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TNF-α mediated NF-kappaB activation is constantly extended by transglutaminase 2

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1. ABSTRACT

Increased levels of transglutaminase 2 (TGase 2) expression have been reported in many inflammatory diseases, as well as in drug resistant cancer cells. Previous reports have shown that TGase 2 is capable of inducing nuclear factor-kappaB (NF-kappaB) activation via depletion of inhibitor of kappaB (I-kappaB)α through polymerization in the absence of I-kappaBalpha kinase activation. This raises the question of whether increased expression of TGase 2 can extend NF-kappaB activation mediated by a canonical activation pathway. In the TGase 2-inducible EcR23/TG cell line, TGase 2 over-expression resulted in sustained activation of NF-κB in the presence of TNF-alpha, for up to 24 hrs, while in the absence of TGase 2 induction, NF-kappaB activity was restored to basal levels within 6 hrs of TNF-alpha treatment. In mice injected with an adenovirus vector expressing TGase 2, NF-kappaB was constitutively activated for up to 5 days, whereas Adeno/GFP-injected mice exhibited attenuated activation of NF-kappaB in response to TNF-α stress. Thus, the presence of increased levels of TGase 2 may exacerbate NF-κB activation in inflammatory states.

2. INTRODUCION

Constitutive activation of nuclear factor-κB (NF-κB) is essential for chronic inflammation (1) as well as cancer progression (2). Tremendous effort has led to our current understanding of the mechanisms of activation of NF-κB by various inducers. NF-κB is activated by biological stress such as bacterial infection (3), physical stress such as UV radiation (4), and chemical stresses such as reactive oxygen intermediates (5) and tumor necrosis factor (TNF)-α-activated signaling pathways (6). Mutations that result in constitutive activation of NF-κB have been reported in certain cancers (1). However, in many types of inflammatory diseases and cancers, NF-κB activation is achieved in the absence of constitutive I-κBα phosphorylation (7,8,3). Even under physiological conditions, a single inducer such as TNF-α demonstrates bimodal signaling kinetics, leading to prolongation of stimulus duration (9). These results suggest that there are intrinsic factors in cells that mediate stable long-term responsiveness to a single NF-κB inducer.

Inflammation induces the expression of hundreds of genes and alters the physiological responses of cells. A

very complex sequence of gene expression is involved in chronic inflammation in various disease states. One of these intrinsic factors is transglutaminase 2 (TGase 2, E.C. 2.3.2.13). TGase 2 is a calcium-dependent cross-linking enzyme that catalyzes the formation of iso-peptide bonds between glutamines and lysines within proteins (10, 11). Increased levels of TGase 2 expression have been reported in autoimmune diseases such as inflammatory myopathies (12, 13) and Celiac disease (14). Increased TGase 2 expression has also has been reported in many inflammatory diseases such as rheumatoid arthritis (15), Simian immunodeficiency virus (SIV) encephalitis (16), and allergic conjunctivitis (17). Additionally, deregulation of TGase 2 expression has been reported in some cancers such as lung cancer (18), breast cancer (11, 19), hepatoblastoma (20), pancreatic cancer (21) and glioblastoma (22). TNF-α expression is upregulated in early hepatic inflammation, and Kuncio *et al* showed that treatment of liver cells with TNF-α results in upregulation of TGase 2 expression (23). However, the precise role of TGase 2 in inflammation is largely unknown, although it has been suggested that TGase 2 activates phospholipase A2 (PLA_2) via intra-crosslinking (17, 24). Recently, we demonstrated that TGase 2 is able to activate NF-κB in the absence of inhibitor of I-κB (IKK)-pathway activation in rat neuroblastoma, mouse microglia, human kidney and human breast cancer cells (10, 11). One possible mechanism is the depletion of free inhibitor of κB (I-κB)α by TGase 2 through polymerization of lysines 22 and 177 and glutamines 266 and 267 of I-κBα (7).

Constitutively elevated TGase 2 expression has been observed in inflammatory diseases as well as in some types of cancer. Interestingly, various NF-κB inducers, including physical, biological, and chemical stresses, are also able to induce TGase 2 (11). We have demonstrated that TGase 2 inhibition markedly reduces inflammatory processes in several inflammatory disease models, including pollen-induced allergic conjunctivitis (17), an LPS-induced brain injury model (25), and an LPS-induced lung injury model (26). In cancer models such as malignant glioblastoma (27) and drug resistant breast cancer (28), TGase 2 inhibition also markedly increases drug sensitivity through down regulation of NF-κB activity (25).

The aim of the current study was to determine whether increased TGase 2 expression activates NF-κB simultaneously and in a cooperative manner with one of the canonical pathways of NF-κB activation, TNF-α. To test this, we used an *in vitro* cell culture system in which TGase 2 expression is induced by tetracycline, and an *in vivo* system of recombinant adenovirus vector-mediated over-expression of TGase 2 in mice. In both systems, TNF-α treatment in combination with TGase 2 over-expression resulted in a profound increase of NF-κB activity that was sustained for over 72 hrs, while in control groups, activity recovered to basal levels within 24 hrs. The results of the current study provide compelling evidence that increased TGase 2 expression levels play an important role in constitutive NF-κB activation.

3. MATERIALS AND METHODS

3.1. Immunoblot analysis

Cytosolic fractions were prepared using a CelLytic NuCLEAR Extraction kit (Sigma) according to the manufacturer's instructions. Samples were subjected to electrophoresis on a NuPAGE 4-12% Bis-Tris gel in MOPS SDS running buffer (Invitrogen) and then transferred to a polyvinylidene difluoride membrane (Bio-Rad) using a semidry blotting apparatus (Hoefer SemiPhor). Membranes were incubated in Tris-buffered saline containing 0.5% Tween 20 (TBS-T) and 5% bovine serum albumin (BSA) for 1 hr at room temperature and then incubated with primary antibody overnight at 4^{o}C. The primary antibodies used in these studies were as follows: anti-I-κB-α (Cell Signaling Technologies), anti-TGase 2 (clone CUB 7402, NeoMarkers), anti-NF-κB (p65, Cell Signaling Technologies) and anti-β-actin (Abcam). After incubation with primary antibody, the membrane was washed 3 times for 20 minutes (min) each in TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) in TBS-T containing 1% BSA for 1 hr at room temperature. The concentration of primary and secondary antibody was 3.0 and 0.1 μg/ml, respectively. Immunoreactive proteins were visualized using enhanced chemi-luminescence (Pierce).

3.2. Plasmid Constructs and Transient Transfection

Single point mutations in full-length I-κBα (K177G, Q255G, Q266G, Q267G, K21G/22G, S32A/36A) were constructed by the recombinant PCR method using the full-length I-κBα cDNA as a template (pCMV- I-κBα; BD Biosciences). Amplified fragments containing point mutations were inserted into pcDNA 3.0 (Invitrogen) at the Hind III and Xba I sites. The sequences of the clones were verified by DNA sequencing. Transient transfection with wild type (1 μg) or mutant I-κBα (1 μg) expression vectors and/or pcDNA-hTG2, or pcDNA 3.0 (Invitrogen) as a control (mock vector), was carried out using lipofectamin^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded at a density of $4x10^5$ cells/well in a 6-well plate. When cells reached 40-50% confluence they were washed with 2 ml of Opti-MEM (Invitrogen) and then incubated with DNA-lipofectamine mixture (1 μg of DNA plus 3 μl of lipofectamine reagent) for 6 hrs in a humidified 5% CO_2 chamber. The medium was replaced with fresh culture medium and the cells were allowed to incubate for an additional 48 hrs. For the secreted alkaline phosphatase (SEAP) reporter assay, cells were transfected with a reporter plasmid in which expression was driven by the NF-κB promoter (pNF-κB-SEAP; BD Biosciences Clontech, 1.0 μg) using 3 μl of Lipofectamine 2000. Cells were co-transfected with pGAL (1.0 μg) to normalize for transfection efficiency. Transfection of a small interfering RNA (siRNA) duplex targeting human TGase 2 (5′-AAGAGCGAGAUGAUCUGGAAC-3′) was carried out using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. Briefly, cells were seeded at a density of $3x10^5$ cells/well in a 6-well tissue culture dish and then incubated overnight. Cells were transfected

with siRNA-Lipofectamine mixture (200 pmol siRNA and 3 μl of Lipofectamine reagent) and then allowed to incubate for 2 days. Transfection complexes were removed and then the cells were incubated in culture medium without antibiotics in preparation for TNF-α treatment. As a control, cells were incubated with the Lipofectamine RNAiMAX Stealth Negative control (Invitrogen).

3.3. Reporter Assays: SEAP Assay, β-galactosidase Assay, and β-lactamase Assay

NF-κB activity was measured using the SEAP reporter system 3 (BD Bioscience Clontech). Cell culture medium was collected 24-48 hours after transfection with pNF-κB-SEAP. The SEAP assay was performed according to the manufacturer's instructions. Briefly, culture medium (25 μL) was mixed with 25 μL of dilution buffer in a 96-well flat-bottomed microtiter plate and then incubated at 65°C for 30 min. The plate was chilled on ice for 2 minutes, after which 97 μL of assay buffer was added to each well. The plates were incubated at room temperature for 5 min, followed by the addition of 3 μL of 1 mM MUP fluorescent substrate, and then the plates were incubated for an additional 60 min in the dark at room temperature. The fluorescence of each sample was measured using a 96-well fluorescent plate reader (SpectraMAX GeminiEM, Molecular Devices) equipped with a 360-nm wavelength excitation filter and a 460-nm wavelength emission filter. To normalize for expression levels in cells transfected with various expression plasmids, cells were co-transfected with pGAL (1 μg). SEAP activity was normalized to β-galactosidase activity. The β-galactosidase assay was performed using a β-Galactosidase Enzyme Assay System (E2000, Promega) (Figs. 1 and 3). To determine NF-κB activity, we used the HEK293T/NF-κB-bla cell system, a live cell reporter assay. The β-lactamase assay was carried out using a β-lactamase loading kit and the CCF4-AM substrate (K1096, Invitrogen), according to the manufacturer's instructions (Figs. 3 and 4). Results represent the averages and standard deviation (SD) of three independent experiments.

3.4. Immunocytochemical Analysis

The translocation of p65 was visualized by immunostaining and confocal microscopy as described previously (Kim, 2006). EcR293/TG cells were seeded in a two-well slide chamber ($1x10^5$ cells per well) and then transfected with mutant I-κBα (K177G, Q266G, Q255G) or wild-type (WT) I-κBα expression constructs, as indicated, for 24 hrs. The cells were treated with tetracycline (1 μg/ml) for 24 hrs to induce TGase 2 expression, after which they were treated with TNF-α (100 ng/ml) for 2 hrs. Cells were incubated in 3.7% paraformaldehyde on ice for 15 min and then washed with 0.1% PBS-T. Cells were permeabilized with 0.5% PBS-T for 5 minutes, washed again in 0.1% PBS-T, incubated for 1 hr in PBS containing 10% calf serum and 0.5% gelatin, and finally washed with 0.1% PBS-T. Fixed cells were incubated with mouse monoclonal anti-p65 antibody (1:100 dilution, Santa Cruz Biotechnology) for 1 hr at room temperature, washed with 0.1% PBS-T, and then incubated with a fluorescent isothiocynate-conjugated anti-mouse IgG (1:200 dilution, Jackson ImmunoResearch Labs) for 1 hr at room temperature. The cells were washed with 0.1% PBS-T, incubated with 4,6-diamindino-2-phenylindole (DAPI, Sigma) nuclear dye for 5 min at room temperature, washed with 0.1% PBS-T again, and then mounted using VectaShield (Vector Laboratories). NF-κB expression was visualized using a Zeiss axiovert LSM510 microscope.

3.5. Construction of an Adenovirus vector Expressing TGase 2

The recombinant adenovirus expressing TGase 2 was constructed by insertion of the full-length TGase 2 cDNA into the adenoviral shuttle vector pAd1020SfidA (OD_{260}, Inc., Boise, ID, USA). Briefly, a fragment containing CMV-multiple cloning sites-pA-CMV-GFP-pA was excised from pAdTrack-CMV (a generous gift from Dr. Bert Vogelstein) and then inserted into pAd1020SfidA to create pAdSfidACMVGFP. The full-length TGase 2 cDNA was cloned into the EcoRI/SalI sites of pAd1020sfidACMVGFP (pAd1020CMV GFP-CMVTG2), and then this construct was digested with SfiI and PacI and the resulting fragment was ligated into AdenoZap1.2 (OD_{260}). HEK293 cells were transfected with the adenoviral vector using Lipofectamine 2000 (Invitrogen). Amplified adenoviruses expressing TGase 2 (Adeno/TG2) were purified by CsCl gradient centrifugation, dialyzed against 1 L of dialysis buffer (789 ml of double-distilled water, 1 ml of 1 mol/L $MgCl_2$, 10 ml of 1 mol/L Tris-HCl (pH 7.5) and 200 ml of 50% glycerol) for 24 hrs at 4°C with three buffer changes and then stored at –80 °C for future use.

3.6. Animal Experiments

Six-week-old BALB/c mice were housed in the animal facilities of the National Cancer Center and cared for in accordance with the Declaration of Helsinki and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were injected with 1.0×10^9 virus particles of Adeno/TG2, or AdCMVGFP as a control, via tail vein injection using a 34-gauge Hamilton syringe (Hamilton). Twenty-four hrs after viral injection, 0.5 μg/g of body weight of TNF-α (R&D Systems Inc) was injected via the tail vein. Five mice were sacrificed 120 hrs after TNF-α injection.

3.7. Electrophoretic Mobility Shift Assay for the detection of nuclear NF-κB

Nuclear extracts were prepared using a CelLytic™ NuCLEAR™ Extraction kit (Sigma). An NF-κB double-stranded oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was purchased from Santa Cruz Biotechnology and used as the probe. The DNA probe was labeled with Redivue adenosine 5'-(γ-^{32}P)triphosphate, Triethylammonium salt (Amersham AA0068; 9.25 MBq, 250 μCi, 10 μCi/μl/25μl) using T4 kinase (Takara) and T4 kinase buffer (10X); radiolabeled probe was purified using MicroSpin G-25 columns (Amersham, 27-5325-01). Labeled probe was incubated with nuclear extracts for 20 min at 37°C. The mixtures were separated on a 6% non-denaturing polyacrylamide gel. The gel was exposed to a BAS-MS2040 plate (FUJIFILM) for 30 min in a BAS cassette2 (FUJIFILM). Signals were detected using a BAS-

2500 Phosphor Imager (FUJIFILM).

4. RESULTS

4.1. TGase 2- and TNF-α-mediated NF-κB activation is reduced by the TGase 2 inhibitor cystamine

To determine whether TGase 2 expression modulated the activation of NF-κB via the canonical TNF-a pathway, we carried out a SEAP gene reporter assay using the TGase 2-inducible cell line EcR293, in which the expression of TGase 2 is induced by tetracycline (Kim, 2006; Lee *et al.*, 2004). Upon tetracycline stimulation, TGase 2 levels increased concomitantly with a decrease in I-κBα protein levels, as assessed by immunoblot analysis (Figure 1B). This decrease in I-κBα levels was also evident as an increase in NF-κB promoter activity (Figure 1A). To determine whether TGase 2 inhibition reversed the prolongation of NF-κB activation, EcR293/TG cells were treated with the TGase 2 inhibitor cystamine (CTM) (Figure 1). Following treatment of cells with varying concentrations of CTM (0.5, 1, 5 mM) for 1 hr, TGase 2 was induced by treatment with tetracycline for 18 hrs, followed by TNF-α treatment for 6 hrs in the presence or absence of CTM. Induction of TGase 2 expression increased NF-κB activity approximately 50%, whereas TNF-α treatment resulted in maximal activation of NF-κB (Figure 1). Induction of TGase 2 expression resulted in constitutive (prolonged) activation of NF-κB activity by TNF-α (Figure 1). Furthermore, NF-κB activity was reduced by the TGase 2 inhibitor CTM in a dose dependent manner (Figure 1). Immunoblot analysis showed that I-κBα levels were also restored by CTM treatment in TGase 2-induced cells in a dose dependent manner (Figure 1). Thus, I-κBα levels decreased in the presence of TNF-α, and this decrease was rescued to a certain extent by CTM. These results suggested that TGase 2 prolongs NF-κB activation by TNF-α. Then, we treated TNF-α for 24 hrs instead of 6 hrs.

4.2. Effect of TGase 2 expression on I-κBα degradation induced by TNF-α treatment and activation of NF-κB signaling pathways

In cells in which TGase 2 expression was induced, NF-κB activity was constitutively increased in the presence of TNF-α. To determine whether this increase in NF-κB activity in the presence of TGase 2 was due to decreased I-κBα levels, EcR293/TG cells were treated with TNF-α with or without induction of TGase 2 for 24 hrs, and then TGase 2 and I-κBα protein levels were analyzed by immunoblot (Figure 2A). In the absence of TGase 2 induction, I-κBα levels almost completely recovered within 6 hrs of TNF-α treatment (Figure 2A). In cells in which TGase expression was induced, I-κBα protein levels failed to recover, even after 24 hrs of TNF-α treatment (Figure 2A). We previously reported that in MCF7/DOX cells (doxorubicin-resistance MCF-7 cells), TGase 2 expression is constitutively elevated as compared to MCF-7 cells (Figure 2B) (11). In MCF-7 cells, I-κBα levels recovered within 6 hrs of TNF-α treatment, whereas in MCF7/DOX cells, the levels of I-κBα remained low even after 24 hrs of TNF-α treatment (Figure 2B). To determine the effect of TGase2 down-regulation on I-κBα levels in MCF7/DOX cells, cells were treated with a TGase 2 siRNA for 2 days. The recovery of I-κBα levels in TGase 2 siRNA-treated MCF/DOX cells reached that of MCF-7 cells within 6 hrs of TNF-α treatment (Figure 2C).

4.3. TGase 2 sustains TNF-α mediated NF-κB activation

To determine the kinetics of NF-κB activation by TNF-α in the presence or absence of TGase 2 expression, we assayed a panel of cell lines (EcR293/TG, MCF-7, MCF/DOX, HEK293T/NF-κB-bla) containing varying levels of TGase 1 (Figure 3). Cells were transfected with pNF-κB-SEAP (and a TGase 2 expression vector, where indicated), and then NF-κB activity was measured by the SEAP gene reporter assay. Dual activation of NF-κB by TGase 2 and TNF-α resulted in a high level of sustained activity as compared to TNF-α alone (Figure 3 A-C). Down-regulation of TGase 2 in MCF7/DOX cells by siRNA treatment shortened the period of sustained NF-κB activation as compared to mock treated MCF7/DOX cells (Figure 3C). These results indicated that TGase 2 plays an important role in the sustained pattern of NF-κB activation observed in many inflammatory diseases.

4.4. Inhibition of TNF-α-induced NF-κB activation by expression of TGase 2 targeting site mutants of I-κBα

To investigate the mechanism by which TGase 2 modulated TNF-α-mediated NF-κB activation, we generated a set of dominant negative mutant forms of I-κBα containing single point mutations in ubiquitination sites (K21/22), IKK phosphorylation sites (S32/36), or TGase 2 cross-linking sites (K177, Q267), as well as a randomly mutated negative control (Q255). HEK293T/NF-κB-bla cells were co-transfected with wild-type or mutant I-κBα and TGase 2 expression vectors for 48 hrs, and then NF-κB activity was measured by β-lactamase reporter assay (Figure 4). NF-κB activity was measured after 6 hrs of TNF-α treatment. I-κBα ubiquitination site mutants and IKK phosphorylation site mutants reduced NF-κB activation by approximately 50% and 40%, respectively (Figure 4A). Mutants containing single amino acid substitutions in the TGase 2 targeting sites (K177 and Q267) reduced NF-κB activity by 60% (Figure 4A). Immunoblot analysis confirmed that I-κBα levels were decreased in the presence of the control mutant (Q255) as compared to the dominant negative mutants of I-κBα (K21/22, S32/36, K177, Q266, Q267) (Figure 4B).

4.5. Nuclear Translocation of NF-κB in response to TNF-α is impaired by TGase 2 targeting site mutants of I-κBα

To examine whether inhibition of TGase 2 affected the translocation of NF-κB into the nucleus in response to TNF-α, EcR293/TG cells were transfected with expression vectors for wild-type I-κBα or the various I-κBα mutants for 24 hrs and then TGase 2 was induced for 12 hrs, followed by treatment with or without TNF-α for 2 hrs. Localization of NF-κB was analyzed by immunocytochemistry using an anti-p65 antibody (Figure

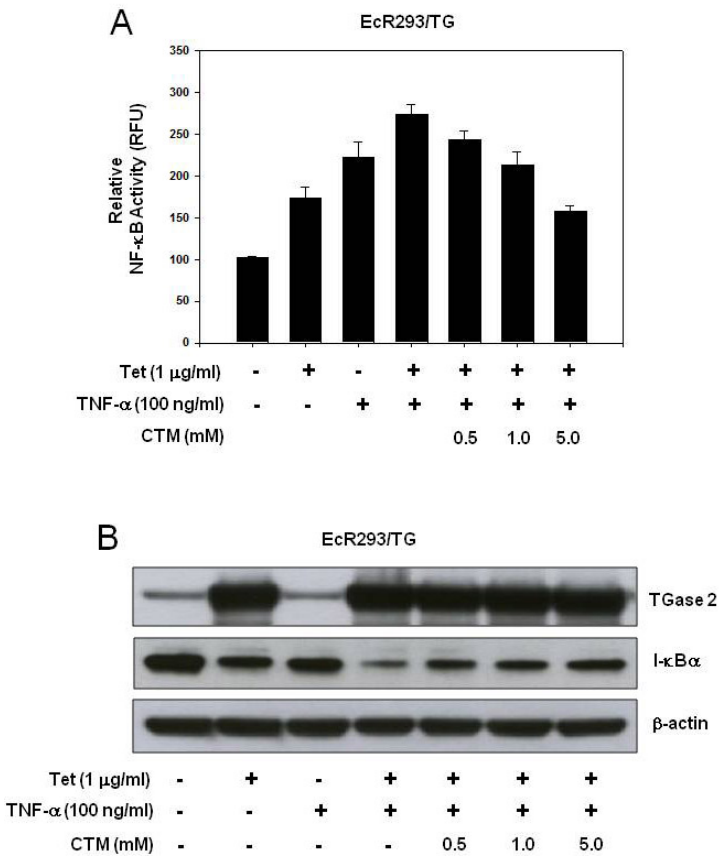

Figure 1. Additive effect of TGase 2 on TNF-α mediated NF-κB activation. (a) SEAP reporter assay using EcR293/TG cells. Cystamine (CTM) was treated in three different concentrations (0.5, 1, 5 mM) for 1 hr before TGase 2 induction, and tetracycline was treated to induce TGase 2 for 18 hrs together with cystamine. After induction, TNF-α (100 ng/ml) was treated for 6 hrs. NF-κB activity induced by TNF-α treatment together with TGase 2 induction was higher than either only with TNF-α or TGase 2 induction. Cystamine reduced the NF-κB activity in a dose dependent manner in TNF-α mediated NF-κB activation. Shown are averages ± SEM of triplicates. (b) Western blots analysis of TGase 2 and I-κBα in EcR293/TG cells. In the same condition as panel *A*, level of I-κBα was recovered by cystamine treatment in a dose-dependent manner. Interestingly, it was rescued decreased I-κBα by 6 hrs of TNF-α treatment.

5). In the presence of wild-type I-κBα, NF-κB was detected in mainly in the nucleus in TGase 2-induced cells (Figure 5, white arrowhead in WT I-κBα). TNF-α treatment combined with TGase 2 induction resulted in prominent translocation of NF-κB into the nucleus as compared to TNF-α alone (Figure 5; white arrowhead in I-κBα wild). Expression of TGase 2 targeting site mutants of I-κBα resulted in a marked reduction of nuclear NF-κB, and an accumulation of NF-κB in the cytosol (Figure 5; red arrowheads in I-κBα K177G and Q266G). Expression of the control I-κBα variant had no effect on NF-κB translocation (Figure 5; white arrowhead in I-κBα Q255G).

4. 6. Profound activation of NF-κB in the mouse liver by combined TGase 2 over-expression and TNF-α treatment

To investigate whether the synergistic activation of NF-κB induced in cultured cells by TGase 2 and TNF-α also occurred *in vivo*, TGase 2 over-expression in mice was achieved by tail vein injection of an adenovirus vector expressing TGase 2 (Adeno/TG2) (Figure 6). Hepatocytes isolated from mice infected with Adeno/TG2 exhibited dramatically elevated levels of TGase 2 expression. Twenty-four hrs after injection of Adeno/TG2, mice were treated with TNF-α through tail vein injection. Five days (120 hrs) after TNF-α treatment, liver tissue was isolated and examined for gross morphology and expression of NF-κB. In TGase 2 over-expressing mice, tissue swelling was evident, and there were substantially increased numbers of NF-κB positive hepatocytes, whereas liver tissue from control mice was normal in appearance and there was no increase in NF-κB positive hepatocytes (data not shown). Immunoblot analysis showed that TGase 2 expression was sustained over the period of 120 hrs after TNF-α treatment (Figure 6A). NF-κB activity was increased approximately 3-fold by the combination of TGase 2 overexpression and TNF-α treatment (as compared to TNF-α alone); NF-κB activity induced by TNF-α alone was increased by approximately 50% over untreated animals (Figure 6B). Cox-2 expression was increased approximately 4-fold in

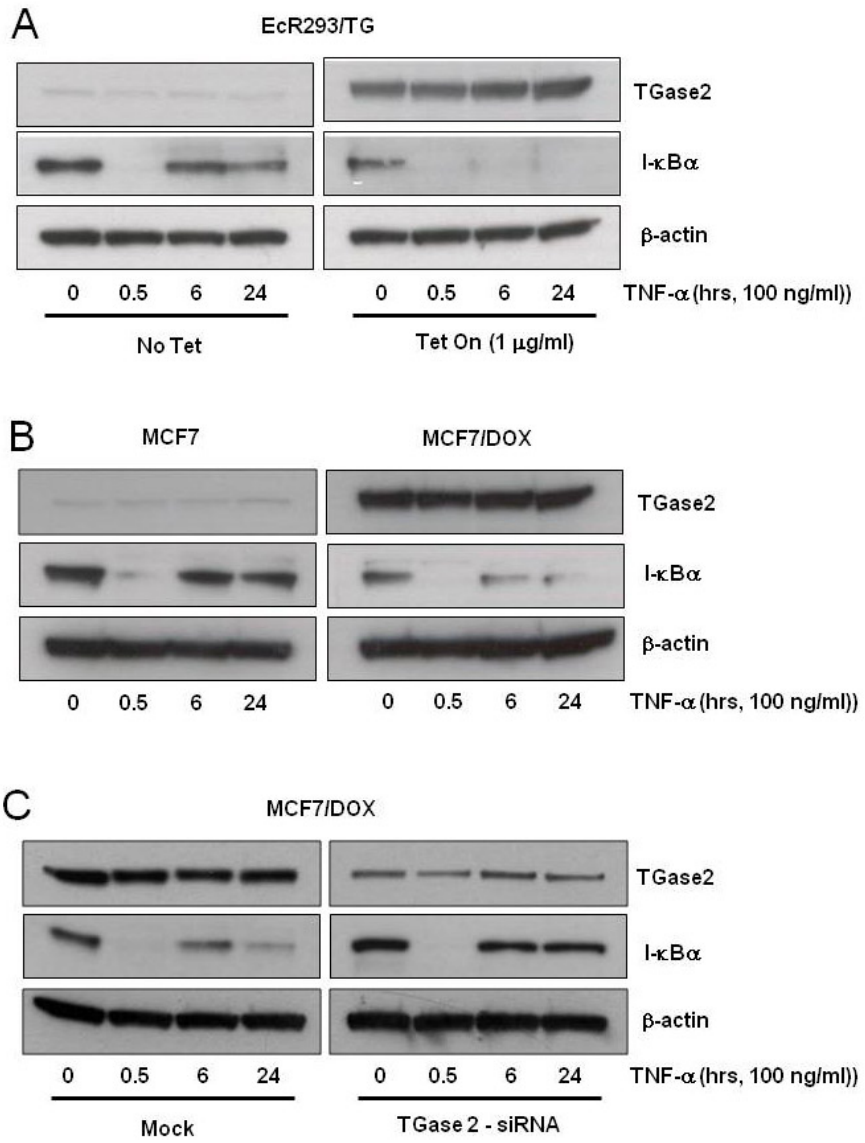

Figure 2. Extended period of I-κBα degradation by TGase 2 in TNF-α induced NF-κB signaling pathway. (a) Analysis of I-κBα by western blot of cytoplasmic fractions in EcR293/TG cells after treatment of TNF-α (100 ng/ml). In the condition of low TGase 2 level, I-κBα decreased until 3 hrs after treatment of TNF-α, recovered until 6 hrs. However, I-κBα level was not recovered even in 24 hrs after TNF-α treatment in the group with TGase 2 induction. (b and c) Analysis of I-κBα regulation by TNF-α and TGase 2 in breast cancer cells. MCF-7/DOX (Doxorubicin-resistant) expresses higher level of TGase 2 than MCF-7. By TNF-α treatment (100 ng/ml), I-κBα level in MCF-7/DOX was not recovered as much as in MCF-7 even in 24 hrs, which inversely correlates with TGase 2 expression. For effect of TGase2 down-regulation on I-κBα regulation by TNF-α in MCF-7/DOX cells. TGase2 was down-regulated by treatment of TGase 2 siRNA (200 pmol) for 2 days. I-κBα level in MCF-7/DOX transfected with TGase 2 siRNA was recovered as much as in MCF-7 in 6 hrs after TNF-α treatment (100 ng/ml).

the combined treatment group (Adeno/TG2 + TNF-α), while TNF-α alone resulted in a 50% increase (Figure 6A).

5. DISCUSSION

The recent demonstration that TGase 2 plays a critical role in NF-κB activation in the absence of IKK activation was a significant advance in our understanding of the biological role of TGase 2 (11, 25). NF-κB is an important component of inflammatory processes and cancer promotion; thus, the involvement of TGase 2 in NF-κB activation could be an important clue to the molecular mechanisms underlying disease etiology. Previously, we demonstrated that specific glutamine and lysine residues at positions 177 and 266 of I-κBα are involved in protein cross-linking by TGase 2 (7). We were interested in whether TGase 2 had an additive effect on NF-κB activation in combination with the canonical TNF-α

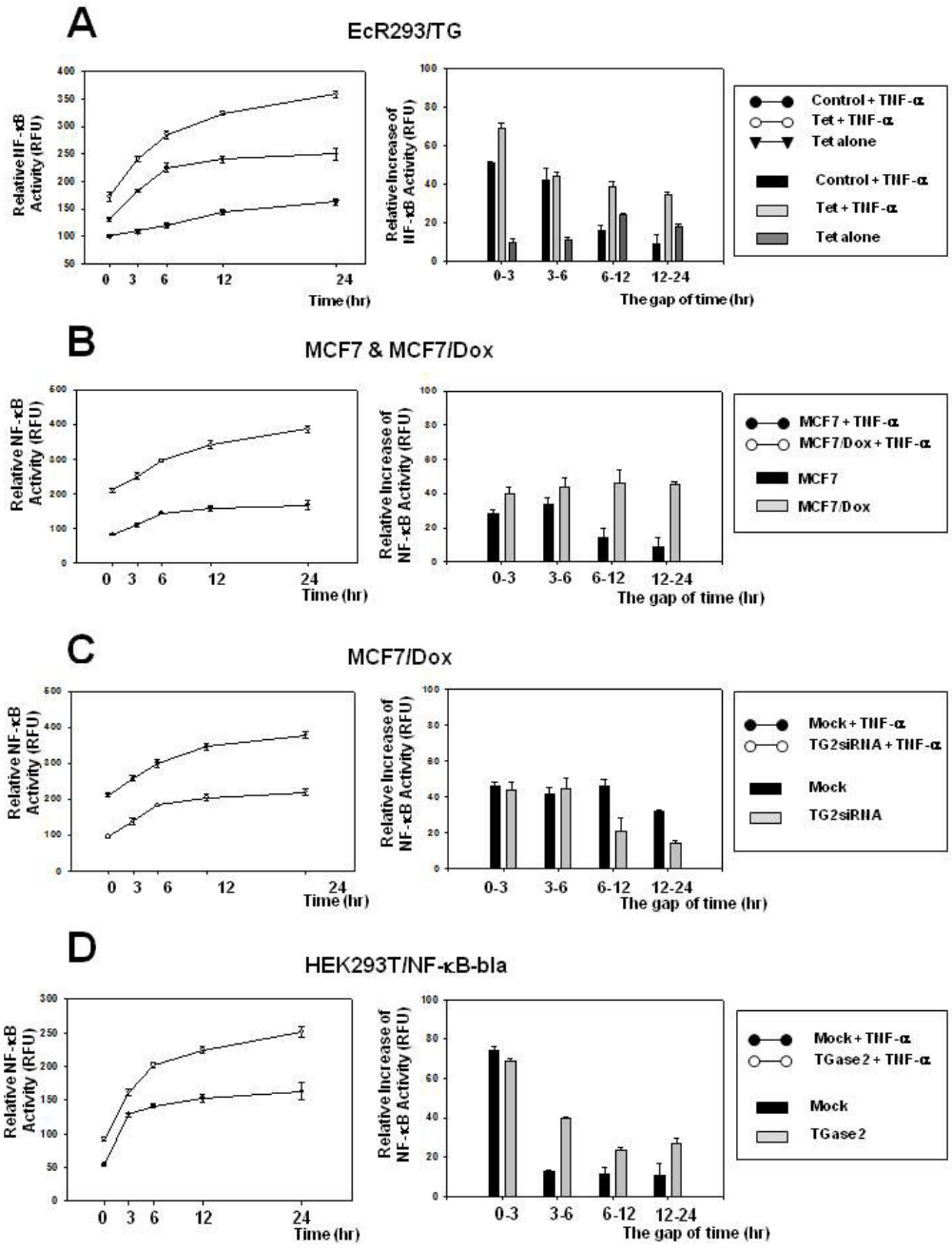

Figure 3. The elongate effect of TGase 2 on TNF-α mediated NF-κB activation. TGase 2 transfection showed elongate effect on TNF-α mediated NF-κB activation in a time dependent manner in the various cell lines. By TNF-α treatment, maintenance of TGase 2 induced NF-κB activation longer than low level of TGase 2 in the various cell lines. Shown are averages ± SEM of triplicates. The relative increase of NF-κB activity was measured between the interval times. (a) NF-κB reporter gene was transfected to 293EcR/TG cell line. After transfection for 24 hrs, TGase 2 induction for 24 hrs by tetracycline treatment and TNF-α (100 ng/ml) treated for 24 hrs. (b) NF-κB reporter gene was transfected to MCF7 and MCF7/Dox cell line. After transfection for 24 hrs, TNF-α (100 ng/ml) treated for 24 hrs. (c) After MCF7/Dox cells treated by siRNA of TGase 2 for 24 hrs, pNF-κB-SEAP was transfected to the cell line for 24 hrs. The treatment time and dose of TNF-α were same as panel A. NF-κB activity induced by TNF-α treatment together with TGase 2-siRNA was lower than only with TNF-α. (d) β-lactamase reporter assay using HEK293T/NF-κB-bla cells. Cells were transfected with TGase 2 (1 μg) for 24 hrs, and were treated for 24 hours with TNF-α (100 ng/ml). Tet, tetracycline.

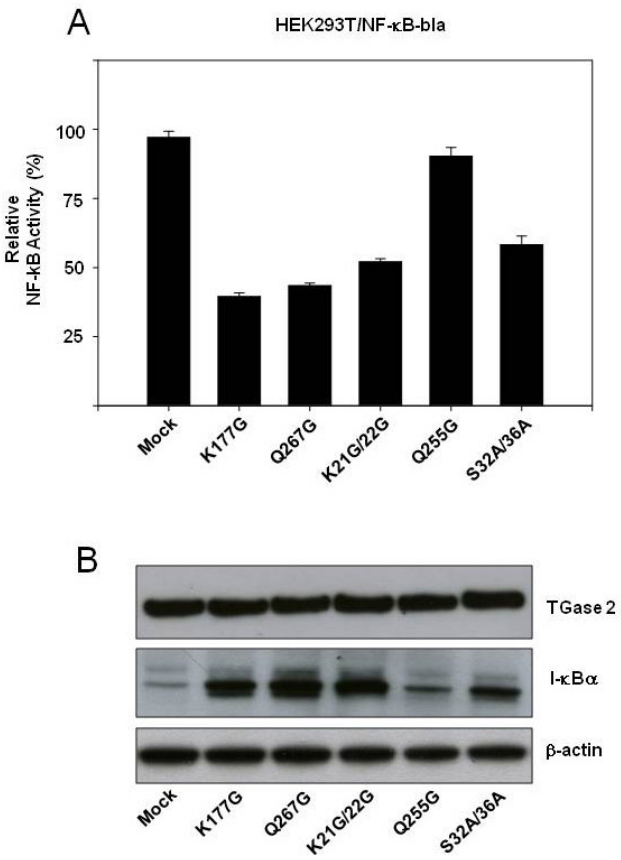

Figure 4. Reversal of TNF-α mediated NF-κB activation by I-κBα mutants at TGase 2 targeting sites. β-lactamase reporter assay was employed in HEK293T/NF-κB-bla cells. Wild type I-κBα and various mutants containing ubiquitination sites (K21/22, phosphorylation sites by IKK, or cross-link sites by TGase 2 were transfected to HEK293T/NF-κB-bla cells. After 48 hrs transfection, TNF-α was treated for 6 hrs. Ubiquitination and phosphorylation mutants inhibited TNF-α mediated NF-κB activation about 40%. However, mutants at cross-link sites inhibited NF-κB up to 60% while random mutant at Q255 showed no effect at all. Recovery of I-κBα by I-κBα mutants accorded to the observation of NF-κB activity. Shown are averages ± SEM of triplicates.

pathway, given the fact that TNF-α can induce TGase 2 expression (23). Here, we showed that TGase 2 expression results in constitutive TNF-α-mediated activation of NF-κB, and that TGase 2 expression activates NF-κB in the absence of IKK activation. Expression of TGase 2 targeting site mutants of I-κBα, in which the TGase 2 cross-linking residues were mutated, restored free I-κBα levels by up to 60% in the cytosol in response to TNF-α. In cells that overexpressed TGase 2, TNF-α treatment resulted in a tremendous increase in NF-κB activity for up to 24 hrs, whereas in control cells that did not overexpress TGase 2, complete recovery was achieved within 6 hrs. These results suggest that increased expression of TGase 2 escalates NF-κB activity for prolonged periods of time, which may exacerbate inflammation and disease processes (Figure 7).

5.1. TGase 2 expression is increased in inflammatory diseases and cancers

Increased TGase 2 expression has been reported in many inflammatory diseases, including inflammatory myopathies (13), Celiac disease (14), rheumatoid arthritis (15), SIV encephalitis (16), and allergic conjunctivitis (17). Abnormally high levels of TGase 2 expression have also been reported in some cancers such as lung cancer (18), breast cancer (11, 19), hepatoblastoma (20), pancreatic cancer (21) and glioblastoma (22). The mechanism of induction of TGase 2 expression in these pathological states is unclear. TGase 2 expression can be induced by TNF-α signaling (23), and TGase 2 can be induced by various stresses, including oxidative stress (29), UV (30), calcium influx in response to calcium ionophore (31) or maitotoxin (32), retinoic acid (RA) (33), glutamate (34), and viral infection (16). However, the induction of TGase under these conditions is normalized within a short time. Epigenetic factors have been implicated in the constitutive activation of TGase 2 expression. TGase 2 expression can be induced by demethylation of specific regions of the *TGM2* promoter (35, 36, 37). This induction of TGase 2 results in constitutive NF-κB activation associated with drug resistance in breast, ovarian, and lung cancers (38). Relatively higher sensitivity to cisplatin is observed in the *TGM2* promoter-methylated cell lines HCC-95 and HCC-

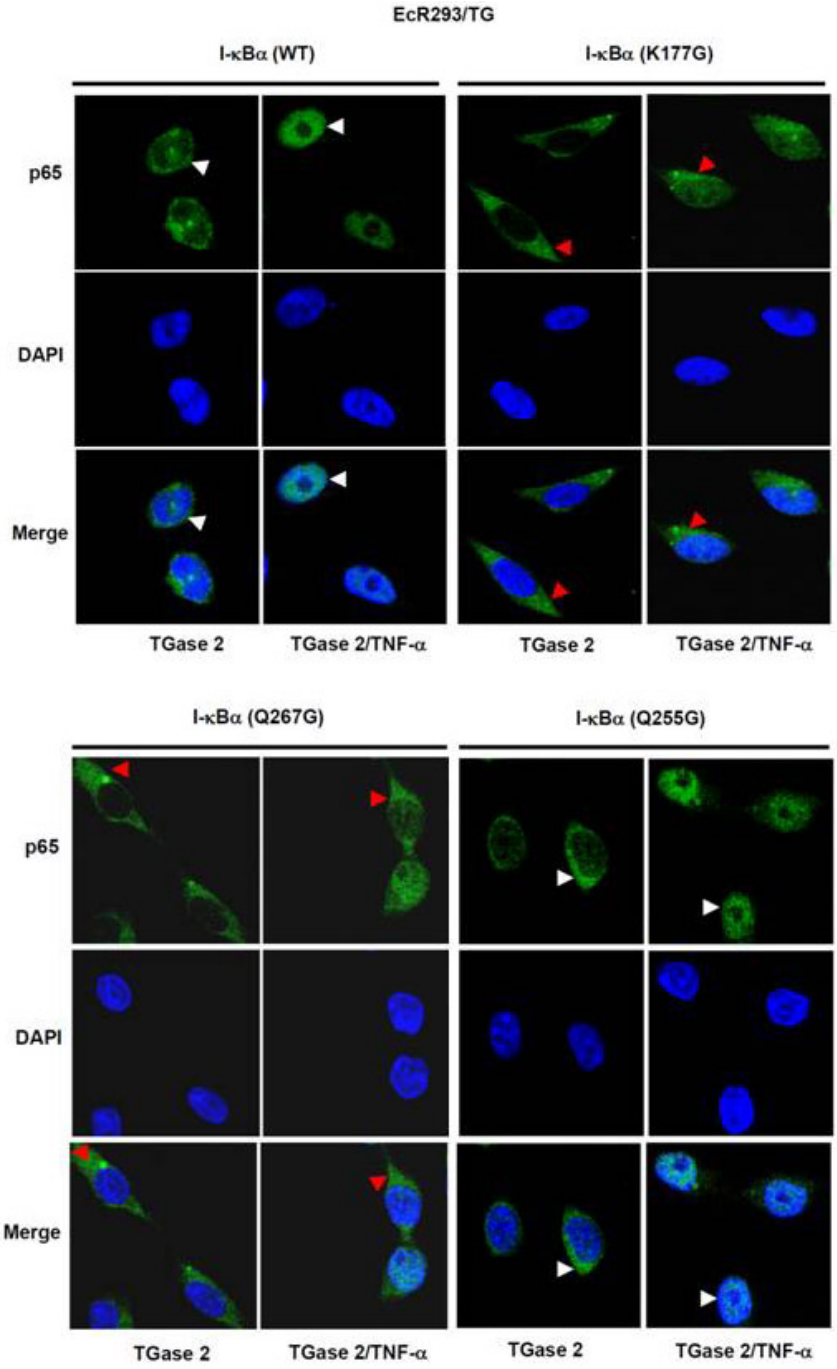

Figure 5. Inhibition of TNF-α mediated NF-κB translocation by I-κBα mutants at TGase 2 targeting sites. Immunocytochemical staining of NF-κB using anti-p65 antibody was performed in EcR293/TG cells. Cells were transfected with wild type (WT) or mutant I-κBαs at TGase 2 targeting sites including K177 and Q266. Mutant at Q255 was used as a negative control. After the 24 hrs, all groups of cells were treated with 1 μg/ml of tetracycline to induce TGase 2 for 24 hrs. Following to the induction, cells were treated with or without TNF-α (100 ng/ml) for 2 hrs. Some NF-κB was detected in nucleus by TGase 2 induction (white arrowhead in I-κBα (WT)). TNF-α treatment with TGase 2 induction showed strong NF-κB translocation in nucleus than TNF-α alone (white arrowhead in I-κBα (WT)). I-κBα mutants containing TGase cross-linking sites markedly reduced NF-κB translocation to nucleus, which resulted in increase of NF-κB detection in the cytosol (red arrowheads in I–κBα (K177G) and (Q266G)). A randomly selected mutant at C-teminus of I-κBα shows no effect on NF-κB translocation (white arrowhead in I-κBα (Q255G)).

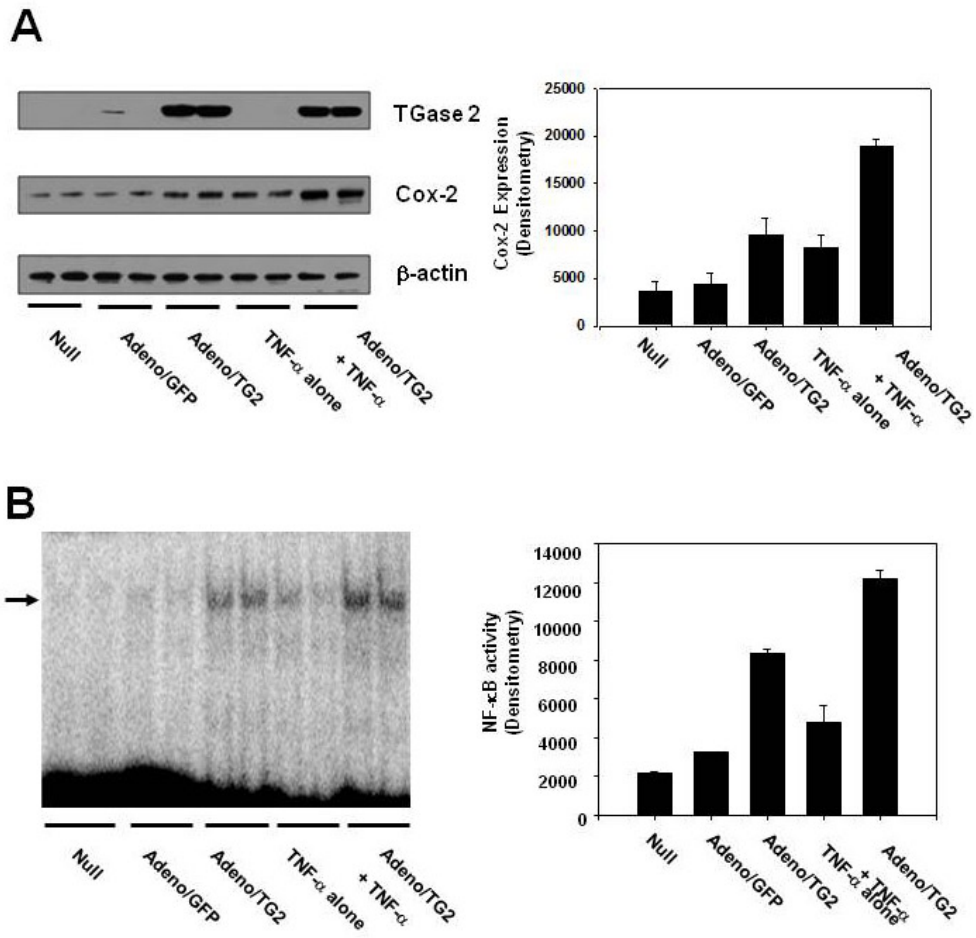

Figure 6. TGase 2 induction using adenovirus system extends TNF-α induced NF-κB activity *in vivo. (*a) 120 hrs after TNF-α treatment following to Adeno/TG2 or mock viral vector infection, the liver tissues from mice were homogenized, and western blotting was performed against TGase 2 and cyclooxigenase 2 (Cox-2). The double treatment group (Adeno/TG2 + TNF-α) showed the highest expressional level of Cox-2. *(*b) For EMSA assay, nuclear extracts corresponding to the analyses above were incubated with a ^{32}P-labeled NF-κB probe, followed by electrophoresis and autoradiography. NF-κB activity was quantitated using densitometry. TNF-α only treatment group showed decreased NF-κB activity that is less than adeno/TGase 2 group. However, the double treatment group showed two times higher NF-κB activity than TNF-α only treatment group showed.

1588 as compared to *TGM2*-expressing cell lines (NCI-H1299 and HCC-1195). The results of down-regulation and over-expression of *TGM2* in non-small cell lung carcinoma (NSCLC) cells also suggest a positive correlation between cisplatin sensitivity and *TGM2* inhibition (35).

5.2. The prolongation effect of TGase 2 on TNF-α-induced NF-κB activation

TGase 2 catalyzes protein cross-linking, and TGase 2 has been recognized as an important factor in matrix formation and cell adhesion. However, the function of TGase 2 in inflammation etiology is not clear. TGase 2 activation results in depletion of free I-κBα through protein polymerization. Here, we demonstrated that induction of TGase 2 reduces the levels of free I-κBα in the cytosol, and this depletion correlates with NF-κB activation (Figs. 1 and 2). We therefore investigated whether TGase 2 over-expression prolongs the activation of NF-κB through a canonical inducer of inflammation. Here, we used TNF-α treatment as an inflammatory stress. Interestingly, TNF-α-induced NF-κB activation gradually increased as TGase 2 expression increased. Moreover, cells in which TGase 2 was over-expressed exhibited sustained NF-κB activation in response to TNF-α as compared control cells (Figure 3). The levels of I-κBα in un-induced cells that did not overexpress TGase 2 recovered to normal levels within 30 min to 1 hr of TNF-α treatment (Figure 2) (9). Surprisingly, I-κBα levels failed to recover from TNF-α treatment even after 24 hrs in the presence of TGase 2 expression (Figure 2). This is an intriguing finding because increased TGase 2 expression is often reported in many inflammatory diseases. In addition, TGase 2 expression has been reported in diseases with associated inflammation such as Parkinson's disease, Alzheimer's disease, Huntington's disease, diabetes mellitus, atherosclerosis and cancers. We cannot determine based on the current results

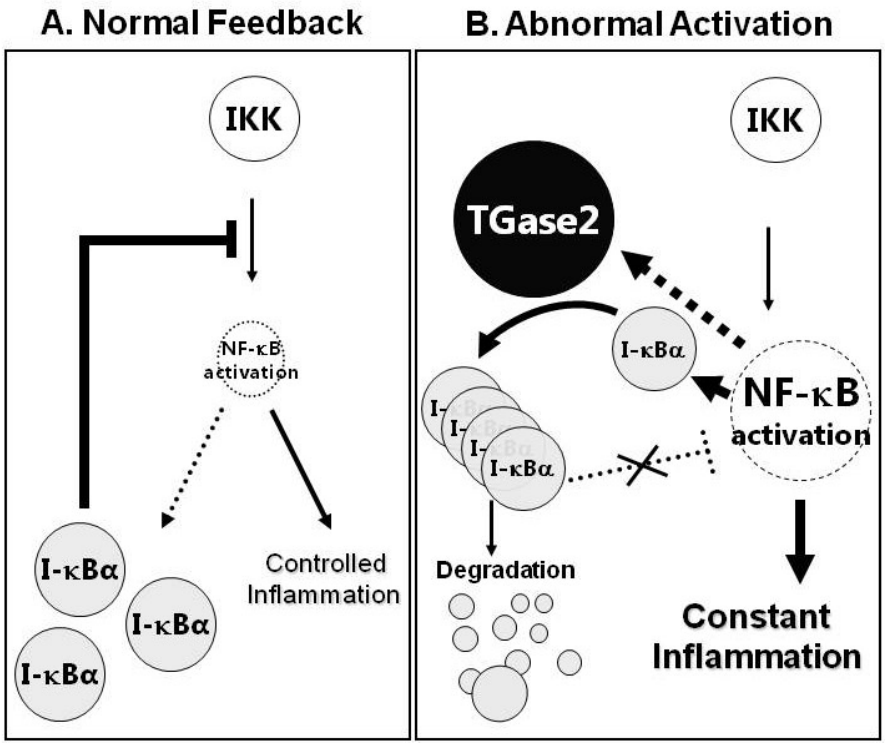

Figure 7. Proposed role of TGase 2 in NF-κB activation (Ouroboros theory of NF-κB activation). In normal condition, NF-κB activation by IKK signaling has feedback mechanism by induced I-κBα. Although TGase 2 can be also induced by NF-κB, the induced I-κBα may be enough to block the pathway in normal condition. However, in case of induced TGase 2 situation before IKK signaling by epigenetic change, TGase 2 appears to deplete I-κBα efficiently, which results in elongated period of IKK dependent NF-κB activation.

whether increased TGase 2 expression leads to a constitutive inflammatory state or constitutive activation of inflammatory signals. However, the results suggest that once TGase 2 expression is increased, it may be sufficient to exacerbate TNF-α induced inflammation.

5.3. Reduction of TNF-α induced NF-κB activation by TGase 2 inhibition

Inhibition of TGase 2 by CTM reversed the kinetics of I-κBα recovery in the presence of TNF-α in a dose-dependent manner, resulting in a reduction of TNF-α-induced NF-κB activity (Figure 1A). Previously we identified the amino acid residues of I-κBα that are targeted by TGase 2 (7). Transient expression of dominant negative mutants of I-κBα in which these TGase 2 cross-linking sites, namely lysine 177 and glutamines 266 and 267, were mutated, reversed NF-κB activity to 40% of that seen in the presence of TNF-α, whereas expression of wild-type I-κBα reduced NF-κB activity by only 10% (Figure 4). These results suggest that TGase 2 in involved in the depletion of I-κBα in response to activation of a canonical pathway of NF-κB activation. In other words, TGase 2 may be responsible for up to 50% of the NF-κB activity induced by TNF-α. These results were confirmed by immunoblot analysis. Furthermore, the vast majority of NF-κB (p65) localized to the nucleus following TNF-α treatment in the presence of wild type I-κBα, co-localizing precisely with DAPI-stained nuclei (Figure 5). In contrast, in the presence of the TGase 2 targeting site mutants of I-κBα (K177G and Q266G), approximately 50% of cellular NF-κB remained in the cytosol after TNF-α treatment (Figure 5).

5.4. Exacerbation of TNF-α-mediated inflammation in the mouse liver by TGase 2 over-expression

To determine whether the synergistic increase in NF-κB activation in cultured cells by TGase 2 and TNF-α also occurred *in vivo*, TGase 2 expression was induced in mice using a recombinant TGase 2-expressing adenoviral vector. Following infection with Adeno/TG2, TNF-α was injected via the mouse tail vein, and then the inflammatory response in the liver in the presence or absence of TGase 2 over-expression was assessed (Figure 6). TGase 2 levels in hepatocytes were markedly increased 120 hrs after infection. Analysis of liver tissue revealed increased NF-κB activity as well as Cox-2 expression in Adeno/TG2-infected animals after 120 hrs (Figure 6). Importantly, NF-κB activity was dramatically increased and markedly sustained for over 120 hrs in the livers of Adeno/TG2-infected animals treated with TNF-α, whereas livers from control animals treated with TNF-α alone exhibited less NF-κB activity than Adeno/TG2-infected animals (Figure 6). TGase-2 and NF-κB were expressed simultaneously within hepatocytes, which suggested that the prolongation of NF-κB activation was due to TGase 2 expression. These

results suggest that increased expression of TGase 2 is involved in the progression of inflammatory diseases, and that inhibition of TGase 2 may be a promising intervention for reducing long term inflammation.

5.5. Model for TGase 2 in NF-κB activation

Constitutive NF-κB activation has been reported in chronic inflammatory diseases, making it a potential target in the development of therapeutics (1). However, constitutive NF-κB activation is not always accompanied by constitutive I-κBα phosphorylation (8). Although several other kinases that deplete free I-κBα have been identified (1), there are other possible mechanisms of induction of NF-κB activity in the absence of I-κBα phosphorylation. The PEST sequence (proline (P), glutamic acid (E), serine (S), threonine (T)) in the C-terminal region of I-κBα plays a very important role in the rapid turnover of I-κBα in the absence of phosphorylation (38). Interestingly, regardless of mechanism, depletion of I-κBα is the main cause of NF-κB activation, as I-κBα is the most effective negative regulator of NF-κB (9). Thus, the various pathways of NF-κB activation must work cooperatively to achieve efficient induction of anti-inflammatory genes. It is unclear whether increased TGase 2 expression is due to NF-κB activation, or vice versa, in disease states. Here, we showed that increased TGase 2 expression exacerbates TNF-α-induced inflammation. Interestingly, constitutive NF-κB activation often accompanies increased TGase 2 expression in inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis, and allergic conjunctivitis, as well as certain cancers such as breast cancer and neuroblastoma (11). TGase 2 expression can be induced by various stresses as well as epigenetic modifications such as de-methylation of the *TGM2* promoter (35). Here, we demonstrated that TGase 2 induction is required for sustained NF-κB activation, which in turn delays the resolution of inflammation. We propose an 'Ouroboros theory of NF-κB activation' (review submitted), in which NF-κB activation induces TGase 2 expression, which in turn exacerbates inflammation (Figure 7). Thus, an inflammatory inducer such as TNF-α may trigger significant activation of NF-κB, particularly in individuals in which TGase 2 expression is elevated.

6. ACKNOWLEDGEMENTS

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Abbreviations: TGase 2, transglutaminase 2; NF-κB, nuclear factor kappa B; I-κBα.Inhibitor of nuclear factor kappa-B alpha; TNF-α, tumor necrosis factor alpha; IKK, I-κB kinase; LPS, Lipopolysaccharide; CTM, cystamine; WT, wild type; Tet, tetracycline.

Key Words: transglutaminase 2, I-κBα, NF-κB, inflammation, TNF-α, Review

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