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Tumor necrosis factor α induces γ-glutamyltransferase expression via nuclear factor-κB in cooperation with Sp1

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ABSTRACT

γ-Glutamyltransferase (GGT) cleaves the γ-glutamyl moiety of glutathione (GSH), an endogenous antioxidant, and is involved in mercapturic acid metabolism and in cancer drug resistance when overexpressed. Moreover, GGT converts leukotriene (LT) C4 into LTD4 implicated in various inflammatory pathologies. So far the effect of inflammatory stimuli on regulation of GGT expression and activity remained to be addressed.

We found that the proinflammatory cytokine tumor necrosis factor alpha (TNFα) induced GGT promoter transactivation, mRNA and protein synthesis, as well as enzymatic activity. Remicade, a clinically used anti-TNFα antibody, small interfering RNA (siRNA) against p50 and p65 nuclear factor-kappaB (NF-κB) isoforms, curcumin, a well characterized natural NF-κB inhibitor, as well as a dominant negative inhibitor of kappaB alpha (IκBα), prevented GGT activation at various levels, illustrating the involvement of this signaling pathway in TNFα-induced stimulation. Over-expression of receptor of TNFα-1 (TNFR1), TNFR-associated factor-2 (TRAF2), TNFR-1 associated death domain (TRADD), dominant negative (DN) IκBα or NF-κB p65 further confirmed GGT promoter activation via NF-κB.

Linker insertion mutagenesis of 536 bp of the proximal GGT promoter revealed NF-κB and Sp1 binding sites at −110 and −78 relative to the transcription start site, responsible for basal GGT transcription. Mutation of the NF-κB site located at −110 additionally inhibited TNFα-induced promoter induction. Chromatin immunoprecipitation (ChIP) assays confirmed mutagenesis results and further demonstrated that TNFα treatment induced in vivo binding of both NF-κB and Sp1, explaining increased GGT expression, and led to RNA polymerase II recruitment under inflammatory conditions.

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1. Introduction

γ-Glutamyltransferase (GGT) (EC 2.3.2.2) catalyzes the transfer of the γ-glutamyl moiety of glutathione (GSH), a tripeptidic natural antioxidant protecting cells against toxins, to acceptor molecules including peptides, amino acids, H_2O or glutathione itself [1].

In human, GGT is encoded by a multigene family of at least seven genes and pseudogenes [2]. Amongst these genes, one locus is of great importance since its transcription generates type I GGT mRNAs [2,3], encoding an enzyme with the same catalytic properties than the enzyme isolated from human tissues [4]. Different subtypes of GGT type I mRNAs exist showing all the same open reading frame (ORF) and coding for the functional GGT protein but differing by their 5′ untranslated region (UTR) [5–8]. GGT mRNA were shown to be induced by cytokines including tumor necrosis factor alpha (TNFα) [9], interferon (IFN)-α and -β [10].

GGT plays multiple roles in human including the conversion of GSH conjugates into mercapturic acid, which is subsequently excreted into bile and urine [11].

Furthermore, tumor cells expressing GGT demonstrated a growth advantage in vivo compared to GGT-negative tumor cells as they use extracellular glutathione as a source for cysteine [12,13].

GGT also catalyzes leukotriene (LT) C4 to D4 conversion which stimulates pro-inflammatory activities such as endothelial cell adherence and chemokine production by mast cells [14]. Moreover, leukotrienes induce asthma and other inflammatory disorders, thereby reducing the airflow to the alveoli [15]. Patients with a genetic deficiency of GGT showed complete lack of LTD4 biosynthesis but increased concentrations of LTC4 [16].

To our knowledge, no studies have until now investigated the effect of an inflammatory environment on GGT expression mechanisms and synthesis. We show here that inflammatory conditions increase synthesis of GGT and provide evidence that TNFα, an inflammatory cytokine, induces GGT synthesis and activity. Furthermore, we demonstrate that TNFα-induced GGT expression is mediated through the nuclear factor-kappaB (NF-κB) signaling pathway, Sp1 and RNA polymerase II recruitment to the GGT promoter.

2. Materials and methods

2.1. Cell culture and reagents

Human Philadelphia chromosome-positive chronic myelogenous leukemia cells (K562 and MEG-01) were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) (Hyclone, Perbio, Erembodegem, Belgium) and 1% (v/v) antibiotic–antimycotic (Lonza, BioWhittaker™, Verviers, Belgium) at 37 °C, in a 5% CO_2, humidified atmosphere. Human recombinant TNFα (PeproTech, Rocky Hill, NJ, USA) was resuspended in a phosphate buffer salt (PBS) 1X sterile solution containing 0.5% bovine serum albumin (MP Biomedicals, Asse-Relegem, Belgium) to reach a final concentration of 10 μg/mL. Remicade® (generic name: infliximab) was purchased from Centocor, Leiden, The Nederlands and curcumin (Sigma, Bornem, Belgium) was dissolved in 100% DMSO (Sigma) at 20 mM.

2.2. GGT enzyme activity

GGT activity was assayed with the method of Sze et al. [17], involving the spectrophotometric measurement of *p*-nitroaniline released from the substrate γ-glutamyl *p*-nitroanilide (γ-GPNA); this method is specific for GGT that associates with the outer side of the plasma membrane and metabolizes extracellular GSH. One unit of GGT activity was defined as the amount of enzyme capable to release 1 mmol *p*-nitroaniline/min from the substrate γ-GPNA [18].

2.3. Protein extractions

After treatment, 10^7 cells were harvested and total or nuclear proteins were extracted as previously described [19]. Protein content was determined for each sample using the Bradford assay (Bio-Rad protein Assay, Biorad, Nazareth, Belgium).

2.4. Western blot analysis

Proteins from total or nuclear extracts were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a Hybond™-P membrane (GE Healthcare, Diegem, Belgium). Membranes were pre-hybridized overnight at 4 °C in PBS 1X containing 0.1% (v/v) Tween 20 (PBS-T) and 5% milk. Hybridizations with primary antibodies, directed against GGT (kind gift from Profs. Pompella and Paolicchi), Grp78 (sc-13968), p50 (sc-7178), p65 (sc-109) or β-actin (Sigma) used as a loading control, were then carried out in PBS-T containing 5% milk or 5% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were then washed and probed with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Proteins of interest were visualized with Ecl Plus Western blotting Detection System (GE Healthcare) reagent using a Kodak Image Station (Analis, Suarlée, Belgium).

2.5. RT-PCR analysis

Isolation of total RNA was performed using TRIzol® reagent (Invitrogen, Merelbeke, Belgium). 5 μg of total RNA were submitted to reverse transcription (RT) using Oligo(dT) primers. The resulting RT products were used as templates for PCR amplification using the Platinium® Taq DNA Polymerase High Fidelity (Invitrogen) and GGT mRNA specific primers (S: 5′-GCCCAGAAGTGAGAGCA-GTT-3′; AS: 5′TCCAGAAAGCAGCTAG-AGGG-3′) (Eurogentec, Liège, Belgium). The amount of cDNA synthesized was evaluated by amplification of the S14 gene (S: 5′-GGCAGACCGAGAT-GAATCCTC-3′; AS: 5′CAGGTCCAGGGGTCTTGGTCC-3′ (Eurogentec, Liège, Belgium) as a standard. After amplification, the PCR products were separated on a 2% agarose gel.

2.6. Transient transfections

Transient transfections of K562 cells were performed by electroporation using the BioRad gene Pulser. For each experiment, 3.75×10^6 cells at a concentration of 1.5×10^7 cells/mL were electroporated at the following settings: 250 V and 500 μF. For each transfection, 250 μL of cells were combined with 5 μg of ph-RG-tk Renilla plasmid (Promega, Leiden, The Netherlands) together with 5 μg of a reporter plasmid, placed under the control of regulatory elements of the human GGT promoter (p536-Luc and p835-Luc) or five NF-κB binding sites (pNF-κB) (Stratagene). The human GGT promoter, inserted in the pGL3 vector, was cloned from cosmid c507 [20]. It comprises the leading exon of placenta type GGT mRNA [21] and either 536 bp (p536-Luc) or 835 bp (p835-Luc) of the promoter sequence.

For co-transfection assays, 5 μg of expression plasmids coding for proteins of the NF-κB pathway (receptor of TNFα-1 (TNFR-1), TNFR-associated factor-2 (TRAF2), TNFR-1 associated death domain (TRADD), inhibitor of kappaB alpha (Iκbα), p65) were added to the electroporation mixture. siRNA assays were performed with siRNA duplexes 1, 2, 3 and 5 for p50 (NFkB1: MU-003520-01, Dharmacon, Erembodegem, Belgium) and with siRNA duplexes 1, 3, 4 and 5 for p65 (RELA: MU-003533-02, Dharmacon, Erembodegem, Belgium). 100 μL of 1X siRNA buffer were added to 2 nmol of siRNA to reach a final concentration of 20 μM and 0.25 nmol of siRNA were used per transfection. siRNA were added to the electroporation mixture containing cells and plasmids. After transfection, electroporation cuves were placed on ice for 10 min.

24 h after electroporation, transfected cells were harvested, resuspended in growth medium to reach a final concentration of 10^6 cells/mL and subjected or not to the treatment in microtitration plates. In order to assay Renilla activity, 75 μL Dual-Glo™ Luciferase Reagent (Promega) were incubated for 10 min at 22 °C and then, 75 μL Dual-Glo™ Stop&Glo® Reagent (Promega) were added to the cells for 10 min incubation at 22 °C. Renilla and Luciferase activities were measured with an Orion microplate luminometer (Berthold detection systems) and results are expressed as a ratio of Luciferase activity normalized to Renilla activity.

2.7. Immunofluorescence microscopy analysis

After transfection, 10^7 cells were fixed with 2% paraformaldehyde and permeabilized in 0.1% Triton X-100/PBS 1X for 8 min. Cells were then incubated with primary antibodies against TRAF2 (sc-876), TRADD (sc-1163), Iκbα (sc-203) or p65 (sc-109) for 1 h at room temperature, and secondary labeled with Alexa Fluor 568 or 488 (Molecular Probes) at room temperature for 30 min. Cells were washed before nuclear staining with 1 μg/mL Hoechst 33342 (Calbiochem) for 15 min at 37 °C. For membrane proteins, like TNFR-1 and GGT, cells were washed and stained with primary antibodies (TNFR-1 (sc-12746) or GGT) for 40 min on ice without fixation and permeabilization of the cells. Secondary antibody (Alexa Fluor 568) was added on cell pellet and incubated on ice in the dark 40 min. Cells were then washed and stained with 1 μg/mL Hoechst 33342 (Calbiochem) 30 min on ice. Results were analyzed using an inverted DMIRB fluorescence microscope (Leica Microsystems, Lecuit, Howald, Luxembourg) equipped with appropriate filter cubes, a CCD camera and the software Firecam 3.0 (Leica Microsystems). Image analysis was performed using NIH Image J software (http://rsb.info.nih.gov/ij/).

2.8. Computer analysis of potential transcription factor binding sites

The sequence of GGT promoter has been analyzed by Matinspector (Genomatix; http://www.genomatix.de).

2.9. Linker insertion mutagenesis

Three potential NF-κB sites (NF-κB site 1, 2 or 3) and a potential Sp1 site, found by the analysis by Matinspector, have been mutated by PCR using Platinum® Pfx DNA Polymerase (Invitrogen) and specific primers containing a mutation in the NF-κB or Sp1 binding sites (NF-κB-1 S: 5′-ACATCC-CACCTGCAG-TGTCTAGAGTGCCTTTAAAGCCTCC-3′; AS: 5′-GGAGGCTTTAAAGGCACTCTA-GACACTGCAGGTGGGATGT-3′, NF-κB-2 S: 5′-GAGAGGGCCTGTTAGG-AGCGAGATCTGG-TGCCCTGGGGTTGTA-3′; AS: 5′-TACAACCCCAGGGCACC-AGATCTCGCTCCTAACAGGCCCTCTC-3′, NF-κB-3 S: 5′-GAAATTGGGGGTC-AACTCTAGACGGTCCTGGATTATGTGA-3′; AS: 5′-TCACATAATCCAGGAC-CGTCTAGAGTTGACCCC-CAATTTC-3, Sp1 S: 5′-GCCTTTAAAGCCTCCCTAC-CCCACTC-TAGACACCCAGGCCACTA-GGG-3′; AS: 5′-CCCTAGTGGCCTG-GGTGTCTAGAGTGGGGTAGGGAGGCTTTAAAGGC-3′). 536 bp of GGT promoter were mutated by introducing a *Xba*I site into the sequence to be mutated. PCR reaction uses 1 μL (50 ng) of DNA (p536-Luc or p875-Luc plasmid), 5 μL of 10X Pfx Amplification buffer, 1.5 μL of sens and antisens primers (0.3 μM each), 1.5 μL dNTP mixture (0.3 mM each), 1 μL MgSO4 (1 mM), 0.4 μL Platinum® Pfx DNA Polymerase (1 unit). PCR amplification is performed in a thermocycler (Eppendorf) under the following conditions: 2 min at 94 °C, 25 cycles of 15 s at 94 °C, 30 s at 55 °C and then 6 min at 68 °C. After verification of PCR products on gel, clones were digested by *Dpn*I for 2 h at 37 °C. 2 μL (3 ng) of purified plasmids (Qiagen) were transformed into XL1-blue bacteria (Stratagene). Three to six clones per mutation are selected and cultivated in LB medium overnight. Mini-preparation are realized the following day, followed by a digestion with the restriction enzymes *Xba*I and *Bgl*II, in order to verify if the mutation is inside the plasmid. Finally, clones were sequenced by GATC biotech (http://www.gatc-biotech.com/de/index.php).

2.10. Chromatin immunoprecipitation (ChIP) assays and PCR analysis

2×10^7 K562 cells were fixed with formaldehyde 1% for 10 min at room temperature before being quenched with 0.125 M glycine for 10 min at room temperature. Cells were washed twice with ice-cold 1X PBS, pelleted by centrifugation, resuspended in cell lysis buffer (5 mM PIPES [pH 8.0], 85 mM KCl, 0.5% NP-40, plus 1X protease inhibitor cocktail (Complete minus EDTA, Roche)) and incubated on ice for 10 min. Nuclei were pelleted by centrifugation, washed with digestion buffer (5 mM Hepes [pH 7.5], 60 mM KCL, 15 mM NaCl, 0.34 mM sucrose, 0.15 mM mercaptoethanol, 0.5 mM spermidine plus

protease inhibitor cocktail), and resuspended in digestion buffer supplemented with 3 mM $CaCl_2$. Suspension was incubated 10 min at 37 °C with micrococcal nuclease (Mnase, Worthington) to shear the chromatin, and the reaction was blocked with 2X stopping buffer (90 mM Hepes [pH 7.9], 10 mM EDTA, 220 mM NaCl, 2% Triton X 100, 0.2% Na-deoxycholate, 0.2% SDS plus 1X protease inhibitor). After centrifugation to remove cell debris, chromatin was precleared by a 1-h incubation at 4 °C with protein A-agarose beads (Upstate). The precleared chromatin was diluted two times in IP dilution buffer (16.7 mM Tris–Cl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS plus 1X protease inhibitor), and 10% of the supernatants were used as inputs. The diluted chromatin was incubated overnight on a rotating platform at 4 °C with antibodies for p50 (sc-7178X), p65 (sc-8008), Sp1 (sc-420X) and RNA polymerase II (05-623, Upstate) or non-specific IgG (Santa Cruz). The immune-complexes were recovered by a 1-h incubation at 4 °C with either protein A- or protein-G-

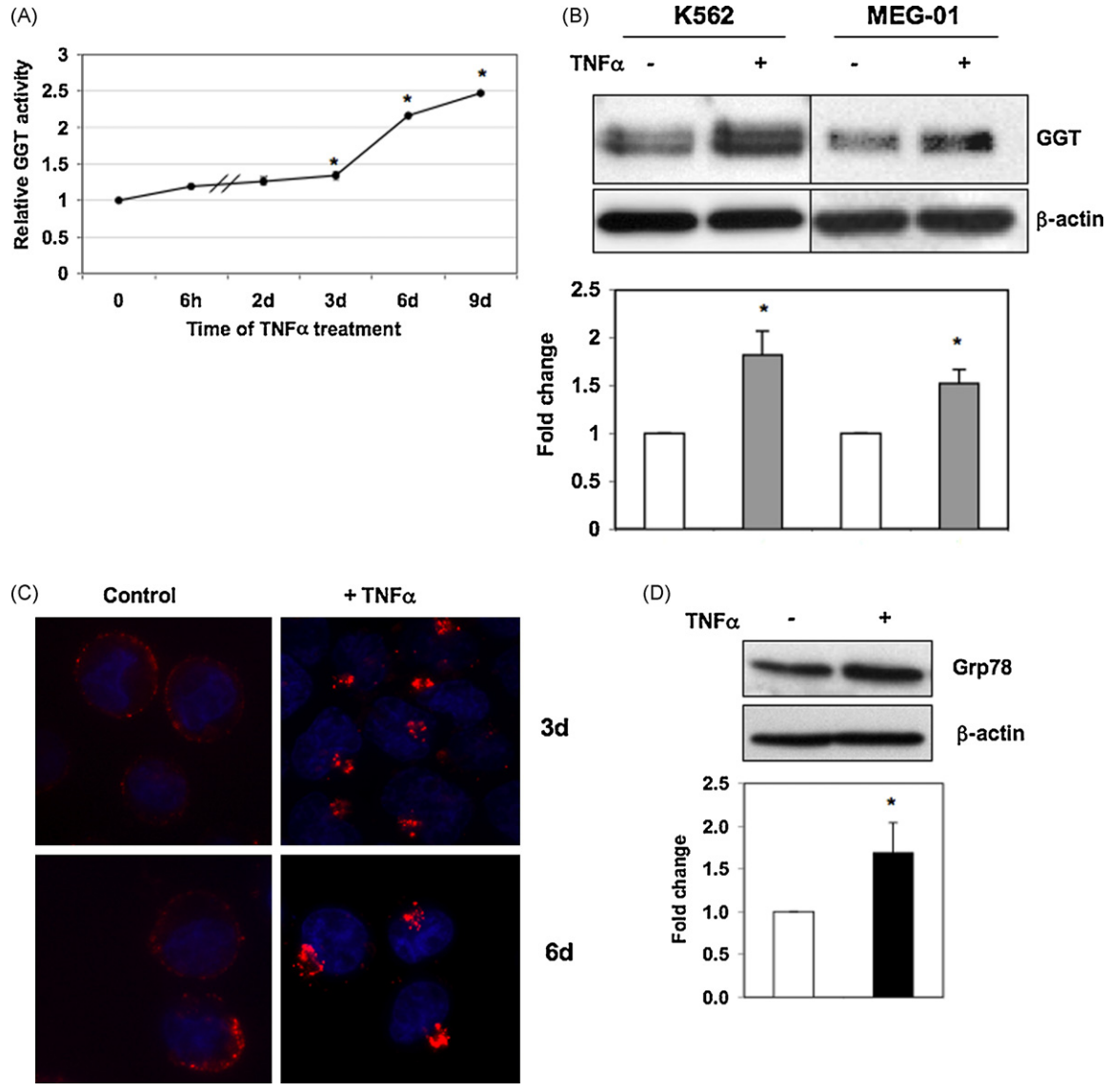

Fig. 1 – Measurement of GGT activity and protein level after TNFα treatment. (A) GGT activity was measured after 6 h, 2, 3, 6 or 9 days (d) of TNFα treatment. The values obtained are normalized and fold induction of treated cells divided by control cells are represented as relative GGT activity. (B) Western blot analysis of GGT protein. K562 and MEG-01 cells were treated with TNFα (6 h, 20 ng/mL) and total proteins were extracted and analyzed by Western blot using an anti-GGT antibody and β-actin as a loading control. Untreated cells are control (−) and a quantification of three independent experiments expressed as a ratio between GGT and β-actin level is shown. (C) Fluorescent microscopy analysis of GGT protein expression. K562 cells were treated with TNFα (20 ng/mL) for 3 and 6 days and immunostained with primary anti-GGT and secondary Alexa Red 568 antibodies. Untreated cells are control. Magnification × 40. (D) Western blot analysis of Grp78 protein. K562 cells were treated with TNFα (6 h, 20 ng/mL) and total proteins were extracted and analyzed by Western blot using an anti-Grp78 antibody and β-actin as a loading control. Untreated cells are control (−) and a quantification of three independent experiments expressed as a ratio between Grp78 and β-actin level is shown. (*) indicates $p < 0.05$ compared to the control.

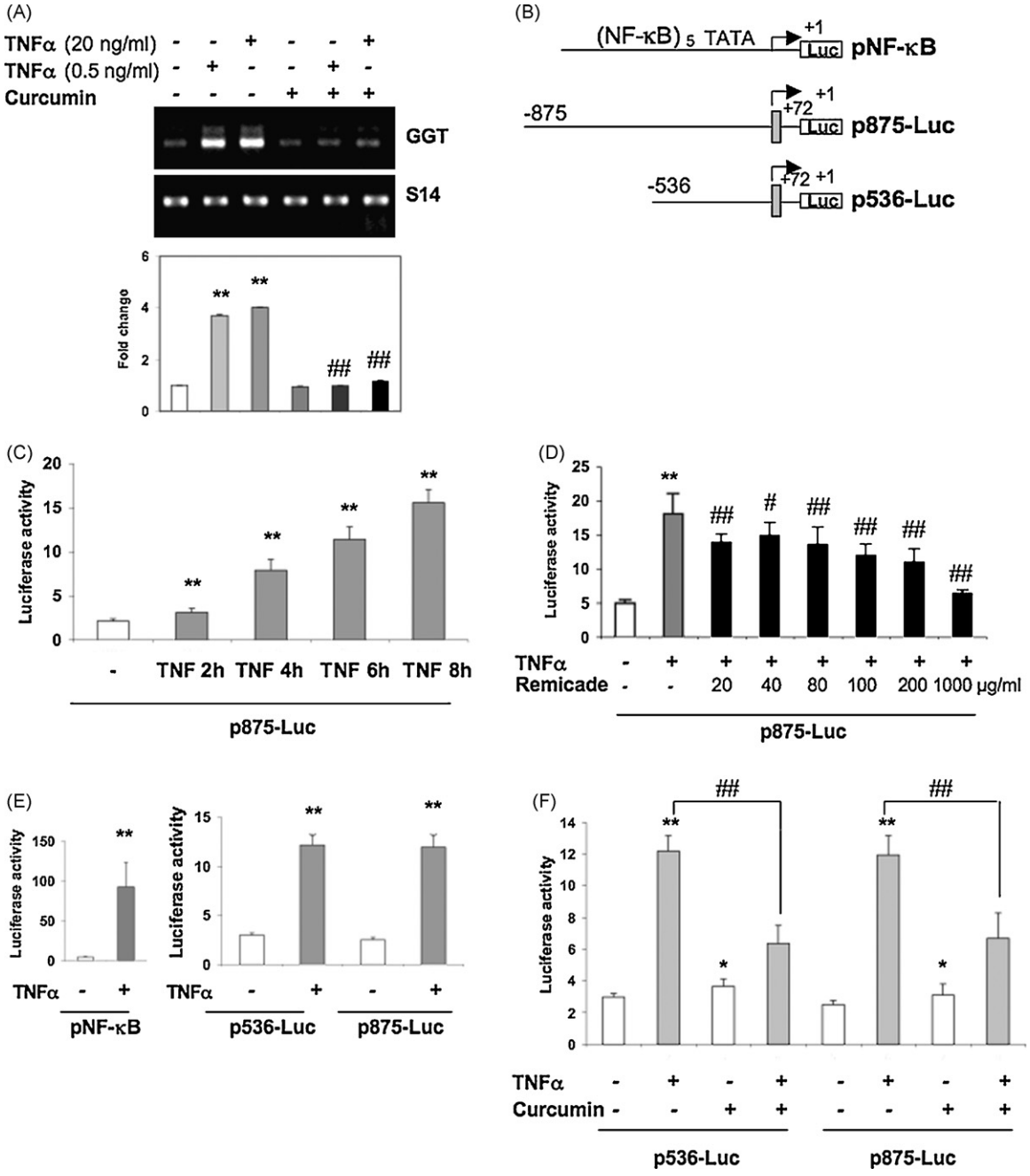

Fig. 2 – GGT mRNA and gene transcription analysis after TNFα treatment. (A) RT-PCR analysis of GGT mRNA. Total RNA was extracted from K562 cells treated with TNFα (6 h, 0.5 and 20 ng/mL), with curcumin (2 h, 20 μM) and with curcumin plus TNFα (8 h with a pre-treatment of 2 h with curcumin). Specific primers targeting GGT mRNA are used and S14 is control. Untreated cells are control and a quantification of three independent gels expressed as a ratio between GGT and S14 mRNA level is shown. (B) Representation of the reporter constructs used for transfection assays. pNF-κB plasmid, used as a positive control, contains five consensus NF-κB binding sites as well as a TATA box upstream of a luciferase gene. GGT plasmids (p875-Luc and p536-Luc) contain either 875 or 536 bp of GGT promoter sequence and 72 bp of coding region upstream of the luciferase gene. (C) Luciferase assay with p875-Luc plasmid. K562 cells were transfected with p875-Luc plasmids and treated during 2, 4, 6 and 8 h with TNFα (20 ng/mL). Untreated cells are control (−). (D) Luciferase assay with p875-Luc plasmid. K562 cells were transfected with p875-Luc plasmids and treated with TNFα (6 h, 20 ng/mL) and with different concentrations of Remicade (20, 40, 80, 100, 200 and 1000 μg/mL, for 30 min prior to TNFα), an anti-TNFα antibody. Untreated cells are control. (E) Luciferase assay with pNF-κB, p536-Luc and p875-Luc plasmids. K562 cells were transfected with pNF-κB, p536-Luc and p875-Luc plasmids before being treated with TNF((6 h, 20 ng/mL). Untreated cells are control (−). (F) Luciferase assay with p536-Luc and p875-Luc plasmids. K562 cells were transfected with p536-Luc and p875-Luc plasmids before being treated with TNFα (6 h, 20 ng/mL), curcumin (2 h, 20 μM) or TNFα plus curcumin (8 h). Untreated cells are control. (*) and (**) indicate $p < 0.05$ and $p < 0.01$ compared to control, respectively, and (#) and (##) indicate $p < 0.05$ and $p < 0.01$ compared to the TNFα treated sample, respectively.

agarose beads (Upstate) depending on the antibody specificity. The beads were washed twice with 1X dialysis buffer (50 mM Tris–HCl [pH 8.0], 2 mM EDTA, 0.2% sarkosyl plus 1X protease inhibitor) and four times with IP wash buffer (100 mM Tris–HCl [pH 9.0], 500 mM LiCl, 1% NP-40, 1% deoxycholic acid). Precipitated chromatin complexes were removed from the beads by incubation with elution buffer (50 mM $NaHCO_3$, 1% SDS), by mild vortexing. Cross-linking was reversed by adding NaCl to a final concentration of 0.3 M and incubation overnight at 65 °C in the presence of RNase A. Samples were then digested with proteinase K at 45 °C for 1.5 h. DNA was purified on Qiaquick® columns (PCR purification kit, Qiagen), eluted in ddH2O, and an aliquot was used for PCR analysis.

QRT-PCRs was performed with 1X Power SYBR® Green PCR Master Mix (Applied Biosystems), and 0.1 μM of each primer specific for human GGT (Fig. 5F). Primers were optimized in order to amplify a DNA fragment carrying the putative NF-κB-1 and Sp1 binding sites. Amplifications were realized using an ABI 7300 real-time PCR system (Applied Biosystems) with the following conditions: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60 °C. Relative differences in QRT-PCR among samples were determined using the $\Delta\Delta C_T$ method. The ΔC_T value for each sample reflects the difference in the amount of immunoprecipitated DNA relative to the amount of input DNA. C_T values were obtained from the means of replicates. The $\Delta\Delta C_T$ was calculated by subtracting control ΔC_T values from the corresponding experimental ΔC_T. The resulting values were converted to fold-changes over control using $2^{-\Delta\Delta C_T}$.

2.11. BLAST

BLAST searching for homologous promoter sequence fragments in the genomic sequences of different species was performed using a 150 base pair promoter sequence, directly upstream of the transcriptional start site, as input for the cross-species megaBLAST tool at the NCBI web site (http://www.ncbi.nlm.nih.gov). Expect value cutoff was set to 10 and low complexity sequence filtering was turned off.

2.12. Statistical analysis

Results from at least three independent experiments were analyzed for statistical significant differences using the Student's *t*-test. They are expressed as the mean ± SD. *p*-Values below 0.05 (* or #) or 0.01 (** or ##) were considered as statistically significant.

3. Results

3.1. TNFα induces γ-glutamyltransferase activity and protein level

TNFα-treated K562 cells showed an increased enzymatic activity of 140% after 3 days, 220% after 6 days and 250% after 9 days of treatment compared to untreated controls (Fig. 1A). In parallel, increased GGT protein expression was shown by Western blot in K562 and MEG-01 cells (Fig. 1B), and a localized overexpression of GGT could be observed after 3–6 days of TNFα treatment by immunofluorescence microscopy analysis (Fig. 1C). Interestingly, GGT expression was stimulated as early as 6 h whereas its increase in activity appeared delayed. GGT is a membrane heterodimeric glycoprotein [22], synthesized as a single precursor, which acquires extensive maturation before reaching the membrane where it is active [23]. In order to investigate if TNFα-induced endoplasmic reticulum (ER) stress could also contribute to this delay, we studied the effect of TNFα on glucose-regulated protein-78 kDa (Grp78) expression, a bona fide ER stress marker (Fig. 1D). Results show that TNFα increased Grp78 expression after 6 h, witnessing ER stress that can contribute to explain slow GGT transport towards the membrane.

3.2. TNFα increases expression of γ-glutamyltransferase gene expression

To assess the effect of TNFα on GGT gene expression, mRNA analysis by RT-PCR was performed and fragments of 386 and 600 bp were amplified. Both fragments correspond to the main as well as an alternatively spliced isoform of the GGT encoding mRNA. Altogether results showed that TNFα increases GGT-encoding mRNA up to four-fold (Fig. 2A).

Curcumin, a natural, well documented inhibitor of many NF-κB driven genes [24–28], prevented TNFα-induced increase of GGT mRNA. These results raised the hypothesis that NF-κB signaling pathway could be involved in the regulation of GGT by TNFα.

To assess the effect of TNFα on GGT promoter transactivation, transiently transfected K562 leukemia cells with a p875-Luc GGT promoter construct (Fig. 2B) were treated with TNFα for various times and results showed a seven-fold induction of the luciferase activity after 8 h indicating that a TNFα responsive element is located within 875 bp 5′ to the transcriptional start site (Fig. 2C). In order to ascertain our results, we co-treated transfected cells with Remicade and obtained concentration-dependent inhibition of TNFα induction (Fig. 2D). Similar results were obtained with promoter construct p536-Luc (Fig. 2B and E). In order to ascertain our experimental conditions, control pNF-κB with five NF-κB response elements were transfected and a 18-fold induction by TNFα was achieved as expected (Fig. 2B and E). Finally, curcumin efficiently prevented TNFα-induced increase of γ-glutamyltransferase promoter transactivation further confirming that NF-κB signaling pathway is involved in TNFα-induction of GGT (Fig. 2F).

To ascertain the involvement of NF-κB in TNFα-induced increase of GGT expression, K562 cells were transfected with the p875-Luc plasmid together with siRNAs targeting p50 or p65 NF-κB mRNAs resulting in decreased TNFα-induced luciferase activity (Fig. 3A and B, upper panel). Control Western blots showed that decreased NF-κB expression is paralleled by a decreased transactivation of the GGT promoter by p50 (Fig. 3A, lower panel) or p65 (Fig. 3B, lower panel). These results clearly show that the two NF-κB isoforms, p50 and p65, are involved in TNFα-induced increase of GGT promoter activity in K562 cells. Experiments using pNF-κB plasmid with the same siRNA confirmed our observations (Fig. 3A and B, middle panel).

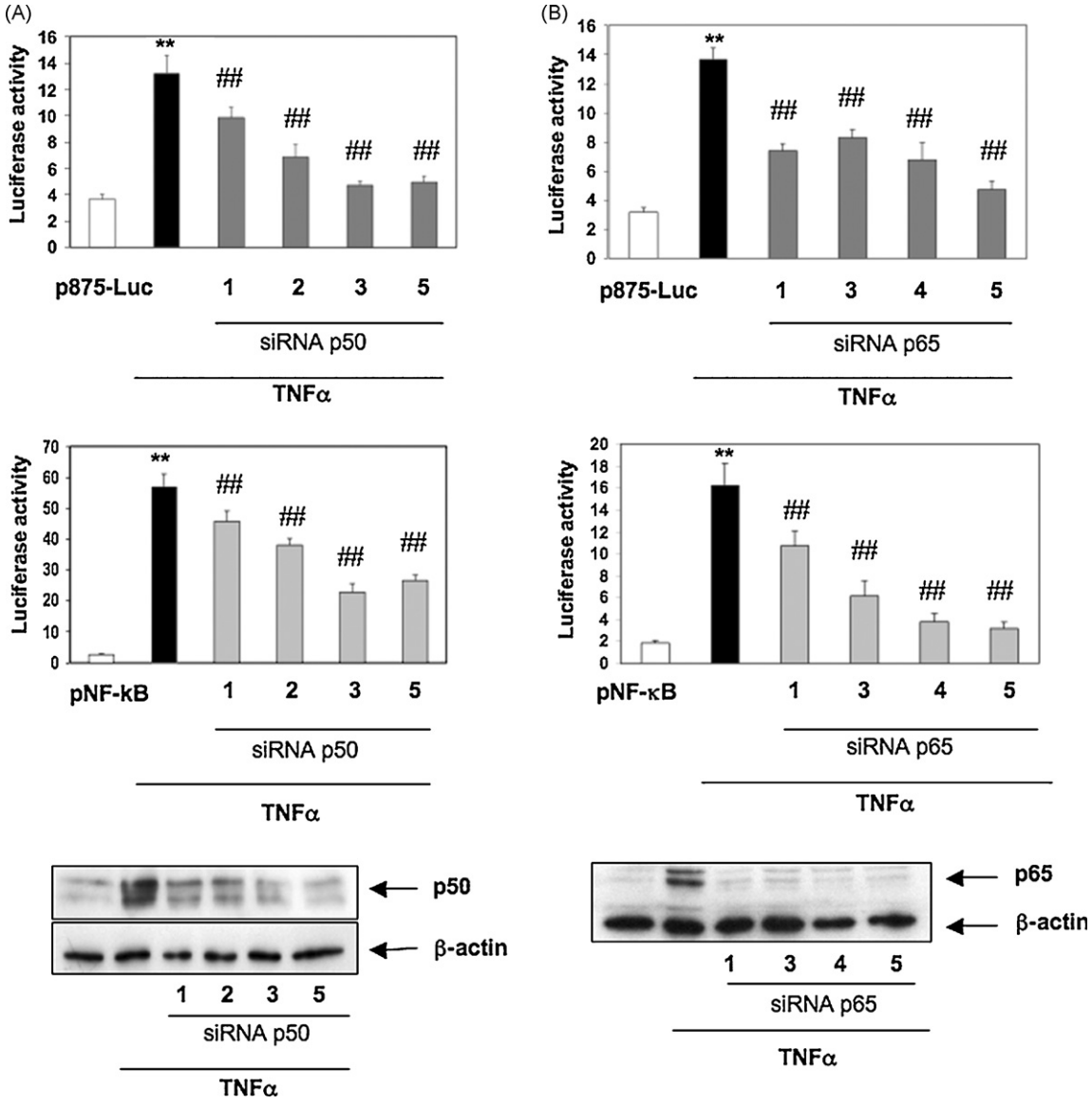

Fig. 3 – Effect of p50 and p65 siRNAs on TNFα-induced γ-glutamyltransferase and pNF-κB promoter activity. (A) p875-Luc and pNF-κB plasmids were transfected into K562 cells together with anti-p50 siRNAs. Nuclear proteins of transfected cells were extracted and analyzed by Western blot using an anti-p50 antibody and β-actin as a loading control. (B) p875-Luc and pNF-κB plasmids were transfected into K562 cells together with anti-p65 siRNAs. Nuclear proteins of transfected cells were extracted and analyzed by Western blot using an anti-p65 antibody and β-actin as a loading control. (**) indicates $p < 0.01$ compared to control and (##) indicates $p < 0.01$ compared to the TNFα-treated sample.

3.3. *TNFα-induced increase of γ-glutamyltransferase promoter activity is mediated by the NF-κB signaling pathway*

To further study the involvement of the NF-κB signaling pathway on GGT promoter activation, we co-transfected K562 cells with p536-Luc GGT promoter construct and expression vectors coding for proteins of the NF-κB signaling pathway, TNFR-1, TRAF2, TRADD, IκBαDN and p65 (Fig. 4A and B). After transfection, cells were treated with TNFα and results showed that overexpression of TRAF2, TRADD and p65 induced GGT promoter activity, while dominant negative IκBα, reversed induction (Fig. 4A). Curcumin efficiently inhibited the activation of p536-Luc by TNFα in cells co-transfected with the NF-κB cell signaling intermediates (Fig. 4B). Similar results were obtained for co-transfected pNF-κB that served as a control (Fig. 4C and D). To assess for transfection efficiency, transfected and control cells were stained with fluorescent antibodies and induced expression levels are shown in Fig. 4E. Altogether, these results show that the NF-κB signaling pathway is implicated in the regulation of GGT.

3.4. *Mutation of a NF-κB binding site inhibits basal and TNFα-induced GGT promoter activity*

As previous results indicated that NF-κB proteins are responsible for TNFα-induced GGT promoter activity in K562

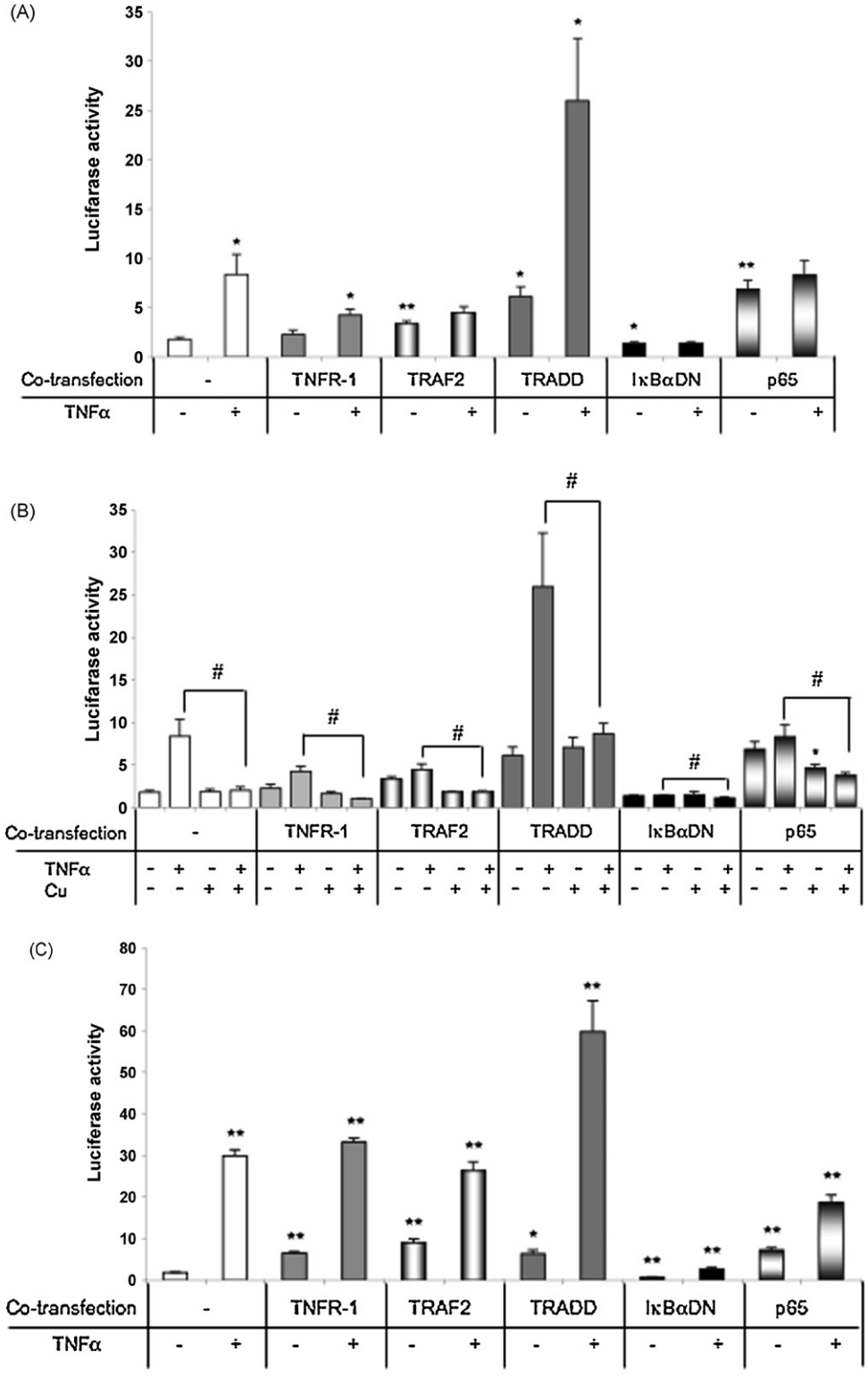

Fig. 4 – Study of the NF-κB signaling pathway on γ-glutamyltransferase and pNF-κB promoter activity. (A) Expression vectors of the NF-κB pathway, TNFR-1, TRAF2, TRADD, IκBα dominant negative (IκBαDN) and p65, as well as the p536-Luc plasmid, were transfected into K562 cells and cells were treated with TNFα (6 h, 20 ng/mL). For each co-transfection (p536-Luc + TNFR-1, + TRAF2, + TRADD, + IκBαDN, + p65), luciferase activity of both, treated (+) and untreated (−) cells is compared to untreated cells transfected only with the p536-Luc plasmid (−). (B) Expression vectors of the NF-κB pathway, TNFR-1, TRAF2, TRADD, IκBα dominant negative (IκBαDN) and p65, as well as the p536-Luc plasmid, were transfected into K562 cells and cells were treated with TNFα (6 h, 20 ng/mL), curcumin (Cu) (2 h, 20 μM) and TNFα plus curcumin (8 h). For each co-transfection (p536-Luc + TNFR-1, + TRAF2, + TRADD, + IκBαDN, + p65), luciferase activity of TNFα + curcumin treated cells is compared to TNFα treated cells. (C) Expression vectors of the NF-κB pathway, TNFR-1, TRAF2, TRADD, IκBα dominant

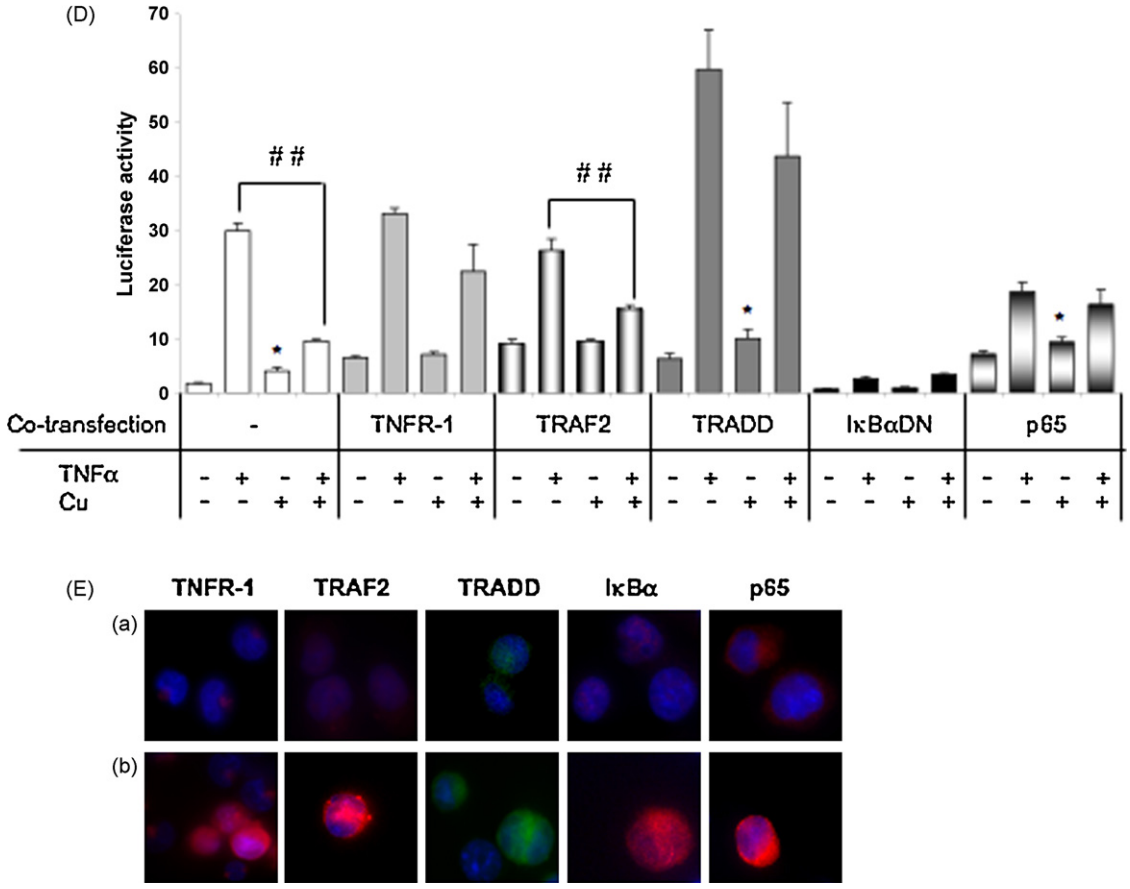

Fig. 4. (Continued).

cells, we scanned the promoter for potential NF-κB binding sites. Software analysis by Matinspector detected three potential NF-κB binding sites named NF-κB-1, -2 and -3, relative to the transcriptional start site (+1) (Fig. 5A). Mutation by linker insertion of the NF-κB-1 site, located at −110/−119 completely abolished basal as well as TNFα-induced GGT promoter activity (Fig. 5B and C, mutant 42), indicating that this sequence in the GGT promoter is responsible for the basal and TNFα-induced promoter activity. Mutation of NF-κB-2 and -3, either mutated alone or together, had no effect (Fig. 5B).

3.5. *Mutation of Sp1 binding site inhibits basal but not TNFα-induced GGT promoter activity*

To ascertain our results, the 536 bp of the proximal GGT promoter were systematically mutated by linker insertion without detecting an additional TNFα-responsive site within this promoter fragment (Fig. 5C and D). Interestingly, the mutation of a site located at −78/−93 in the GGT promoter (Fig. 5C, mutants 45 and 46 and E), strongly reduced basal GGT promoter activity without loosing TNFα induction. Computer analysis identified this site as a consensus Sp1 site.

3.6. *NF-κB, Sp1 and RNA polymerase bind to the GGT promoter in vivo*

To confirm the binding of NF-κB and Sp1 to the GGT promoter in vivo, we performed chromatin immunoprecipitation (ChIP) assays using primers targeting the region containing the NF-κB-1 and Sp1 binding site (Fig. 5F). Results showed that p50, p65, Sp1 and the RNA polymerase II bind to this specific region of the GGT promoter in control cells (Fig. 5G). TNFα treatment

negative (IκBαDN) and p65, as well as the pNF-κB plasmid, were transfected into K562 cells and cells were treated with TNFα (6 h, 20 ng/mL). For each co-transfection (pNF-κB + TNFR-1, + TRAF2, + TRADD, + IκBαDN, + p65), luciferase activity of both, treated (+) and untreated (−) cells is compared to untreated cells transfected only with the pNF-κB plasmid (−). (D) Expression vectors of the NF-κB pathway, TNFR-1, TRAF2, TRADD, IκBα dominant negative (IκBαDN) and p65, as well as the pNF-κB plasmid, were transfected into K562 cells and cells were treated with TNFα (6 h, 20 ng/mL), curcumin (Cu) (2 h, 20 μM) and TNFα plus curcumin (8 h). For each co-transfection (pNF-κB + TNFR-1, + TRAF2, + TRADD, + IκBαDN, + p65), luciferase activity of TNFα + curcumin treated cells is compared to TNFα treated cells. (E) Fluorescent microscopy analysis of expression level of NF-κB proteins before (a) and after transfection (b) into K562 cells. (*) and (**) indicate $p < 0.05$ and $p < 0.01$ compared to control, respectively, and (#) and (##) indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the TNFα treated of each co-transfection.

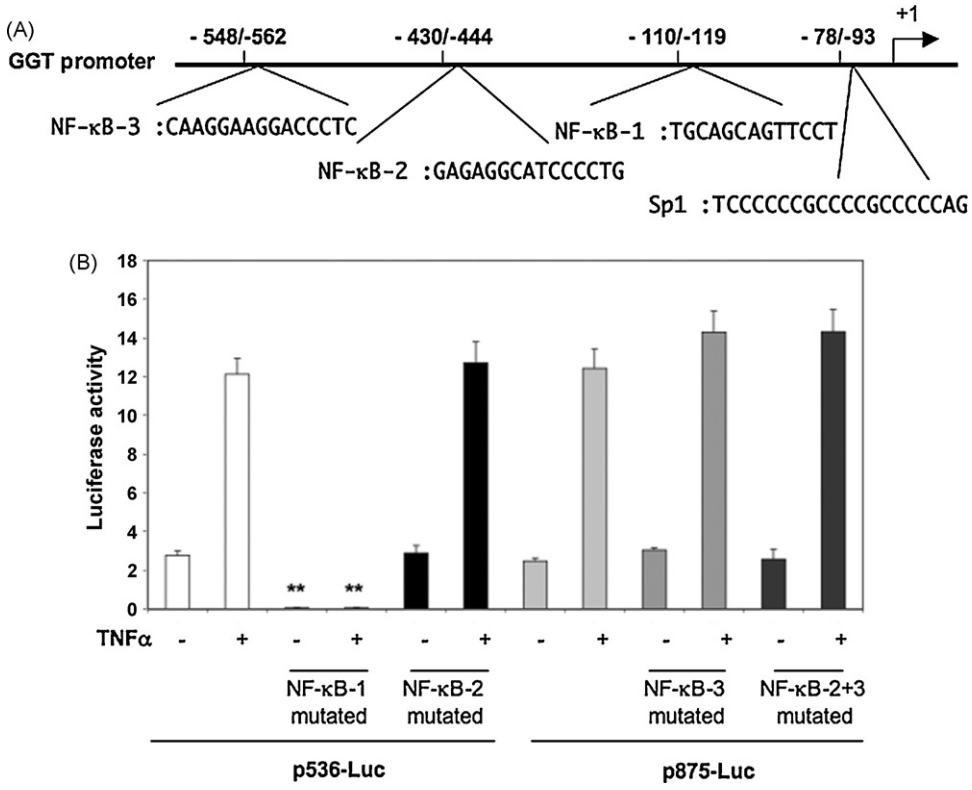

Fig. 5 – GGT promoter analysis. (A) Representation of γ-glutamyltransferase promoter with three potential NF-κB and one Sp1 binding sites. The sequence of the different binding sites is shown as well as their position relative to the transcriptional start site (+1). (B) Effect of mutated NF-κB binding sites on γ-glutamyltransferase promoter activity. K562 cells were transfected with the p536-Luc plasmids, either mutated in the NF-κB site 1 or NF-κB site 2 and with the p875-Luc plasmids, either mutated in the NF-κB site 3 alone or double mutated in NF-κB sites 2 and 3. Cells were treated with TNFα (6 h, 20 ng/mL) and not mutated p536-Luc and p875-Luc plasmids are control. (C) Linker insertion mutagenesis of GGT promoter. 536 bp of GGT promoter were mutated each 10 bp along GGT promoter sequence (from -536 bp to +1). Cells were then transfected with 53 mutants and treated with TNFα (6 h, 20 ng/mL) and wild type p536-Luc plasmid is control. (D) Representation of important sequences found by linker insertion mutagenesis with a scale relative to the transcription start site. (E) Effect of mutated Sp1 binding site on γ-glutamyltransferase promoter activity. Cells were transfected with the p536-Luc plasmid, mutated in the Sp1 site and treated with TNFα (6 h, 20 ng/mL). Wild type p536-Luc plasmid is control. (F) Sequence of the GGT promoter region (−200 to +150 bp) containing NF-κB and Sp1 elements. The sequences of the pair of PCR primers used to perform ChIP assay are shown. (G) Chromatin immunopreciption (ChIP) assay of γ-glutamyltransferase promoter. Chromatin extracted from control K562 cells was immunoprecipitated with antibodies against p50, p65, Sp1 and RNA polymerase. ChIP results are divided by IgG and expressed as relative occupancy. (H) Chromatin extracted from TNFα-treated (1 h, 20 ng/mL). K562 cells were immunoprecipitated with antibodies against p50, p65, Sp1 and RNA polymerase and results are represented as fold induction of treated cells divided by control cells. (*) and (**) indicate $p < 0.05$ and $p < 0.01$ compared to control, respectively.

increased specifically binding of p50 and Sp1 (Fig. 5H), compared to the control cells, and also strongly induced RNA polymerase II recruitment to the promoter, eventually explaining TNFα-induced GGT expression.

4. Discussion

Our studies showed that TNFα, an inflammatory cytokine [29], increased GGT activity, protein and mRNA expression as well as GGT promoter activity through NF-κB signaling pathway in K562 cells, a chronic myeloid leukemia model. We showed here that TNFα induced GGT activity significantly after 3, 6 and 9 days of TNFα treatment. This finding was confirmed in astroglial cells [30] and by using other cytokines, including IFN-α and -β [10] and interleukin 1β [31], that induced up-regulation of GGT activity after 3 days. Authors suggested that interleukin 1β-induced up-regulation of GGT activity is partially mediated via activation of NF-κB.

GGT is a membrane heterodimeric glycoprotein [22], synthesized as a single precursor, which acquires co-translationally high mannose core glycosylation [23]. During its maturation in the endoplasmic reticulum the precursor is cleaved, yielding a light subunit, with the active site and a heavy subunit. The processed enzyme is transferred to the Golgi where it acquires complex type oligosaccharides before

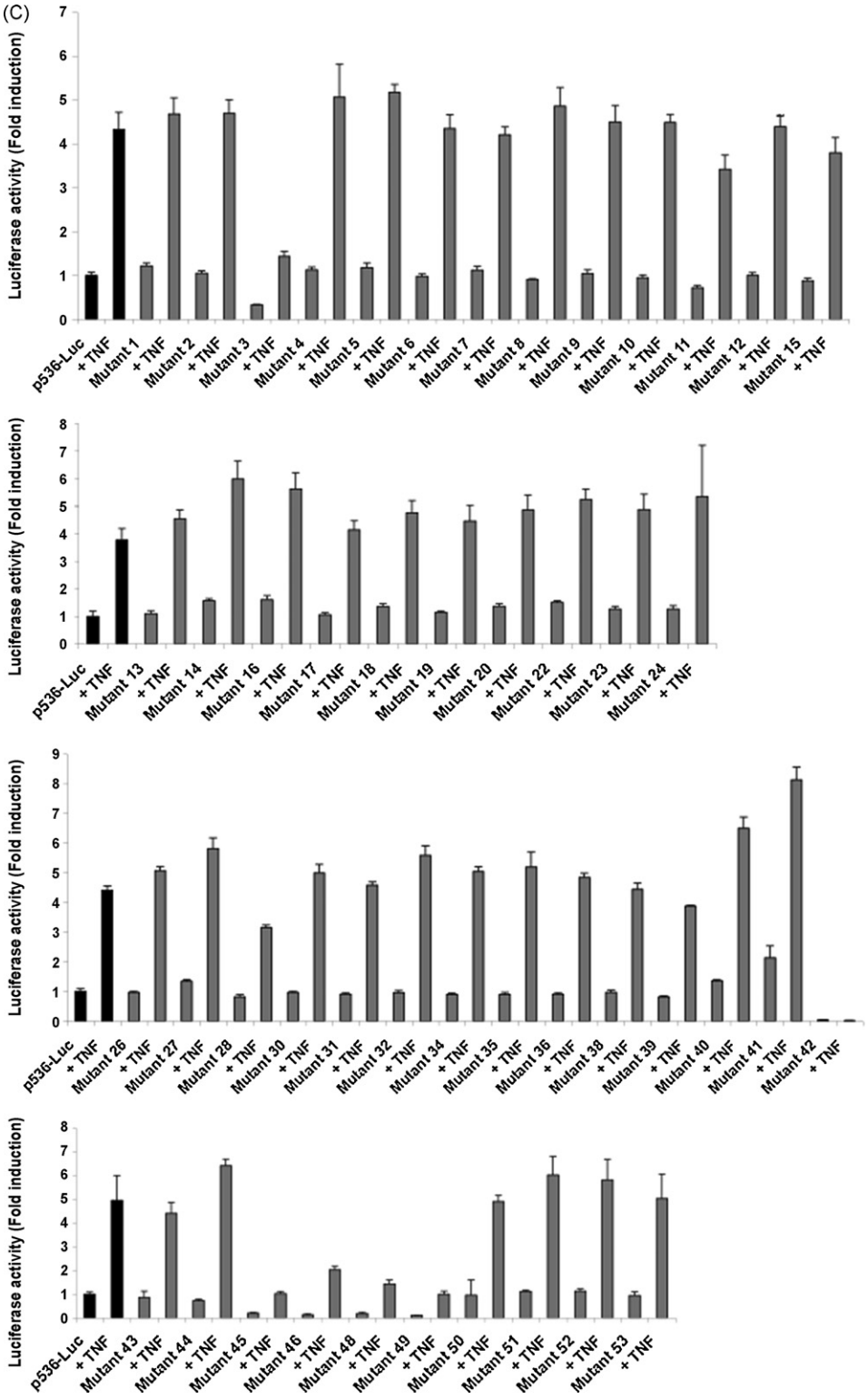

Fig. 5. (Continued).

it reaches the plasma membrane [23]. This extensive maturation explains in part the delay between GGT synthesis and GGT activity as we observed induced GGT synthesis after 6 h of TNFα treatment whereas a significant increase in GGT activity was only observed after 3 days. In order to investigate if ER stress, caused by TNFα, could also delay transport to the membrane [32], where the enzyme exerts its full activity, we investigated the effect of TNFα on a typical ER stress marker, the Grp78 protein [33]. Effectively, an increase of the Grp78 protein was observed after 6 h of TNFα treatment, most probably contributing to the observed delay.

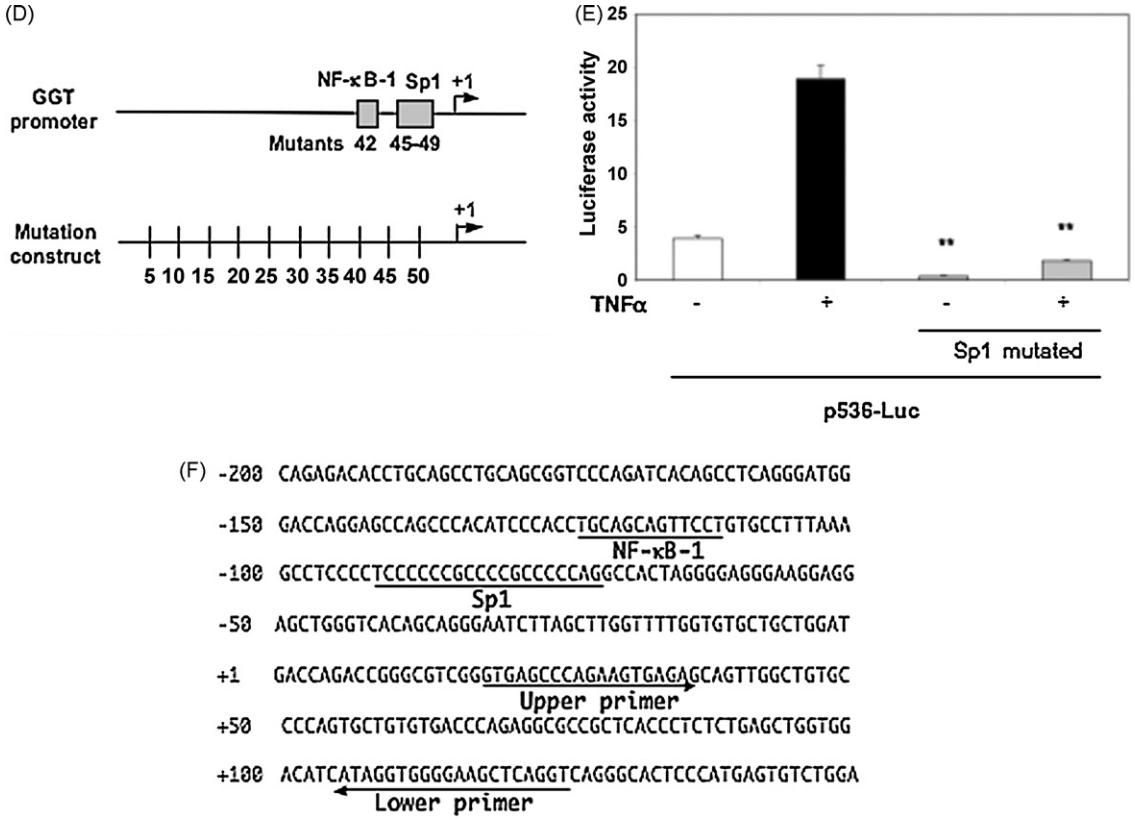

Fig. 5. (Continued).

Immunofluorescence analysis of GGT protein revealed that TNFα induced GGT expression at focal points, whereas in control cells, a uniform plasma membrane localization was observed. This particular pattern resembles a microdomain or lipid raft morphology but further experiments are needed to study subcellular GGT localization. However, a similar pattern was published for TRADD, which was recruited to the plasma membrane after TNFα treatment, where it binds to TNFR-1 [34]. Moreover, TNFR-1 is localized to lipid rafts at cell surface [35] (our results Fig. 4E) and thus, we cannot exclude that GGT is co-localized with TNFR-1 in lipid rafts.

We further show that TNFα induced up-regulation of GGT mRNA. In human, GGT is a multigene family of at least seven genes and pseudogenes [2]. Among these genes, one locus is of greater importance since its transcription generates type I GGT mRNAs [2,3], encoding an enzyme with the same catalytic properties than the enzyme isolated from human tissues [4]. Three subtypes of GGT type I mRNAs exist, mRNA A, B and C, showing all the same open reading frame and coding for the functional GGT protein but differing in their 5′ UTR [5–8]. Daubeuf et al. [9] confirmed up-regulation of mRNA A by TNFα in HepG2 cells while Bouman et al. [10] detected up-regulation of mRNA A with IFN-α and -β whereas type B and C were increased only upon IFN-β treatment.

Curcumin, a natural compound, isolated from the plant Curcuma longa, prevented TNFα-induced GGT mRNAs expression in K562 cells. Curcumin is used as a traditional medicine, well documented in Ayurveda for the treatment of numerous inflammatory conditions. It mediates its anti-inflammatory effects through the down-regulation of the transcription factor NF-κB [36,37], TNFα [38], interleukines 6 and 8 [24,39], adhesion molecules [40], inducible nitric oxide synthase [41], matrix metalloproteinase-9 [42], cyclooxygenase-2 [43] and 5-lipoxygenase (5-LOX) [44]. Curcumin was shown to bind to an

active site in 5-LOX [45]. As curcumin is well known to act as an NF-κB inhibitor [36,37] and prevented TNFα-induced GGT expression, we supposed that the NF-κB signaling pathway is involved in TNFα-induced increase of GGT expression. We used siRNA directed against two major isoforms of NF-κB, p50 and p65, and results showed that GGT induction was reduced. In order to further confirm the involvement of the NF-κB signaling pathway in GGT stimulation by TNFα in K562 cells, we overexpressed proteins of the NF-κB signaling pathway together with GGT reporter plasmids containing a fragment of GGT promoter. Results showed that the NF-κB proteins, TRAF2, TRADD and p65, stimulated GGT promoter activity, whereas the inhibitor of NF-κB, IκBα, decreased it, further implicating this pathway in GGT induction. The canonical NF-κB signaling pathway is triggered in response to microbial and viral infections as well as pro-inflammatory cytokines, like TNFα [46]. TRADD is an adaptor protein that interacts with TNFR-1 [47] and with another adaptor protein, TRAF2 [48], to activate NF-κB signaling. The IKK complex, composed of the two catalytic subunits (IKKα and IKKβ) [49,50] and the scaffolding protein IKKγ/NEMO [51], is recruited to the TNFR-1 adaptor proteins where it is activated, and subsequently phosphorylates the specific inhibitors of NF-κB, the IκB proteins. In resting, non-stimulated cells, p50/p65 dimers are retained in the cytoplasm by the IκB proteins, but translocate to the nucleus in response to pro-inflammatory stimuli.

In order to localize the TNFα response element responsible for GGT promoter induction, we performed mutations of three potential NF-κB binding sites detected in the GGT promoter sequence. Mutation of the NF-κB binding site 1, completely, reduced basal as well as TNFα-induced promoter activity, indicating that this specific sequence is responsible for the transcriptional activity as well as for the TNFα-induced induction of GGT promoter in K562 cells. BLAST searching for homologous promoter sequence fragments in different species revealed that the NF-κB-1 binding site motif is conserved in *Macaca Mulatta* and *Pan troglodytes*.

ChIP assays confirmed binding of the transcription factor p50 and p65 to this specific site in the promoter and showed that the RNA polymerase II is recruited as well. Additionally, we mutated an Sp1 site located in the GGT promoter and found a complete reduction of the basal but not TNFα-induced promoter activity. The binding of an Sp1 transcription factor to the GGT promoter has been confirmed by ChIP assays and indicates that both transcription factors NF-κB and Sp1 are responsible for basal promoter activity. A functional Sp1 binding site has also been shown in the GGT promoter 2 of rats [52].

TNFα treatment induced binding of p50 and Sp1 and stimulated strongly the recruitment of the RNA polymerase II to the promoter, explaining stimulated transcription of the GGT promoter after TNFα treatment in K562 cells. The Sp1 transcription factor, which is binding more abundantly to the promoter in TNFα treated cells compared to control cells, is not sufficient to increase GGT transcription alone, but requires p50 to recruit RNA polymerase II to the promoter and increase transcription. Cooperation between NF-κB and Sp1 for the assembly of a functional transactivation complex was also shown by others [53–56]. Results with siRNA against p65 reduce GGT promoter activity but no induction of this transcription factor is observed by ChIP after TNFα treatment, so we conclude that p65 plays an indirect role in GGT stimulation. In fact, it is known that the dimer p50/p65 regulates the expression of the p50 gene itself, of IκBα as well as of TRAF2 [57–59], all three proteins shown to play a role in GGT regulation. The knock-down of p65 with siRNA could thus decrease gene expression of these three proteins and thereby reduce GGT expression.

Altogether our results show that TNFα induces GGT activity, protein and mRNA expression as well as its transcription trough NF-κB signaling in K562 cells. Furthermore, we identified a functional transactivation complex including NF-κB, Sp1 and RNA polymerase II.

A biological significance for our results could be that TNFα induces GGT expression as a defensive mechanism as observed by several authors in conditions of oxidative stress [60], or that TNFα induces GGT expression in order to induce leukotriene synthesis and thus to mediate inflammation. Moreover, TNFα-induced GGT stimulation could prolong NF-κB activation in the cells as GGT itself is capable of promoting NF-κB activation and nuclear translocation [61].

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