3 and (Nelson et al., 2004). However, for a pulse stimulation there is only one peak of NF-κBn, see Figure 3.

The internalised receptor (Complex2) is formed after a lag-phase, leading to the activation of caspases (see Casp3a), consistent with the degradation of IAP (Salvesen and Duckett, 2002). For a continuous stimulation, the cell survives for more than 4h, until a large amount of IAP has been degraded (Lewis et al., 2004). In the pulse case, there remains enough IAP to protect the cell from apoptosis, see Figure 3.

Simulations with different TNF-α stimuli and over different simulation horizons qualitatively very well reproduce the results of the cytotoxicity assays, Figure 2. Especially, the in-silico cell shows an apoptotic phenotype 5h after stimulation with a continuous intermediate TNF-α concentration as experimentally observed, Figure 2(b).

6. SENSITIVITY ANALYSIS

Sensitivities describe how compounds are influenced by changes of the parameters:

$$S_{i,j}^{N}(t) = \frac{\partial \boldsymbol{c}_i(t)}{\partial \boldsymbol{p}_j} \cdot \frac{\boldsymbol{p}_j}{\boldsymbol{c}_i(t)}. \qquad (3)$$

As we are interested in the dynamical case, the sensitivity matrix S^N needs to be calculated along a trajectory starting at $S_{i,j}^{N}(0) = 0$, i.e.

$$\frac{d\boldsymbol{c}(t)}{dt} = \boldsymbol{f}(\boldsymbol{c}(t)), \qquad (4a)$$

$$\frac{d\tilde{S}^{N}(t)}{dt} = \frac{\partial \boldsymbol{f}(\boldsymbol{c}(t), \boldsymbol{p})}{\partial \boldsymbol{c}(t)}\tilde{S}^N + \frac{\partial \boldsymbol{f}(\boldsymbol{c}(t), \boldsymbol{p})}{\partial \boldsymbol{p}}, \quad (4b)$$

$$S_{i,j}^{N}(t) = \tilde{S}_{i,j}^{N}(t) \cdot \frac{\boldsymbol{p}_j}{\boldsymbol{c}_i(t)}. \qquad (4c)$$
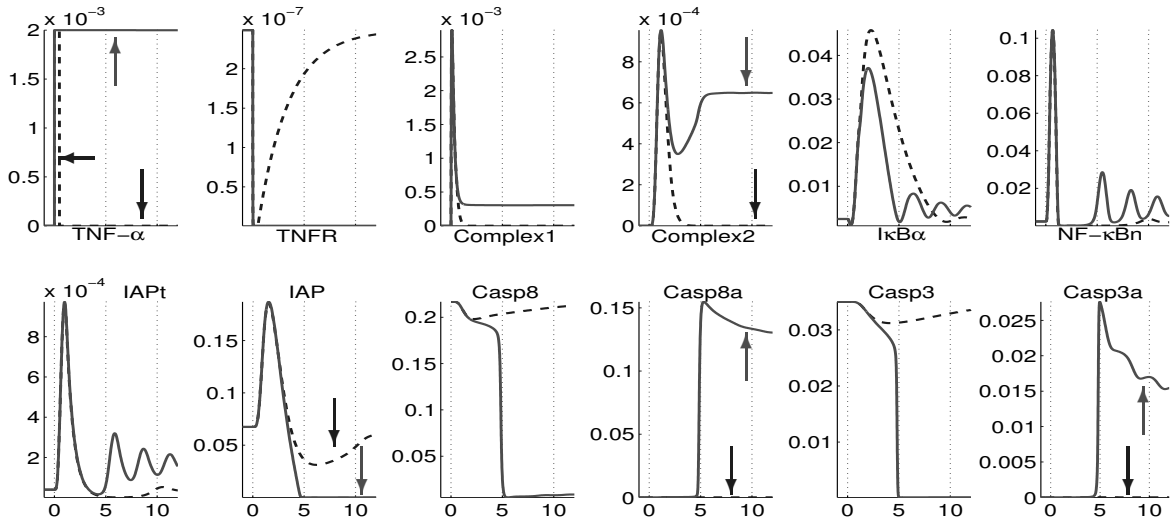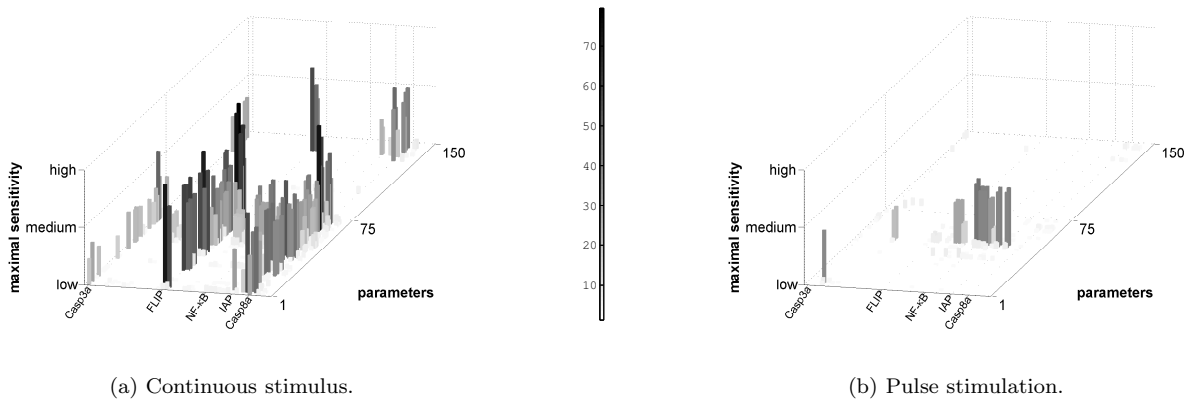
Fig. 3. Dynamics of important compounds over a period of 12 hours for persistent (continuous line) TNF-α stimulation and a TNF-α-pulse of 30 min starting at 0 h (dashed line).

(a) Continuous stimulus.

(b) Pulse stimulation.

Fig. 4. Maximal absolute values of the sensitivities S^N of the system, simulated over 8h.

The maximal absolute values of the sensitivities are depicted in Figure 4(a) for a continuous stimulation of TNF-α and in Figure 4(b) for a pulse stimulation. The sensitivity analysis uncovers the most critical parameters in both stimulation protocols. The results are summarised in Table 1.

Sensitive parameters are present in all three parts of the network, in particular the parameters of (1) reactions influencing the formation of the two receptor complexes, (2) upstream reactions of the NF-κB pathway including IKK activation, and (3) reactions directly involved in the activation and inactivation of caspases.

As can be seen in Figure 4, less parameters are sensitive in the pulse case and, furthermore the maximal sensitivity is almost twice as large in the continuous case.

7. SUMMARY AND OUTLOOK

For a better understanding of the behaviour of complex biological systems such as TNF-α in-

duced signalling, mathematical modelling and model analysis are essential. In this study, we present quantitative experimental data showing that for intermediate concentrations of TNF-α, different signalling pathways are activated, depending on the length of the stimulus. We propose a mathematical model for the TNF-α induced pro- and anti-apoptotic signalling. The model consists of three modules: the receptor signalling, the NF-κB pathway and the caspase activation pathway as well as their interconnection.

Simulation results are in good qualitative agreement with experimental data, in particular, the model can discriminate well between continuous and pulse stimulations of different lengths. The model analysis via a dynamical sensitivity analysis uncovers the most critical components of this complex crosstalk. Such an analysis will prove helpful for uncovering the most important components in interacting signalling networks. The model at hand and the sensitivity analysis allows to quickly test biological hypotheses helping to decide which one to further test in experimentation.

System module	Compound	Most sensitive parameter at	
		pulse TNF-α	continuous TNF-α
Receptor	TNF-α-TNFR	degradation	less sensitive
NF-κB pathway	NF-κBn	• degradation of (cytoplasmic & nuclear) IκBα • nuclear im- / export of IκBα	• (cytoplasmic & nuclear) IκBα degradation • export from the nucleus
	nuclear IκBα	idem	idem
	mRNAs of A20, IκBα, IAP & FLIP	idem	less sensitive
	FLIP	less sensitive	• receptor synthesis • IKK activation • transport of NF-κB into nucleus • (cytoplasmic & nuclear) IκBα degradation • nuclear im- / export of IκBα • FLIP transcription & translation • Casp8 activation by Complex2 & Casp3a • inactivation of Casp3a by IAP
	Complex2$\sim$FLIP	less sensitive	idem
Apoptosis pathway	Casp3	less sensitive	• Casp8 activation by Complex2 & Casp3a • inactivation of Casp3a by IAP
	Casp3a	less sensitive	idem
	Casp8	less sensitive	activation of Casp8 by Complex2

Table 1. Summary of the sensitivity analysis, comparing pulse and continuous stimulation with TNF-α.

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