

# Chemosensitization by diverging modulation by short-term and long-term TNF- $\alpha$ action on ABCB1 expression and NF- $\kappa$ B signaling in colon cancer

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**Abstract.** Multidrug resistance (MDR) is a major cause for cancer chemotherapy failure. Among the numerous strategies to overcome persistent action of proinflammatory cytokines, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) permits down-regulation of MDR-associated genes, including ATP-binding cassette, subfamily B 1 gene (ABCB1). A key regulator of ABCB1 expression is the transcription factor nuclear factor  $\kappa$  light chain enhancer (NF- $\kappa$ B)/p65. We analyzed diverging short- and long-term effects of TNF- $\alpha$  regarding modulation of NF- $\kappa$ B/p65 signaling and ABCB1 expression in colon cancer cells. Highly resistant ABCB1 overexpressing human HCT15 colorectal carcinoma cells were subjected to short- (30-120 min) or long-term (24-96 h) TNF- $\alpha$  treatment. TNF- $\alpha$  mediated modulation of ABCB1 expression was analyzed by real-time RT-PCR and western blot analysis. The TNF-mediated chemosensitization was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. The involvement of TNF receptors and of NF- $\kappa$ B/p65 signaling was analyzed by western blot analysis, ABCB1 promoter analysis and electrophoretic mobility shift assay (EMSA). The study revealed, that long-term, but not short-term TNF- $\alpha$  treatment leads to TNF-receptor 1 (TNFR1) mediated downregulation of ABCB1 resulting in

sensitization towards drug treatment. It dampens NF- $\kappa$ B/p65 activation and nuclear factor of  $\kappa$  light polypeptide gene enhancer in B-cells inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ) resynthesis, associated with reduced nuclear accumulation of NF- $\kappa$ B/p65 and reduced binding to its consensus sequence in the ABCB1 promoter. The study reveals the diverging effects of short- or long-term TNF- $\alpha$  action and provides novel insights on downregulation of ABCB1 expression by TNF-mediated repression of NF- $\kappa$ B signaling.

## Introduction

The phenomenon of multidrug resistance (MDR) still represents a major obstacle for successful chemotherapy of cancer (1-4). The emergence of the MDR phenotype is mainly caused by the increase in expression of MDR-associated genes, such as ATP-binding cassette, subfamily B 1 gene (ABCB1) (MDR1), ABCC1 (MRP1) and ABCG2 (BCRP1). Numerous efforts have been made to develop strategies for modulation of MDR-associated genes. The classical type of MDR is mediated by overexpression of the P-glycoprotein (PgP), the gene product of the ATP-binding cassette (ABC) transporter ABCB1. Therefore reversal approaches target the expression and function of ABCB1/PgP (4). Among these approaches the use of cytokines has shown promise for downregulation of ABCB1 in association with increased drug uptake and chemosensitization of tumor cells (5). Several studies have shown, that cytokines, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\gamma$  (IFN- $\gamma$ ), leukoregulin and interleukin-2 (IL-2) are capable of reducing ABCB1 gene expression and increasing chemosensitivity of different cancer cell lines *in vitro*, and more recently also for endothelial cells in the blood-brain barrier (6-12). It has been shown, that particularly persistent treatment with these cytokines is of importance to achieve significant reduction in ABCB1 gene expression (6,7,9,13). Consistent with the pre-clinical findings of cytokine-mediated chemosensitization, clinical trials have demonstrated synergism of combinations of cytostatic drugs with TNF- $\alpha$  or interferones in cancer patients (14,15). Among all cytokines used for ABCB1 expression modulation, TNF- $\alpha$  has shown the highest efficiency *in vitro*, *in vivo* and in clinical approaches (9,11,13,16). Although the TNF- $\alpha$  mediated effects

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*Abbreviations:* ABCB1, ATP-binding cassette, subfamily B 1 gene; EMSA, electromobility shift assay; MDR, multidrug resistance; NF- $\kappa$ B, nuclear factor  $\kappa$  light chain enhancer; I $\kappa$ B $\alpha$ /I $\kappa$ B $\beta$ , nuclear factor of  $\kappa$  light polypeptide gene enhancer in B-cells inhibitor,  $\alpha/\beta$ ; PgP, P-glycoprotein; TNFR, TNF-receptor; TNF- $\alpha$ ; tumor necrosis factor  $\alpha$

*Key words:* TNF- $\alpha$ , multidrug resistance, ATP-binding cassette, NF- $\kappa$ B, colon cancer, chemosensitization

on ABCB1 expression were repeatedly shown at functional level, it is still not fully understood, what mechanism in cell signalling is responsible for these effects.

TNF- $\alpha$  exerts its intracellular activities via TNF-receptor 1 (TNFR1, p55) and TNF-receptor 2 (TNFR2, p75). Among other factors, the receptor-mediated intracellular signalling cascade requires nuclear factor  $\kappa$  light chain enhancer (NF- $\kappa$ B) as one key mediator and TNF- $\alpha$  is one of the most potent activators of NF- $\kappa$ B signaling (17).

Activation of NF- $\kappa$ B, which is sequestered in the cytoplasm as an inactive factor, is mediated via the nuclear factor of  $\kappa$  light polypeptide gene enhancer in B-cells inhibitor (I $\kappa$ B) kinase (IKK) complex, which is triggered by proinflammatory cytokines including TNF- $\alpha$  or cytostatic drugs (18). IKK phosphorylates the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ , which is then degraded by the 26S proteasome complex. NF- $\kappa$ B is released, unmasking its nuclear localization signal and enters the nucleus to activate transcription of specific target genes, including of its own inhibitor I $\kappa$ B $\alpha$ . Within this signaling cascade I $\kappa$ B $\alpha$  mediates both, rapid activation of NF- $\kappa$ B and strong negative feedback (19).

The link between NF- $\kappa$ B and ABCB1 gene expression regulation has been analyzed in several studies (20). These studies demonstrate that transient induction of NF- $\kappa$ B is associated with increase in ABCB1 gene expression and inversely, inhibition of NF- $\kappa$ B leads to downregulation of ABCB1 expression (20-23). Promoter analyses revealed that the human ABCB1 promoter harbors NF- $\kappa$ B responsive elements, which bind NF- $\kappa$ B to mediate regulation of ABCB1 gene expression (24). However, other studies revealed a more complex picture of TNF- $\alpha$  triggered NF- $\kappa$ B activation and its target gene regulation. They provide detailed insights into the diverging nature of TNF- $\alpha$ /NF- $\kappa$ B signaling. This is strongly dependent on duration of pathway activation, showing rather contrary effects of early transient and of late persistent phase effects, mediated either by I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$  (25). Interestingly, such differential action can lead to negative feedback mechanisms of TNF- $\alpha$ /NF- $\kappa$ B signaling which dampen the initial early transient phase effects, in which I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  are important regulators (26-28).

In this context our study analyzed both, the transient and more importantly persistent TNF- $\alpha$  mediated effects on ABCB1 expression in human colorectal cancer cells as diverging action of this cytokine. We show that NF- $\kappa$ B/p65 exerts signal transduction via TNFR1, which is strictly time-dependent and tightly associated with the modulation of ABCB1 expression. These findings extend the picture of previously described diverging nature of TNF- $\alpha$  action. They provide a potential link to chronic inflammation, persistent TNF- $\alpha$  release and drug sensitivity in TNF- $\alpha$  exposed cells or tissues, as is clinically observed in e.g. inflammatory intestine diseases (29-32). More importantly, this study might open new insights for targeted interventions of MDR reversal in the treatment of colon cancer.

## Materials and methods

**Cell culture.** The human colorectal carcinoma cell line HCT15 was cultured at 37°C, 5% CO<sub>2</sub> in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA), containing 10% FCS.

This cell line endogenously expresses high-level ABCB1 and possesses a strong MDR phenotype (33). Authentication of the cell line was performed by STR DNA typing (DSMZ, Braunschweig, Germany).

**Treatment with TNF- $\alpha$ .** Briefly, 5x10<sup>4</sup> cells were seeded into 24-well plates and grown for 24 h. Then, cells were treated with 30 ng TNF- $\alpha$ /ml (Invitrogen, Carlsbad, CA, USA) for 5-120 min in short-term incubations and 24-72 h in long-term incubations. The cells were harvested for further analyses at indicated time points (5, 10, 15, 30, 60 and 120 min; 24, 48 and 72 h). In the long-term incubations in addition cells were harvested shortly after TNF- $\alpha$  application at the respective days, indicated as 24, 48 and 72 h.

**Blocking of TNF-receptors.** The blocking of TNF-receptors was performed in cells treated for 72 h with TNF- $\alpha$ . For this, 5x10<sup>4</sup> cells were seeded into 24-well plates and grown for 24 h. One hour before each treatment with 30 ng TNF- $\alpha$ /ml (Invitrogen) cells were incubated with 8 or 20  $\mu$ g mouse anti-TNFR1 monoclonal antibody (R&D Systems, Inc., Minneapolis, MN, USA) and 4 or 12  $\mu$ g mouse anti-TNFR2 monoclonal antibody (R&D Systems, Inc.) respectively, to specifically block the receptor (as recommended by manufacturer). The control cells were treated with 30 ng TNF- $\alpha$ /ml only, or remained untreated.

**Real-time quantitative RT-PCR (qRT-PCR).** Total RNA from cells was isolated using the TRIzol™ method (Invitrogen). Reverse transcriptase (RT) reaction was performed with 50 ng of total RNA (MuLV reverse transcriptase; Perkin-Elmer, Weiterstadt, Germany). Each quantitative real-time PCR (95°C for 10 sec, 45 cycles of 95°C 10 sec, 60°C 30 sec and 72°C 1 sec) was done using the LightCycler (LightCycler Fast Start DNA master hybridization probes kit; Roche Diagnostics GmbH, Mannheim, Germany). Expression of human ABCB1, NF- $\kappa$ B/p65, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and of the housekeeping gene glucose-6-phosphate dehydrogenase (G6PDH) was determined in parallel from the same RT-reaction, each done in duplicate per sample. For ABCB1 a 167 bp amplicon (forward, 5'-CCCATCATTGCAATAGCAGG-3'; FITC-labeled probe forward, 5'-CACTGAAAGATAAGAAAGAACTAGAAAGGTGCT-3'; LCRed640-labeled probe forward, 5'-GGAAGATCGCTACTGAAGCAATAGAAAAC-3' and reverse, 5'-GTTCAAACTTCTGCTCCTGA-3'); for NF- $\kappa$ B/p65 a 365 bp amplicon (forward, 5'-AGATCAATGGCTACACAGGA-3' and reverse, 5'-GATGGGATGAGAAAGGACA-3'); for I $\kappa$ B $\alpha$  a 354 bp amplicon (forward, 5'-CCGAGACTTTCGAGGAAT-3' and reverse, 5'-GTGAGCTGGTAGGGAGAATA-3'); for I $\kappa$ B $\beta$  a 417 bp amplicon (forward, 5'-AGTACATGGACCTGCAGAAT-3' and reverse, 5'-GGACCATCTCCACATCTTTg-3'), and for G6PDH a 123 bp amplicon was produced, which were detected by gene-specific fluorescein- and LCRed640-labeled hybridization probes [(primers for ABCB1, NF- $\kappa$ B, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ ; BioTeZ, Berlin, Germany); (probes for ABCB1; TIB Molbiol, Berlin, Germany); (primers and probes for G6PDH; Roche Diagnostics GmbH)]. The calibrator cDNA, derived from the human ABCB1 expressing cell line HCT15, was employed in serial dilutions (in duplicate) simultaneously in each run.

**Western blotting.** Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1X complete protease inhibitor cocktail (Roche Diagnostics GmbH). Cytoplasmic and nuclear extracts were prepared from cells by using the NE-PER extraction kit according to manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL, USA). The protein content was quantified by using the Coomassie Plus protein assay according to manufacturer's instructions (Pierce Biotechnology, Inc.). Precast NuPAGE 4-12% gradient polyacrylamide gels (Invitrogen) were loaded with 50 µg protein of either total cell lysates, cytoplasmic extracts, or 10 µg of nuclear extracts and electrophoresed at 200 V, 60 min. The gels were blotted onto nitrocellulose filter (Hybond-C Extra; Amersham, Freiburg, Germany). The filters were blocked 1 h at room temperature in TBS blocking buffer (50 mM Tris, 150 mM NaCl, pH 7.5, 5% fat-free dry milk) and washed in TBST (0.05% Tween-20 in TBS buffer) at RT.

For detection anti-human-TNFR1 mouse IgG monoclonal antibody (1:100), anti-human-TNFR2 mouse IgG monoclonal antibody (1:250) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-human PgP monoclonal mouse IgG-antibody C219 (1:100; Calbiochem, San Diego, CA, USA), and anti-human NF-κB/p65 mouse monoclonal IgG-antibody (1:500), anti-human IκBα mouse monoclonal IgG-antibody (1:500), anti-human IκBβ mouse monoclonal IgG-antibody (1:100) (all from Santa Cruz Biotechnology, Inc.), anti-human β-tubulin mouse monoclonal IgM antibody (1:500; BD Pharmingen, Heidelberg, Germany), anti-human nuclear matrix protein (NMP) p84 mouse monoclonal IgG antibody (1:2,000; Abcam, Cambridge, UK) and anti-human GAPDH goat polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Inc.) were used. As secondary antibodies HRP-labeled goat anti-mouse IgG-antibody (1:6,000; Pierce Biotechnology, Inc.), goat anti-mouse IgM-antibody (1:5,000; Sigma-Aldrich, St. Louis, MO, USA) or mouse anti-goat antibody (1:5,000; Santa Cruz Biotechnology, Inc.) were used. All antibodies were diluted in TBST containing 5% BSA. After incubation with the respective primary and secondary antibodies, the filters were washed in TBST. After washing, the respective protein was detected using ECL-solution (Amersham) and exposed to Kodak X-Omat AR film.

**Electrophoretic mobility shift assay (EMSA).** The nuclear extracts were prepared from cells by using the NE-PER extraction kit according to manufacturer's instructions (Pierce Biotechnology, Inc.). The EMSA was performed with 3 µg of nuclear extract using biotin end-labeled double-stranded oligonucleotides harboring the NF-κB/p65-binding site of the wild-type human ABCB1 promoter (sense, 5'-GCTGCTCTG GCCGCGATGGGCACTGCAGGGGCTTTCCTGTGCGCG GGTCTCCAGCATCT-3' and antisense, 5'-AGATGCTGGA GACCCCGCGACAGGAAAGCCCCTGCAGTGCCCATC GCGCCAGAGCAGC-3'); or mutant (sense, 5'-GCTGCTCT GCGCGATGGGCACTGCAGTCTTTCCTGTGCGCG GGGTCTCCAGCATCT-3' and antisense, 5'-AGATGCT GGAGACCCCGCGACAGGAAAGCGAGTGCAAGTCC CATCGCGCCAGAGCAGC-3'). In competition experiments unlabeled double-stranded oligonucleotides were used to control binding specificity of NF-κB/p65. For the supershift

experiments, the NF-κB/p65 mouse monoclonal IgG-antibody (0.5 or 1.0 µg; Santa Cruz Biotechnology, Inc.) was used. For the EMSA 6% TBS pre-cast gels (Invitrogen) were used. Gels were blotted onto nitrocellulose filters (Amersham). After UV cross-linking shifted and supershifted bands were detected by the LightShift kit (Pierce Biotechnology, Inc.) according to manufacturer's instructions and exposed to Kodak X-Omat AR film.

**Drug uptake assay.** After the pretreatment with 30 ng TNF-α/ml (Invitrogen) for 2 or 72 h, respectively, cells were incubated with doxorubicin (50 µM; Sigma, Taufkirchen, Germany) in phenol red-free RPMI-1640 medium for 3 h at 37°C, and were washed with phenol red-free medium and kept on ice. Fluorescence intensity of 1x10<sup>4</sup> cells was measured in duplicate per sample by using the FACSCalibur (Cell Quest program; Becton-Dickinson, San Diego, CA, USA). The drug uptake is expressed as fold-increase compared to untreated cells, which did not receive TNF-α.

**Cytotoxicity assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).** For the cytotoxicity assay, 5x10<sup>3</sup> cells were seeded into 96-well plates. The short- or long-term TNF-α effects on drug sensitivity of cells was determined by 2 or 72 h pretreatment with 30 ng TNF-α/ml. Thereafter cells were washed and treated with 50, 100, 200, 400, 1,000, 1,250 and 1,500 ng doxorubicin/ml (Sigma) for 72 h at 37°C. Then, MTT (5 mg/ml; Sigma) was added and absorbance was measured in triplicates at 560 nm in a microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Values are expressed as percent of untreated controls. Cells treated 2 or 72 h with TNF-α only served as additional control.

**Cell signalling reporter assay.** To determine TNF-α mediated short- and long-term induction via NF-κB/p65, we used the Cignal NF-κB/p65 *Renilla*/firefly dual luciferase reporter assay kit (SABiosciences, Frederick, MD, USA). For each well of a 96-well plate 100 ng Cignal NF-κB reporter or Cignal negative control plasmid-DNA, respectively, were reverse transfected with FuGENE HD (Roche Diagnostics GmbH) into 1x10<sup>4</sup> cells and seeded in serum-free conditions, as recommended by the manufacturer. Then, 24 h after transfection medium was replaced with RPMI-1640 medium +10% FBS and treatment with 30 ng TNF-α/ml (Invitrogen) was started. For short-term TNF-α affected reporter expression cells were incubated 2, 4, 6, 8, 10 and 12 h, washed with PBS and lysed in passive lysis buffer (Promega). For evaluation of TNF-α affected reporter expression in long-term treatment, transfected cells were incubated for 8, 24, 48 and 72 h with 30 ng TNF-α/ml. In addition, time points of 8 h after the respective TNF-α treatment were included, indicated as 24, 48 and 72 h+. Cells were washed with PBS and lysed in passive lysis buffer. The luciferase assay was performed in triplicates with Dual-Luciferase Reporter Assay system (Promega) using 3 µl cell lysate in triplicates and using the Centro LB 960 luminometer (Berthold Technologies GmbH & Co., Bad Wildbad, Germany).

**ABCB1 promoter driven luciferase reporter assay.** To analyze the short- and long-term effects of TNF-α on the human ABCB1 promoter activity, the NF-κB/p65 consensus harboring 990 bp

promoter sequence was PCR-cloned (24). This sequence spans the first exon and intron, and was used to drive the pGL3 plasmid luciferase reporter expression (Promega).

For the assay 150 ng ABCB1 promoter luciferase or promoterless pGL3 plasmid-DNA as negative control respectively were reverse transfected in  $1 \times 10^4$  cells in each well of a 96-well plate with LTX-RG (Invitrogen) in serum-free conditions. Medium was replaced with RPMI-1640 medium and 10% FBS 24 h after transfection and TNF- $\alpha$  treatment was started. For determination of short-term TNF- $\alpha$  affected reporter expression cells were incubated with 30 ng TNF- $\alpha$ /ml (Biosource) for 2, 4, 6, 8, 10 and 12 h, washed with PBS and lysed passive lysis buffer (Promega). For evaluation of TNF- $\alpha$ -affected reporter expression in long-term treatment, transfected cells were incubated for 8, 24, 48 and 72 h with TNF- $\alpha$ , washed with PBS and lysed in passive lysis buffer. In addition, cells were harvested 8 h after the respective TNF- $\alpha$  application in the long-term treatment, indicated as 24, 48 and 72 h+. The luciferase activity was quantified by luciferase reporter assay in triplicates by the Centro LB 960 luminometer.

**Statistical analysis.** Analyses for statistical significance were performed with GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). Comparison of several groups was done by one-way analysis of variance (ANOVA) and Bonferroni post hoc multiple comparison. Statistical significance was set at P-values <0.05.

## Results

**TNF- $\alpha$  mediated regulation of ABCB1 expression, drug uptake and cytotoxicity.** The TNF- $\alpha$  mediated effects on ABCB1 expression were analyzed in HCT15 human colon carcinoma cells, which is an intrinsically high expressor of ABCB1. First, we evaluated the effects of human TNF- $\alpha$  on ABCB1 expression and ABCB1-mediated resistance. The treatment with 30 ng TNF- $\alpha$ /ml significantly reduced ABCB1 expression at mRNA level starting from 24-72 h (Fig. 1A). This long-term treatment caused 1.9-fold reduction in mRNA expression after 24 h, 3.8-fold reduction after 48 h and 6-fold reduction after 72 h, whereas short-term treatment (0.5-2 h) did not reduce ABCB1 expression. Similarly, western blot analysis revealed reduced PgP-expression at 24 h up to 96 h of TNF- $\alpha$  treatment (Fig. 1B). The short-term treatment (5 min to 2 h) however, did not alter PgP-expression. In a next step we determined possible functional effects of TNF- $\alpha$  mediated ABCB1 downregulation. For this, the uptake of the fluorescent drug doxorubicin as PgP substrate was determined (Fig. 1C). TNF- $\alpha$  treatment for 72 h leads to significant up to 3.8-fold increase in doxorubicin uptake, whereas 2 h TNF- $\alpha$  treatment caused only 1.4-fold increase in drug uptake. We next asked if the increased drug uptake after long-term TNF- $\alpha$  treatment has impact on doxorubicin cytotoxicity. Long-term treatment did increase cytotoxicity leading to 64% growth inhibition at highest dose of 1,500 ng/ml (Fig. 1E). By comparison, 2 h TNF- $\alpha$  treatment did not exert any effect on doxorubicin cytotoxicity even at highest drug doses (Fig. 1D). Taken together, we demonstrated in our model that long-term TNF- $\alpha$  treatment downregulated ABCB1 expression at mRNA and protein levels in colorectal

cancer cells. This leads to high drug accumulation and sensitization towards doxorubicin, reflected by increased cytotoxicity.

**TNFR signaling and PgP expression.** Next we determined, whether TNFR1/p55 or TNFR2/p75 is essential in mediating the observed TNF- $\alpha$  effects on ABCB1 expression. Western blot analysis revealed, that both receptors are present in the colorectal cancer cells (Fig. 2A). For blocking the TNF- $\alpha$  binding to either TNFR1 or TNFR2 we pre-treated the cells with anti-TNFR1 or anti-TNFR2 antibody (Fig. 2B). As shown, addition of anti-TNFR1 antibody prevented PgP downregulation of the long-term 72 h TNF- $\alpha$  treatment, whereas addition of the anti-TNFR2 antibody could not prevent downregulation of PgP. In control experiments sole addition of the antibodies, however, did not alter PgP expression. This excluded any antibody mediated unspecific effect on PgP expression (Fig. 2C). Thus, binding of TNF- $\alpha$  to TNFR1 is essential for TNF- $\alpha$  mediated downregulation of ABCB1 expression after long-term TNF- $\alpha$  treatment.

**Effects of short- and long-term TNF- $\alpha$  mediated NF- $\kappa$ B/p65 signaling on ABCB1.** TNFR1 mediates signaling via the NF- $\kappa$ B pathway. Therefore, we determined the effect of short- and long-term TNF- $\alpha$  on NF- $\kappa$ B/p65 and on I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  as regulators of NF- $\kappa$ B/p65 (Fig. 3A and B). The short-term (5-120 min) TNF- $\alpha$  mediated effects show strong cytoplasmic reduction in I $\kappa$ B $\alpha$  levels after 10 min of TNF- $\alpha$  treatment, whereas, no detectable alteration for cytoplasmic NF- $\kappa$ B level was observed (Fig. 3A). As early as 10 min of TNF- $\alpha$  treatment the NF- $\kappa$ B/p65 protein starts to accumulate in the cell nuclei. This differs from what is observed for long-term (24-72 h) TNF- $\alpha$  mediated effects. Here I $\kappa$ B $\alpha$  levels remain at very low level and NF- $\kappa$ B shuttling to the nucleus is reduced. This is paralleled by reduction in PgP levels in the same experiment (Fig. 3B). Therefore, long-term TNF- $\alpha$  treatment reduces shuttling of NF- $\kappa$ B/p65 to the nucleus and prevents re-appearance of I $\kappa$ B $\alpha$  to levels of control cells in the cytoplasm. The analysis of I $\kappa$ B $\alpha$  mRNA expression revealed that this reduction in I $\kappa$ B $\alpha$  for long-term TNF- $\alpha$  treatment is the result of significant up to 3-fold reduced transcription (Fig. 3C). The cytoplasmic NF- $\kappa$ B level, however, seems to be unaffected by long-term TNF- $\alpha$  action. Even if analyzing the time point of 30 min after re-application of TNF- $\alpha$  (indicated as 24, 48 and 72 h+), no increase in nuclear accumulation of NF- $\kappa$ B/p65 is seen. For NF- $\kappa$ B/p65 shows only slight and insignificant reduction in mRNA expression by the long-term TNF- $\alpha$  treatment as seen in Fig. 3C.

Furthermore, we analyzed the fate of I $\kappa$ B $\beta$  in long-term TNF- $\alpha$  treatment where I $\kappa$ B $\beta$  protein persists in the cytoplasm, however, for 24-72 h TNF- $\alpha$  treatment at slightly lower level (Fig. 3C). The expression analysis also revealed an up to 2.5-fold reduction of I $\kappa$ B $\beta$  mRNA for the long-term TNF- $\alpha$  treatment, which correlates with the reduction at protein level (Fig. 3B and C).

**NF- $\kappa$ B/p65 binding to its consensus sequence in the ABCB1 promoter after long-term TNF- $\alpha$  treatment.** Since long-term TNF- $\alpha$  reduced nuclear NF- $\kappa$ B/p65 we determined if this is associated with reduced binding of NF- $\kappa$ B/p65 to its consensus

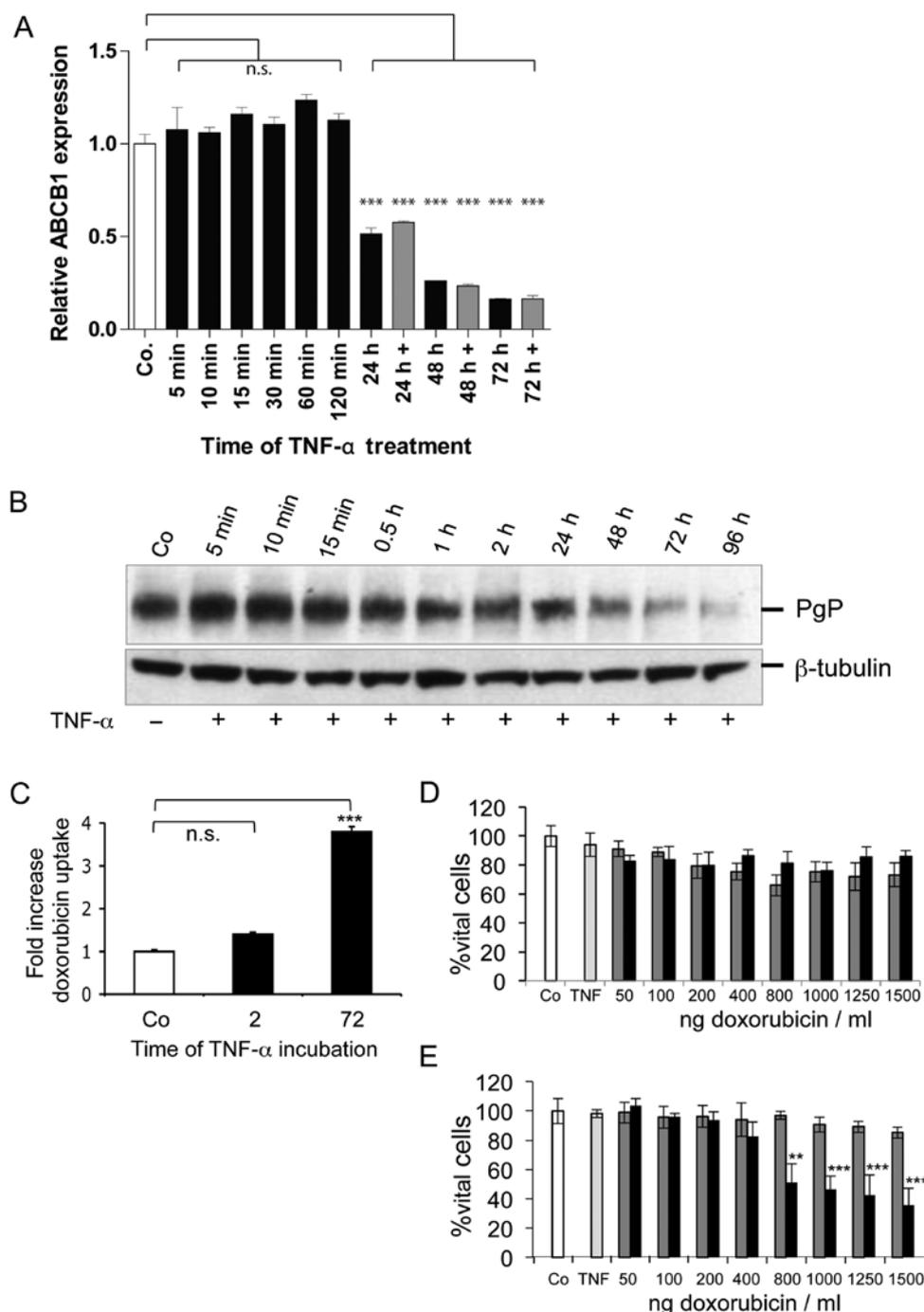


Figure 1. Effect of short- and long-term TNF- $\alpha$  treatment on ABCB1 expression, drug uptake and sensitivity towards doxorubicin in human HCT15 colon carcinoma cells. Incubation with 30 ng/ml TNF- $\alpha$  leads to decrease in ABCB1 expression at mRNA-level (A) and at protein level (B), starting at 24-h incubation with TNF- $\alpha$ . The time points indicated as 24, 48 and 72 h+ show qRT-PCR from cells, which were collected 30 min after addition of TNF- $\alpha$  at the respective day (A). (C) Effect of short- (2 h) and long-term (72 h) TNF- $\alpha$  treatment on doxorubicin uptake. Sensitization of HCT15 cells towards doxorubicin treatment, determined in the MTT cytotoxicity assay after short- (2 h; D) or long-term (72 h; E) treatment with TNF- $\alpha$ . The error bars represent SD. The asterisks mark level of statistical significance: \*P<0.01; \*\*P<0.001; \*\*\*P<0.0001.

sequence in the human ABCB1 promoter. In the EMSA we first analyzed, if binding to the NF- $\kappa$ B consensus sequence of the ABCB1 promoter is specific (Fig. 4A). Under non-stimulated conditions there is binding of the nuclear extract, which by addition of excess nonlabeled oligo disappears due to competition. The mutated oligo however shows no binding. After treatment with TNF- $\alpha$  for 30 min the mutated oligo shows binding of nuclear extract. However, addition of anti-NF- $\kappa$ B/p65 antibody does not lead to supershift. By contrast, use

of the wild-type oligo shows binding of the nuclear extract, and supershift bands after addition of the anti-p65 antibody. This indicates, that the short-term TNF- $\alpha$  treatment induces NF- $\kappa$ B binding to the consensus sequence. Next we analyzed the impact of long-term TNF- $\alpha$  action on NF- $\kappa$ B binding to its consensus sequence within the human ABCB1 promoter by supershift experiment using the anti-p65 antibody. As positive control, the 30 min TNF- $\alpha$  stimulation again induced NF- $\kappa$ B binding, verified by appearance of a supershift band (Fig. 4B).

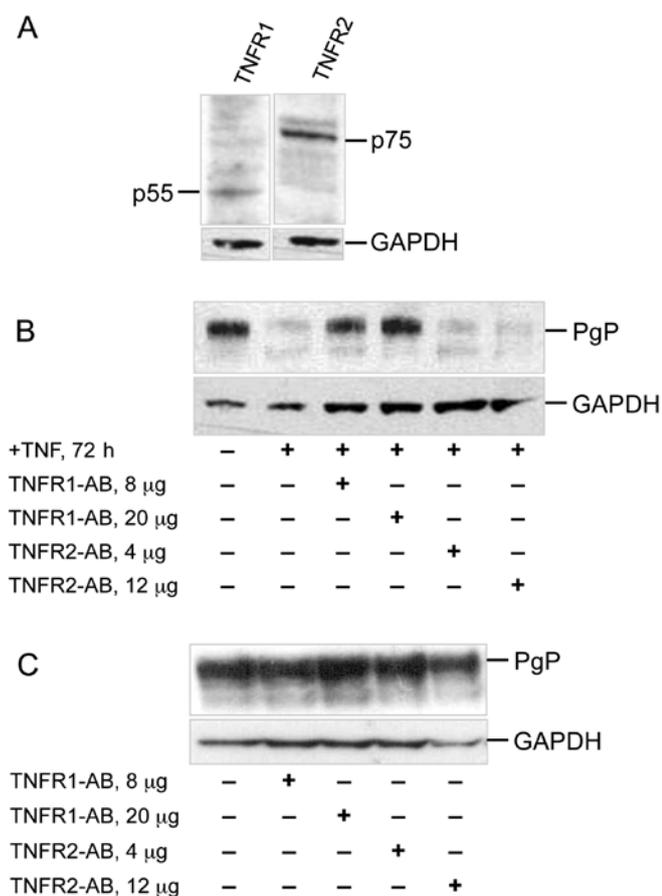


Figure 2. Analysis of TNFR1 and TNFR2 expression and effect of TNFR-blocking on TNF- $\alpha$  mediated long-term effect of PgP expression inhibition. (A) Western blot analysis for TNFR1 and TNFR2 expression in HCT15 cells. (B) Influence of TNFR1 or TNFR2 blocking by specific antibodies on long-term (72 h) TNF- $\alpha$  mediated reduction in PgP expression. The antibodies were applied 1 h prior to addition of 30 ng/ml TNF- $\alpha$  to block receptor function. (C) Effect of addition of TNFR1 or TNFR2 specific antibody for 72 h on PgP expression in HCT15 cells. All loading controls were performed using GAPDH-specific antibody.

This supershift disappeared when TNF- $\alpha$  was added for 24, 48 and 72 h, respectively. Interestingly, this is also seen for the samples, collected 30 min after each re-application of TNF- $\alpha$ , indicated as 24, 48 and 72 h+. This suggests that long-term TNF- $\alpha$  treatment desensitizes the colorectal cancer cells to TNF- $\alpha$  mediated NF- $\kappa$ B signaling and prevents NF- $\kappa$ B from binding to its consensus sequence within the ABCB1 promoter. This in turn leads to downregulation of ABCB1 expression.

*Diverging effect of TNF- $\alpha$  mediated regulation on ABCB1 promoter activity.* Since long-term TNF- $\alpha$  action prevents nuclear translocation and binding to consensus sequence of NF- $\kappa$ B, determination of functional consequences of this is important. First, we analyzed the effects of short- and long-term TNF- $\alpha$  treatment using the Signal NF- $\kappa$ B/p65 *Renilla*/firefly dual luciferase reporter assay, which harbors tandem repeats of NF- $\kappa$ B transcriptional response element (Fig. 5). In this assay short-term application of TNF- $\alpha$  induces reporter expression peaking at 8 h with a 1.7-fold increase compared to the control (Fig. 5A). The long-term effects of TNF- $\alpha$  dramatically differ from this. Here, persistent TNF- $\alpha$  application leads to significant downregulation of reporter expression compared to

the short-term TNF- $\alpha$  effects (Fig. 5B). This is reflected by the 3- to 5-fold decreases in promoter activity at 24-72 h of TNF- $\alpha$  application. Even at the time points of 8 h after each respective re-application of TNF- $\alpha$  (indicated as 24, 48 and 72 h+) the reporter system remains unresponsive towards TNF- $\alpha$  mediated induction. To further validate this observation, we used the NF- $\kappa$ B consensus sequence containing human ABCB1 promoter for luciferase expression. These assays revealed that short-term TNF- $\alpha$  application slightly reduced basal promoter activity in the colorectal cancer cells (Fig. 5C). The picture again drastically changes for long-term TNF- $\alpha$  treatment. The results show significant 2- to 9-fold decreases in ABCB1 promoter activity (Fig. 5D). At 8 h after respective TNF- $\alpha$  re-application (indicated as 24, 48 and 72 h+) this reporter system remains also unresponsive for TNF- $\alpha$  mediated induction and the reporter expression is even further reduced. Taken together, these analyses support that long-term TNF- $\alpha$  inhibits ABCB1 promoter activity. This results in reduced ABCB1 transcription and PgP expression (Fig. 1) leading to MDR reversal in the resistant cells.

## Discussion

MDR still represents a leading obstacle for successful chemotherapy of cancer. Many different approaches are used to overcome MDR including the use of MDR reversing drugs or cytokines (1,5,34). One long known molecule, which mediates MDR in cancer is the ATP binding cassette ABCB1/PgP transporter. This membrane protein is responsible for the drug extrusion from cancer cells causing drug resistance. Therefore great efforts are aimed at downregulation of ABCB1 expression to sensitize tumor cells towards chemotherapy. One such approach is the use of pro-inflammatory cytokines, such as TNF- $\alpha$  or INF- $\gamma$  for chemosensitization of resistant tumor cells (7,35). From numerous studies it is well accepted that particularly TNF- $\alpha$  is able to modulate the ABCB1 expression. In this regard several studies report, that treatment with TNF- $\alpha$  mediates ABCB1 downregulation, which in turn leads to improved drug accumulation in association with increased cytotoxicity. This has been found in different tumor models, including glioblastoma, colon, breast and hepatocellular cancer, and more recently also for endothelial cells in the blood-brain barrier (5,8-12). By contrast there are reports with the opposite observations, stating that treatment with TNF- $\alpha$  or other factors, which lead to activation of the NF- $\kappa$ B pathway rather act as inducers of ABCB1 expression (20,36-38), thus, interference in NF- $\kappa$ B signaling by inhibitors has been shown to downregulate ABCB1 expression, which then sensitizes tumor cells towards chemotherapy (20).

Due to the complex and partially contradictory picture on TNF- $\alpha$  mediated effects we were interested to determine the interplay between short- and long-term TNF- $\alpha$  treatment, NF- $\kappa$ B signaling and the resulting modulation in this signaling, which leads to ABCB1 repression in colon cancer cells.

In this study we determined in more detail the impact of short-term and of long-term action of TNF- $\alpha$  on the expression regulation of ABCB1 in intrinsically resistant human HCT15 colon carcinoma cells. We have shown, that persistent treatment with TNF- $\alpha$  for 24-72 h leads to significant downregulation of ABCB1 in association with

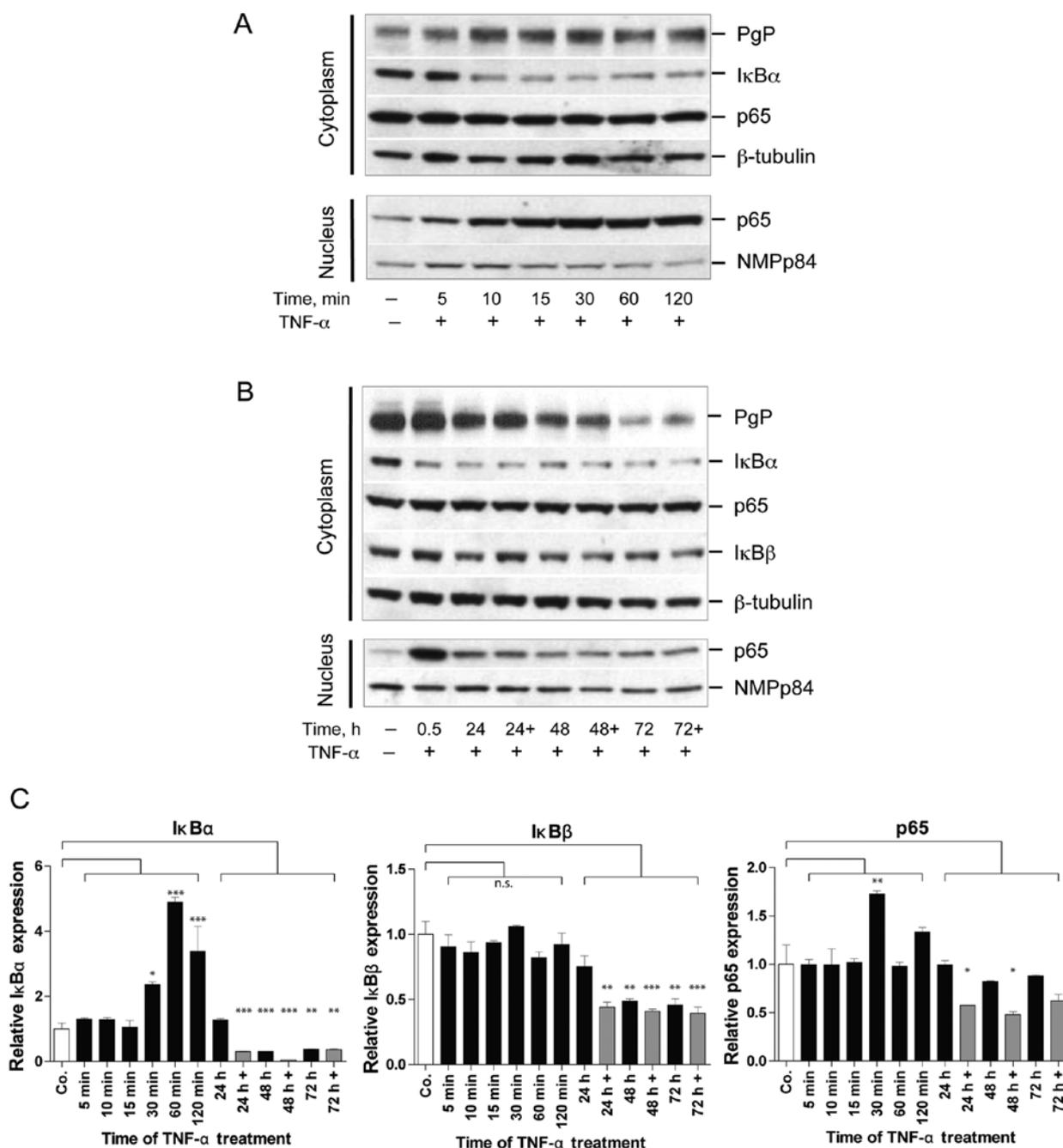


Figure 3. Effect of short- (2 h) and long-term (72 h) TNF- $\alpha$  treatment on NF- $\kappa$ B/p65, I $\kappa$ B $\alpha$  and Pgp in HCT15 human colon carcinoma cells. The western blot analysis shows NF- $\kappa$ B/p65, I $\kappa$ B $\alpha$  and Pgp in cytoplasm and nuclear fraction of cells treated with 30 ng/ml TNF- $\alpha$  for 2 h (A) and for 72 h (B). The time points indicated as 24, 48 and 72+ show lysates from cells, which were lysed 0.5 h after addition of TNF- $\alpha$  at the respective day. The loading controls for cytoplasm were performed using the  $\beta$ -tubulin antibody and for the nuclear fractions by using the NMPp84 antibody. (C) Real-time RT-PCR analyses of NF- $\kappa$ B, I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in HCT15 cells treated with 30 ng/ml TNF- $\alpha$  for the indicated times, expressed as relative expression level in relation to the untreated controls. The error bars represent SD. The asterisks mark level of statistical significance: \*P<0.01; \*\*P<0.001; \*\*\*P<0.0001. The time points of 0.5 h after TNF- $\alpha$  re-application are indicated as 24, 48 and 72 h+.

sensitization of these cells towards drug treatment. This is in line with previous publications, in which TNF- $\alpha$  leads to MDR reversal (5,9).

Apart from these studies, the question remained, what mechanism is responsible for the TNF- $\alpha$  mediated downregulation of ABCB1, which is mostly observed for persistent treatment. We therefore evaluated by which TNFR-signaling such modulation of ABCB1 expression is permitted. This analysis revealed, that TNFR1 mediated signaling is

important for this process. Since TNF- $\alpha$  triggers NF- $\kappa$ B activation and nuclear translocation via TNFR1, we focused on this pathway under conditions of short- and of long-term TNF- $\alpha$  activation. The human ABCB1 promoter harbors NF- $\kappa$ B binding sites linking NF- $\kappa$ B signaling and ABCB1 expression regulation (24). Our study shows short-term TNF- $\alpha$  treatment triggers NF- $\kappa$ B activation. This is demonstrated by nuclear accumulation of NF- $\kappa$ B, accompanied by disappearance of I $\kappa$ B $\alpha$  from the cytoplasm, and binding of the



The absence of I $\kappa$ B $\alpha$  leads to delayed and reduced cytokine-induced NF- $\kappa$ B activation (27). Here we show, that the persistent TNF- $\alpha$  action prevents re-appearance of I $\kappa$ B $\alpha$  due to reduced I $\kappa$ B $\alpha$  transcription, which is then unavailable for maintenance of ABCB1 expression. In addition, we did also observe significant reduction in I $\kappa$ B $\beta$  transcription and reduced protein level in the long-term TNF- $\alpha$  treated HCT15 cells. This reduction however, does not reach the extent observed for I $\kappa$ B $\alpha$ . It is suggested that the remaining I $\kappa$ B $\beta$  level might be sufficient for dumping the long-term oscillations in NF- $\kappa$ B signaling, as described by others (26). In this context almost unaltered presence and action of I $\kappa$ B $\beta$  might overrule the remaining small I $\kappa$ B $\alpha$  activity and hinder NF- $\kappa$ B activation, which finally results in ABCB1 downregulation.

As we observed, persistent TNF- $\alpha$  treatment not only reduces nuclear translocation of NF- $\kappa$ B, but also DNA binding to its consensus sequence in the ABCB1 promoter, explaining why ABCB1 transcription is reduced. This is further supported by the reporter assays. They clearly show, that long-term TNF- $\alpha$  treatment has inhibitory effects, whereas for short-term TNF- $\alpha$  treatment induction in reporter gene expression was seen. Interestingly, even re-application of TNF- $\alpha$  in the long-term treatment was unable to restimulate reporter gene expression, from either the Cignal NF- $\kappa$ B/p65 *Renilla*/firefly dual luciferase reporter system or the authentic ABCB1 promoter construct.

In conclusion, our study provides new perspectives on the mechanism how long-term treatment of TNF- $\alpha$  is reducing NF- $\kappa$ B signaling, which results in the downregulation of ABCB1. This strongly supports the MDR reversing potential of the pro-inflammatory cytokine, which in turn mediates effective chemosensitization of colon cancer cells. Furthermore, this mechanism might also apply to the phenomena observed in situations of chronic inflammation of the gut associated with persistent presence of TNF- $\alpha$ , such as ulcerative colitis. These conditions are reported to be associated with reduced expression of ABC transporters, including ABCB1 (29,30). Interestingly, apart from colon cancer such observation has recently also been made in an inflammation model for microglia parenchymal cells, where ABCB1 expression is reduced pointing to a potential more general property of TNF- $\alpha$  on regulation of ABCB1 (41).

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