Differential activation of nuclear factor-κB by tumour necrosis factor receptor subtypes. TNFR1 predominates whereas TNFR2 activates transcription poorly

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Abstract Tumour necrosis factor-α (TNF-α) signals though two receptors, TNFR1 and TNFR2. TNFR1 has a role in cytotoxicity, whereas TNFR2 regulates death responses or proliferation. TNF activates pro-inflammatory transcription factor nuclear factor-κB (NF-κB) by uncertain signalling mechanisms. Here we report the contribution of each TNFR towards the NF-κB activation processes. In human cells expressing endogenous or exogenous TNFR2, in addition to TNFR1, we found both TNFRs capable of activating NF-κB, as measured by IκBα (inhibitor of NF-κB) degradation, electrophoretic mobility shift assay and NF-κB gene reporter assays. TNFR2 activation did not degrade IκBβ. However, TNF-effects on NF-κB activation occurred predominantly through TNFR1, with TNFR2 activating the transcription factor poorly.

Key words: Cytokine; Receptor; Subtype; Signal transduction; Kinase; Tumour

1. Introduction

Tumour necrosis factor-α (TNF) is a pro-inflammatory cytokine important for functioning of the immune system, tissue homeostasis and embryonic development. TNF exerts its cellular effects such as differentiation, proliferation and cell survival or cytotoxicity through signalling of two distinct TNF receptors, TNFR1 and TNFR2 [1]. Type I TNFR, TNFR1 (also known as p55TNFR, TNFRSF1a, CD120a), has a molecular mass of 50–60 kDa, whereas TNFR2 (also known as p75TNFR, TNFRSF1b, CD120b) has a molecular mass of 70–80 kDa. Both receptors are based on four cysteine-rich repeat domains in their extracellular region and belong to the extended TNF/nerve growth factor receptor superfamily. Intracellularly, TNFR1 and TNFR2 are structurally different, indicating that the receptors have distinct biological functions. TNFR1 intracellular sequence contains a death domain motif that is found in some receptors of the TNF superfamily and allows the recruitment of death domain-containing adapter proteins implicated in cell death signalling in many cell types [2]. Unlike TNFR1, TNFR2 does not contain a death domain motif but still recruits adapter proteins to perform the receptor's intracellular signalling activities. As well as being proliferative in some cell types, engagement of TNFR2 has been found to have pro-apoptotic effects of varying magnitude [3]. TNFR2-mediated apoptosis has been described as by having a direct effect [4–6], as a ligand passing effect [7,8], as due to endogenous induction of TNF [9], and as a co-operative effect with non-triggered TNFR1 [1].

TNF exerts its effects through TNFR1 and TNFR2, which signal and initiate activation of a multitude of kinase pathways [10]. Studies have demonstrated that TNF can also activate the NF-κB transcription factor that serves as a critical regulator of inducible expression of many genes [11]. Transcription factor NF-κB belongs to a family of ubiquitously expressed Rel-related transcription factors and is activated by proteolytic degradation of inhibitor of NF-κBs (IκBs) in the cytoplasm of the cell. NF-κB is localised in the nucleus and sequestered in the cytoplasm of most cells. Sequestration of NF-κB is regulated by the IκB proteins which binds to NF-κB and inhibits its transportation to the nucleus to activate transcription [12].

NF-κB binds an inhibitory protein, IκBα, which regulates DNA binding and intracellular localisation of NF-κB [13,14]. On binding of TNF to TNFRs, the signal may be transduced through the TNFR-associated death domain protein (TRADD) [15], TNFR-associated factor 2 (TRAF2) [16] and NF-κB-inducing kinase (NIK) [17], which eventually activates the IκB kinases (IKKα, IKKβ and IKKγ) [18–22]. Activated IKKα and IKKβ phosphorylate serine residues in the N-terminal region of IκB, resulting in the release of NF-κB from IκB, allowing NF-κB to translocate to the nucleus and promote gene transcription. IκBα becomes phosphorylated in response to TNF treatment and leads to IκBα being rapidly degraded. IκBβ and IκBε are also degraded to some extent [12].

The mechanism by which TNFRs can activate NF-κB is not fully understood. TNFR1 through its FADD/TRADD interaction, is capable of NF-κB activation. Much work has

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzene sulfonyl fluoride; EMSA, electrophoretic mobility shift assay; (e)GFP, (enhanced) green fluorescent protein; hrGFP, humanised renilla green fluorescent protein; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; NIK, NF-κB-inducing kinase; RIP, receptor-interacting protein; TNF, tumour necrosis factor-α; TNFR, TNF receptor; TNFR1, type I 55 kDa TNFR; TNFR2, type II 75 kDa TNFR; TRAF, TNFR-associating factor

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focused on the ability of TRAF-2 adapter protein to activate NF-κB [23]. TRAF-2 binds directly to TNFR2 and indirectly to TNFR1 so both receptor isotypes are capable of NF-κB transcriptional activity. Upstream kinases that activate NIK are still being delineated and the ability of each TNFR to activate such kinases is presently uncertain. Indeed, a role for NIK in TNFR triggering of NF-κB has been called into question as NIK-deficient mice are still capable of TNF-stimulated NF-κB activity [24,25]. More recently, a role for receptor-interacting protein (RIP), which contains kinase capabilities, has been shown as a means for NF-κB activation processes [23]. RIP and TRAF-2 either directly or indirectly interact with both TNFRs [26]. RIP has also been suggested to be important in signalling switching by both TNFRs between c-Jun N-terminal kinase (JNK) activity and NF-κB transcription activation mechanisms, resulting in proliferative or apoptotic responses [27].

Surprisingly, little work has addressed the relative contribution each of the TNFRs has towards TNF-induced NF-κB activation, with only indirect assessments [28–37]. This is partly due to the experimental limitations of efficiently activating TNFR2 which, unlike TNFR1, is poorly activated by soluble TNF (the form most commonly used experimentally) [38]. Efficient activation of TNFR2 requires membrane-bound TNF (as is presented in vivo) but can be overcome in the laboratory with the use of agonistic antisera, such as MR2-1 mAb as used here. TNFR subtype-selective stimulation was also achieved with mutated protein ('mutein') TNFs (R32WS86T TNFR1-specific mutein 'R1-TNF' or D143-NA145R TNFR2-specific mutein 'R2-TNF' [39]). Here, not only by measuring exogenously generated TNFR2 actions, but with endogenously expressed TNFRs too, we evaluated the relative contribution of efficiently activated TNFR1 and TNFR2 towards NF-κB activation processes.

2. Materials and methods

2.1. Cells

HEK293 wild-type cells were a gift from Mike Ashford, Aberdeen, UK, and KYM-1 cells were a gift from Terje Espevik, Trondheim, Norway. Stably expressing HeLa-p75TNFR cells were generated by injecting human p75TNFR (provided by Werner Lesslauer, Basel, Switzerland) and pBABE hygromycin-resistant cDNAs into HeLa cells with an Eppendorf InjectMan microinjection and micromanipulation system as described [40]. Stably transfected HEK293 cells containing 3×NF-κB-luciferase reporter construct (provided by Andrew Paul, Strathclyde University, UK, and Ron Hay, St. Andrew's, UK [41]), or NF-κB-luciferase plus p75TNFR expression, were created by transfection of the cDNA with pBABE hygromycin resistance cDNA, using Lipofectamine transfection reagent (Gibco BRL). Colonies of stable transfectants were selected in Dulbecco's modified Eagle's medium (DMEM) containing 100 μg/ml hygromycin-B (Boehringer Mannheim). Stably transfected cell lines were maintained in a culture of DMEM (1000 mg/ml glucose/no sodium pyruvate) with 10% foetal calf serum (Helena Biosciences), 4 mM L-glutamine and 50 U/ml penicillin, 50 μg/ml streptomycin, using standard sterile techniques. KYM-1 cells were grown in RPMI 1640 supplemented with 4 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Transient transfections of cells with 0.1 μg NF-κB-dependent reporter, 0.1 μg TNFR2 cDNA or 1 μg of peGFP (enhanced green fluorescence protein; provided by Gwyn Gould, Glasgow, UK) was introduced into the cells by Lipofectamine according to the standard transfection protocol for 3 h before reintroduction to serum-containing medium. After an overnight incubation, the culture media was replaced with fresh DMEM containing 10% foetal calf serum. 48 h post-transfection, the cells were treated with various types of stimuli before being assayed as described below, or viewed directly on a Bio-Rad μradiance confocal system, as was the case with cells transfected with the peGFP cDNA construct.

2.2. Fluorescence-activated cell sorting (FACS) analysis

HeLa cells stably over-expressing TNFR2, KYM-1 cells and HEK293 cells stably expressing NF-κB-luciferase reporter construct or NF-κB-luciferase plus TNFR2 were grown to approximately 70% confluency and dissociated from their culture vessels with 2 ml of trypsin-free cell dissociation solution (Sigma). Cells were analysed for TNFR1 or TNFR2 content using mouse monoclonal anti-human htr-9 or utr-1 respectively, as described [42].

2.3. JNK activity

JNK activity was measured by assessing phosphorylation of its substrate c-Jun (5-89) linked to a glutathione-*S*-transferase fusion protein as described [40].

2.4. Confocal fluorescence microscopy

All cells were treated for the indicated time with either 50 ng/ml recombinant human TNF, R32WS86T mutated protein TNF (TNFR1-specific 'mutein' termed R1-TNF [39]), D143NA145R TNF (TNFR2-specific mutein termed R2-TNF) or with TNFR2-specific agonistic MR2-1 monoclonal antibody (1 μg/ml)(kindly provided by Wim Buurman, Maastricht, Netherlands) [43]. Cells were then fixed and analysed as described previously [40].

2.5. Western analysis

Cells were treated with stimuli for the indicated times before Western analysis as previously described [40].

2.6. EMSA (electrophoretic mobility shift assay)

For preparation of nuclear extracts, cells were seeded into 6-well plates at a concentration of 2×10^5 cells/ml culture medium and incubated overnight prior to a 1 h treatment with 50 ng/ml recombinant TNF, R1-TNF, R2-TNF or R2-TNF plus 1 μg/ml MR2-1 antibody. Cells were washed in ice-cold PBS and harvested in 100 μl buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2% Nonidet P-40, 0.5 mM AEBSF, 1 mM sodium orthovanadate, 1 mM NaF, 0.1 mM aprotonin), centrifuged and the cell pellet resuspended in 50 μl of buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol) and incubated on ice for 10 min with occasional mixing. Samples were centrifuged and the concentration of nuclear extract contained in the supernatant was determined by protein assay (Bio-Rad). 5 μg of nuclear extract was used for the EMSA. High-performance liquid chromatography-purified NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGCGCCTGGGAAAGTCCCCT-CAACT-3′), obtained from Oswel laboratories, Southampton, UK, was end labelled with [^{32}P]γ-ATP using T4 polynucleotide kinase. 5 μg of nuclear extract, 2 μg poly (dI-dC), 200 ng of labelled probe and water were incubated in a final volume of 10 μl for 20 min at room temperature. The samples were separated by 17% PAGE in TBE buffer.

2.7. NF-κB luciferase or GFP activity measurements

HEK293 cells, HeLa cells or HeLa-TNFR2 stable expressing cells were transiently transfected with NF-κB-luciferase reporter construct using the Lipofectamine transfection protocol outlined above, with receptor-stimulated luciferase transcription being measured 48 h post-transfection. Stably expressing NF-κB-luciferase or NF-κB-luciferase plus TNFR2 HEK293 cells were plated into 24-well plates at a density of 1×10^5 cells/ml culture media. The cells were incubated for 24 h to reach 80% confluency before a 6 h treatment with 50 ng/ml recombinant TNF, R1-TNF, R2-TNF or R2-TNF plus 1 μg/ml MR2-1 antibody. NF-κB-stimulated luciferase activity was detected by washing the cells twice, with ice-cold PBS, adding 200 μl of ice-cold lysis buffer (25 mM Tris–phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 15% glycerol) and incubating on ice for 5 min. Cell extracts were scraped into 1.5 ml Eppendorf tubes, centrifuged to pellet cell debris and 100 μl of the supernatant used to measure luciferase induction by injecting it with an equal volume of luciferase buffer (lysis buffer containing 1 mM ATP, 0.25 mM luciferin (Molgfecular Probes), 1% BSA) in a Berthold LB9501 Lumat luminometer. Novel NF-κB–humanised renilla GFP (hrGFP) reporter construct cDNA (Stratagene) was transfected into HeLa-TNFR2 cells.

24 h post-transfection, TNFR-induced GFP expression was measured with a fluorescence/visible light microscope set-up or by FACS analysis, indicating cells which report NF-κB-stimulated gene activity.

3. Results

3.1. Cellular TNFR subtype levels

HeLa human cervical epithelial carcinoma cells and HEK293 human embryonic kidney cell lines express TNFRs which are predominately of the TNFR1 subtype. KYM-1 human rhabdomyosarcoma cells endogenously express high amounts of both TNFR1 and TNFR2, as judged by htr-9- and utr-1-specific monoclonal FACS analysis respectively and isoform-specific [^{125}I]TNF radioligand binding experiments [40]. HeLa-TNFR2 cell line was generated to stably over-expresses exogenous TNFR2, so as to maximise TNFR2 signalling responses. HeLa, HeLa-TNFR2 and KYM-1 cells possess approximately 200, 60 000 and 12 000 TNFR2 receptors/cell respectively. HeLa and HeLa-TNFR2 express some 3000–4000 TNFR1 receptors/cell, whereas KYM-1 cells express similar levels of TNFR1 with 8000 TNFR1 receptors/cell [40].

3.2. JNK and NF-κB activation by TNFRs

Soluble TNFs efficiently stimulate TNFR1, however poorly

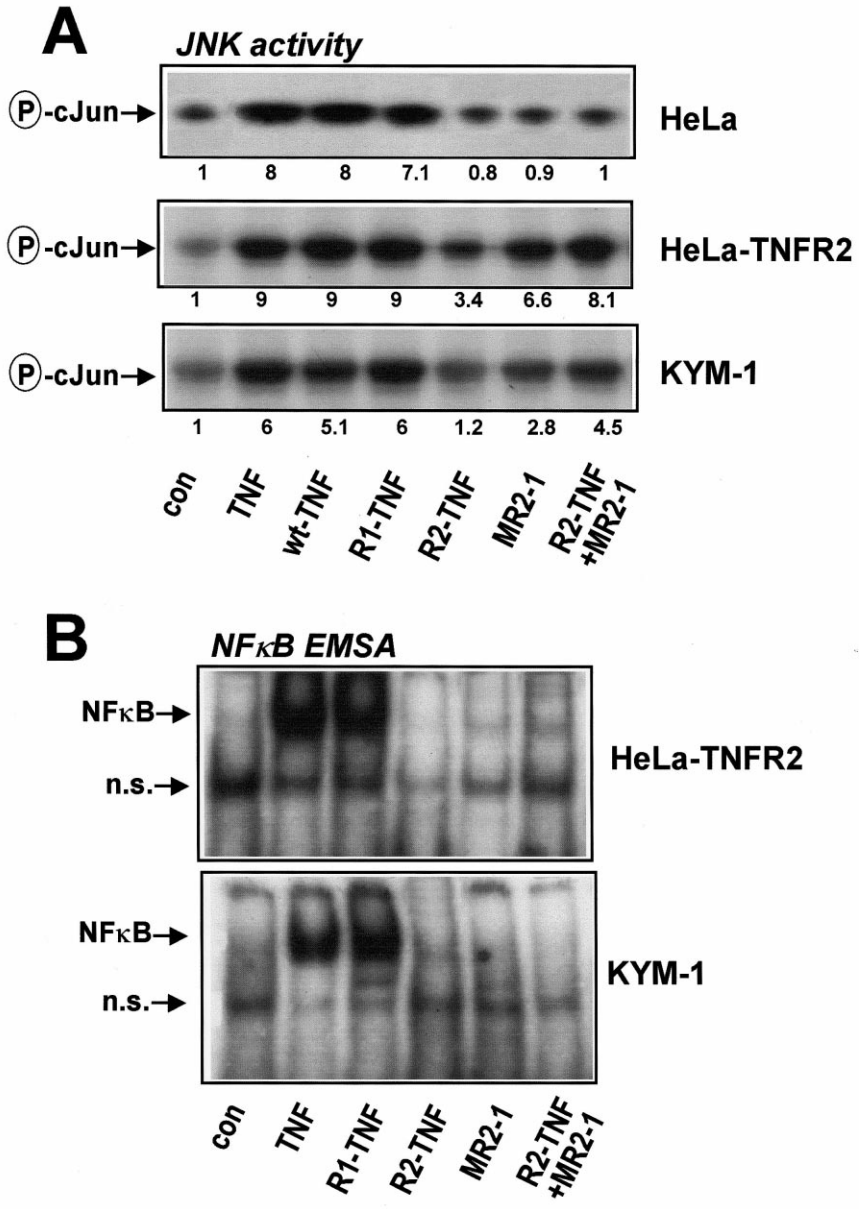

Fig. 1. TNFR1- and TNFR2-stimulated JNK and NF-κB DNA-binding activities. A: Wild-type HeLa, HeLa-TNFR2 stable cell line or KYM-1 cell lines were analysed for TNFR1- and TNFR2-stimulated JNK activity as described in Section 2. TNF (from a commercial source), wild-type TNF (wt-TNF) or mutein TNFs (50 ng/ml) stimulation of JNK activity after 15 min treatment. MR2-1 TNFR2-agonistic monoclonal antisera was also added where indicated (2 μg/ml). The results shown are from experiments that are representative of a least three independent assessments. B: HeLa-TNFR2 stable cell line or KYM-1 cell lines were analysed for TNFR1- and TNFR2-stimulated NF-κB DNA-binding activity by EMSA as described in Section 2. TNF, wt-TNF or mutein TNFs ± MR2-1 (at the above concentrations) stimulation of JNK activity after 60 min treatment. The results shown are from experiments that are representative of a least three independent assessments with essentially the same findings.

activate TNFR2 which requires the membrane-bound form of TNF for maximal stimulation, however, TNFR2-specific agonistic antisera can be used to mimic membrane-bound TNF and stimulate TNFR2 [38]. To test the ability of our receptor subtype-specific agonistic muteins and antisera, we measured activation of the stress kinase JNK by TNFR1 or TNFR2 (Fig. 1A). Stimulation of TNFR1 leads to efficient JNK activation, whereas TNFR2 is capable of efficiently activating JNK activity to maximal levels as judged with 200 nM anisomycin stimulus (not shown). Significant TNFR2-mediated JNK activation is observed in HeLa-TNFR2 and KYM-1 cells (which possess reasonable levels of TNFR2) using the R2-TNF mutein and MR2-1 agonistic antisera stimuli (Fig. 1A). The TNFR-induced JNK activity assays also display the

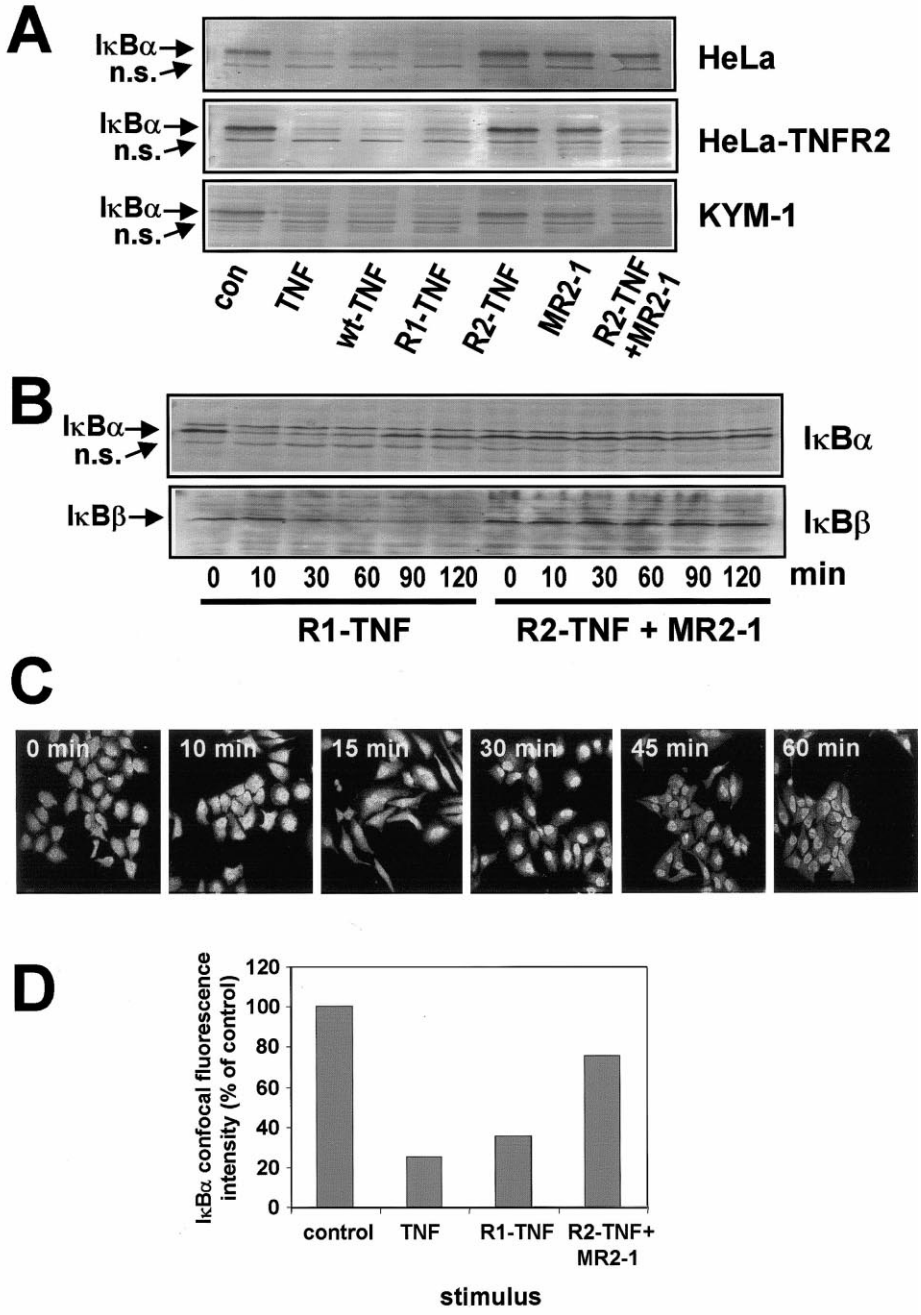

Fig. 2. TNFR1- and TNFR2-stimulated IκB degradation. A: Wild-type HeLa, HeLa stably expressing TNFR2 and KYM-1 cells were stimulated for 30 min by TNF (50 ng/ml) and/or MR2-1 antisera (2 μg/ml) before measurement of IκBα protein degradation by Western analysis, as described in Section 2. A non-specific (n.s.) band generated by the secondary detection antisera is also indicated for comparison. B: Time course of IκBα and IκBβ protein degradation stimulated by TNFR1 or TNFR2 in HeLa-TNFR2 cells using 50 ng/ml R1-TNF or R2-TNF plus 1 μg/ml MR2-1 antisera respectively. The results shown are from an experiment that is representative of a least three independent assessments. C: HeLa-TNFR2 cells were stimulated the indicated times with 50 ng/ml TNF and subcellular IκBα protein degradation measured by confocal microscopy, as described in Section 2. D: Quantified cytoplasmic confocal fluorescence intensities of IκBα protein degradation stimulated by TNFR1 or TNFR2 using 50 ng/ml R1-TNF or R2-TNF plus 2 μg/ml MR2-1 antisera respectively. The results shown are from an experiment that is representative of a least two independent assessments.

reduced ability of soluble TNFRs to activate TNFR2 and the requirement for agonistic antisera to efficiently activate TNFR2-mediated JNK activation.

EMSA DNA-binding assays were used to study the activation of NF-κB protein when induced by TNF or subtype-specific muteins. Fig. 1B shows the EMSA results of NF-κB activation in KYM-1 cells and HeLa cells over-expressing TNFR2 (HeLa-TNFR2), when the cells were treated with recombinant TNF, TNFR1-specific mutein TNF, TNFR2-specific mutein TNF alone or in combination with TNFR2-agonistic antibody MR2-1. Both cell types showed a specificity of NF-κB DNA binding complexes, and was determined by competition with a 10-fold excess of unlabelled probe, but not randomised-sequence probe (not shown). The shift of the NF-κB specific band indicated NF-κB DNA-binding activity in both cell lines by treatment with TNF and TNFR1-specific mutein TNF. There was little observed effect in either cell when TNFR2 activation was performed by mutein and/or antisera stimuli ($<5\%$ of the activity observed with TNF).

3.3. IκBα and IκBβ activation

Efficient stimulation of TNFR2 is achieved by stimulatory agonistic antisera used either alone or in combination with TNFR2-specific mutein TNF. In Fig. 2, we observed the action of maximal stimulatory concentrations of TNF, R1-TNF, R2-TNF or R2-TNF plus MR2-1 antibody on the activation/degradation of IκBα and IκBβ. Fig. 2A,B shows the ability of the TNFR isoform-specific stimuli to effect IκBα degradation as measured by changes in protein abundance of IκBs. A useful non-specific immunoreactive band helps to confirm equal protein loading and the specificity of the IκBα antisera band. HeLa-TNFR2 and KYM-1 cells were treated for 30 min with maximal concentrations of various treatments and it was noted that both cell types showed IκBα degradation at 30 min treatment with recombinant TNF (from a commercial source), wild-type TNF and R1-TNF. In HeLa-TNFR2 and KYM-1 cells, but not wild-type HeLa cells, there was degradation of IκBα by TNFR2-specific mutein and agonistic antisera MR2-1. TNFR2 degraded IκBα, but not to the same complete extent as seen with TNFR1. Curiously, KYM-1 cells showed slightly better TNFR2-induced IκBα degradation than HeLa-TNFR2 cells (Fig. 2A), but less TNFR2-induced NF-κB DNA binding capability in the EMSA assay (Fig. 1B).

These cell lines were treated over varying times with TNFR1-specific mutein TNF or TNFR2-specific mutein TNF plus agonistic antibody MR2-1, to observe at which times each TNFR isotype was having an effect. Treatment of the HeLa-TNFR2 cell type with R1-TNF revealed a transient pattern of IκBα degradation observed within 10 min. IκBα protein returned to control levels after 90 min of TNFR1 activation with the R1-TNF treatment. The IκBα degradation in HeLa-TNFR2 cells when treated with TNFR2-specific stimuli was much less pronounced, with IκBα protein being slightly degraded by TNFR2, but if anything occurring in a more delayed time-frame than observed with TNFR1 stimulation (Fig. 2B). HeLa-TNFR2 cells were treated with similar treatments to observe their effect on IκBβ degradation. TNFR1 mutein showed marked degradation of IκBβ over 120 min of treatment, with degradation occurring less rapidly than is seen with IκBα protein, but no effect on IκBβ degradation was observed when cells were treated with TNFR2-specific mutein over the same time course (Fig. 2B). These observations were confirmed using FACS analysis to measure IκBβ degradation; furthermore, similar patterns of TNFR1 and TNFR2-induced IκB protein degradations were observed in the KYM-1 cell line (data not shown).

Confocal microscopy was used to measure ligand-induced subcellular redistribution and degradation of IκBα protein. As can be seen in HeLa-TNFR2 cells (Fig. 2C,D), treatment with TNF or R1-TNF mutein show degradation of specific IκBα immunofluorescence staining from the cytoplasm of the cells after 30 min treatment. When cells were treated with TNFR2-specific stimuli, less cytoplasmic IκBα degradation was observed, even after 60 min of treatment (not shown). From these results, it would indicate that signalling mostly through the TNFR1 by TNF and TNFR1-specific mutein TNF is required for the noted degradation of IκBα protein. It would appear that the maximal amount of TNF-induced IκBα degradation requires both TNFRs to be activated fully, with TNFR1 activation not giving 100% of the TNF response, and TNFR2 activation always giving some minimal effect. This is in spite of TNFR2 being fully active when measuring TNFR2-mediated JNK activation in HeLa-TNFR2 and KYM-1 cells (Fig. 1A).

3.4. TNFR-stimulated NF-κB transcription

To observe the level of NF-κB gene transcriptional activity, we transiently transfected NF-κB-luciferase gene reporter construct into our cells and assayed for TNFR-stimulated luciferase activity (Fig. 3A). We also looked at transient transfection of HEK293 human embryonic kidney cells which give enhanced transfection and transcription of cDNA vectors. HEK293 cells express endogenous TNFR1 protein. Also included in our transient transfection studies was HEK293 cells stably expressing human TNFR2 (HEK293-TNFR2). Transient transfection of NF-κB reporter construct into HeLa-TNFR2, HEK293 or HEK293-TNFR2 cells revealed that the majority of NF-κB gene transcription is once again stimulated by TNFR1, with a lesser amount of activation (consistently $<15\%$) stimulated by TNFR2. Even though TNFR2 stimulation could efficiently trigger JNK activity, we wanted to be sure that low levels of TNFR2 expression (as is the case with parental HeLa and HEK293 cells) was not the cause for the lesser NF-κB activity observed by TNFR2 compared to TNFR1-stimulated responses.

Unfortunately, our attempts to transiently transfect KYM-1 cells were unsuccessful. We discovered that KYM-1 cells did not produce high levels of NF-κB activity, so we investigated the transfection efficiency of all our cell lines with peGFP (red shifted variant, eGFP) cDNA. The peGFP construct was easily visualised with confocal microscopy when excited with blue light (excitation 488 nm/emission 509 nm). HeLa-TNFR2 cells showed on average $\sim 60\%$ transient transfection efficiency up to 48 h post-transfection, however, KYM-1 cells showed little (typically $<1\%$) transfection efficiency. HEK293 cells showed on average $>90\%$ transfection efficiency with peGFP and so transient transfection of the NF-κB-luciferase construct would pose no significant problems with either the HeLa TNFR2 stable cell line or wild-type HEK293 cell line. Efforts to transiently transfect KYM-1 cells were fruitless. Equally, repeated efforts to stably transfect NF-κB-luciferase reporter into KYM-1 cells resulted in zero cell survival. A similar pattern of receptor isotype-specific NF-κB

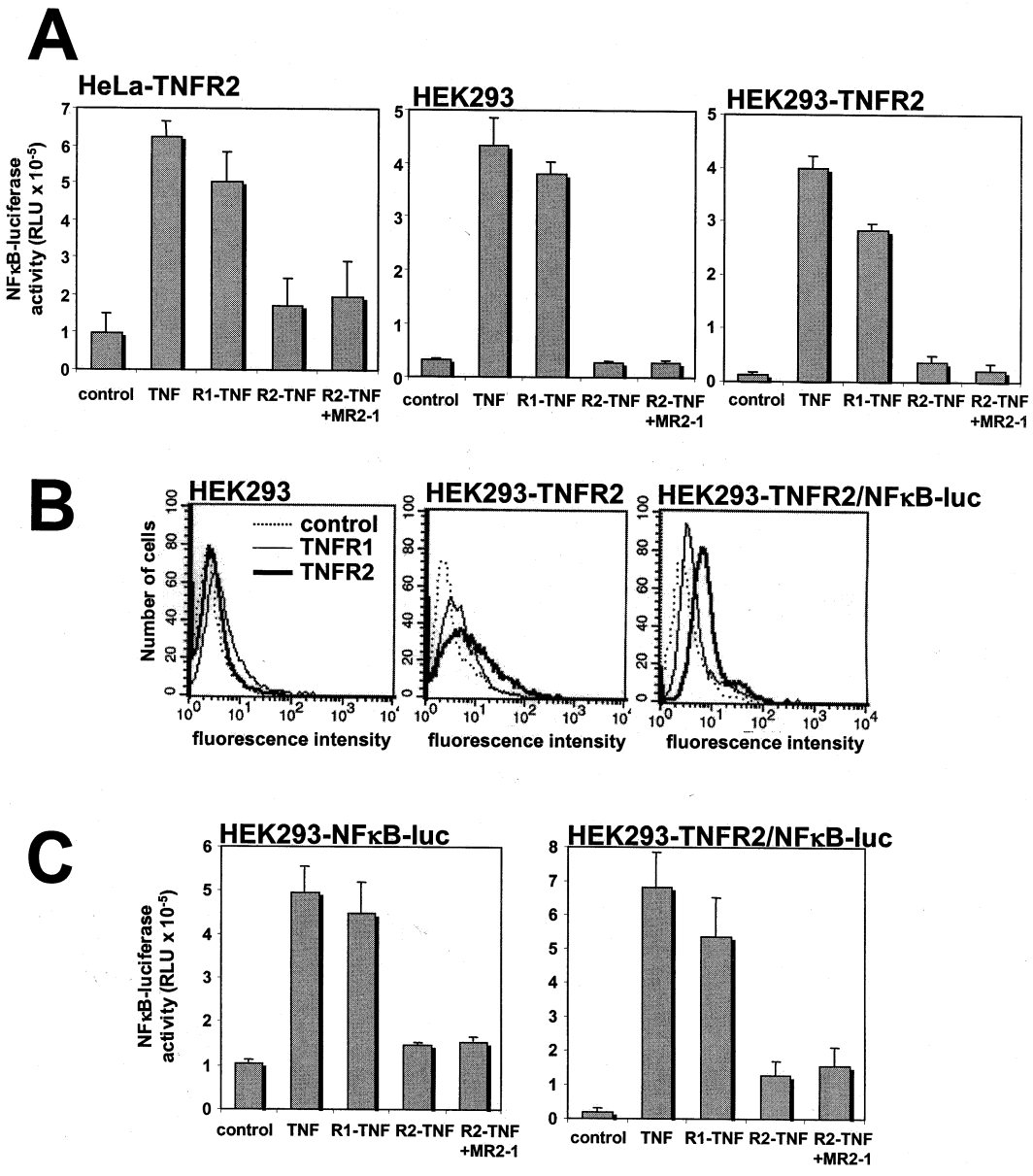

Fig. 3. TNFR1- and TNFR2-stimulated NF-κB-luciferase gene transcription activity. TNFR1- and TNFR2-stimulated NF-κB-luciferase reporter construct measurements (A) by transient transfection reporter construct measured 48 h post-transfection, as described in Section 2, or (C) in stable NF-κB-luciferase reporter construct-containing cells. The TNFR1- and TNFR2-specific stimuli, at the same concentrations as used in Fig. 1, were applied for 6 h before NF-κB-driven luciferase measurement in relative light units (RLU). The results shown are the mean ± S.D. of quadruplicate determinations from experiments that are representative of a least three independent assessments. None of the TNFR2-stimulated responses above control report levels were found to be statistically significant ($P > 0.05$, Student's t-test). B: FACS analysis of TNFR1 and TNFR2 cell surface protein expression of parental HEK293 cells or HEK293 cells stably expressing TNFR2 or TNFR2 plus NF-κB-luciferase reporter construct.

activation was measured by transient transfection of NF-κB-luciferase reporter construct into HEK293 cells or HEK293-TNFR2 cells, with very little detectable TNFR2-stimulation of NF-κB-luciferase activity (Fig. 3A). The majority of stimulation of NF-κB-luciferase being mediated by TNFR1 in HEK293 or HEK293-TNFR2 cells.

As KYM-1 cells are vulnerable to the toxic actions of Lipofectamine, we chose to stably transfect HEK293 cells and HEK293-TNFR2 cells with NF-κB-luciferase reporter construct, as a comparison to HeLa-TNFR2 cells (Fig. 3B,C), so as to be certain that the inability of TNFR2 to efficiently activate NF-κB gene transcription in our cells was not due to poor expression of TNFR2 levels (or NF-κB reporter construct. Fig. 3B shows by FACS analysis the stable expression of TNFR2 in our HEK293-TNFR2 cells, which is not present in parental HEK293 cells. No TNF-stimulated luciferase activity is detectable in HEK293 cells not expressing NF-κB reporter construct (not shown). The NF-κB-luciferase results from both types of transfections of HEK293 cells, either transiently or stably, gave very similar results to those gained for the HeLa-TNFR2 cells transiently transfected with the NF-κB-luciferase construct, i.e. TNFR2-stimulation accounted for

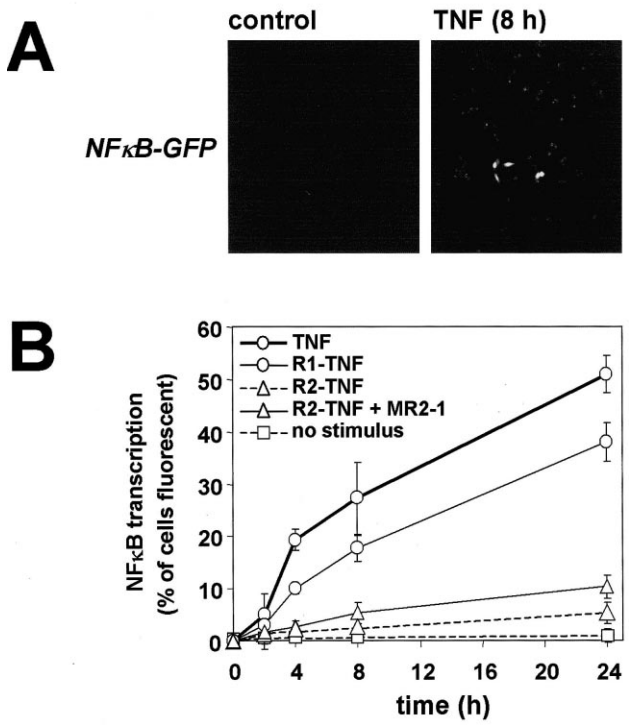

Fig. 4. TNFR1- and TNFR2-stimulated NF-κB–hrGFP gene transcription activity. TNFR1- and TNFR2-stimulated NF-κB–hrGFP reporter construct measurements (A) by transient transfection reporter construct measured 24 h post-transfection, as described in Section 2. B: The TNFR1- and TNFR2-specific stimuli were applied for the indicated time before NF-κB-driven hrGFP measurement by counting the percent of total cells displaying fluorescence. Stimuli at the same concentrations used in Fig. 1, were applied as indicated. The results shown are the mean ± S.D. of triplicate determinations.

approximately only 10% of the achievable TNFR-stimulated NF-κB activity.

Similar profiles of TNFR-induced NF-κB reporter construct activity was seen using transient transfection of cDNAs expressing hrGFP gene rather than firefly luciferase (Fig. 4). This mode of reporter construct has the advantage that the cells do not need to be destroyed to measure the report (as is the case with luciferase activity), but the report is measured in living cells in a more 'in situ' circumstance, and subsequent cellular actions (e.g. apoptosis) can be observed from the reporting cell. These data in Fig. 4 show that HeLa-TNFR2 cells treated with TNFR1- or TNFR2-specific stimuli lead to NF-κB gene activity in a time-dependent manner with significant stimulation of NF-κB-dependent transcription observable in as little as 2 h of TNF treatment. FACS analysis of TNFR-stimulated GFP expression again revealed predominately TNFR1-stimulated activity with markedly less TNFR2-activated NF-κB gene stimulation (not shown).

4. Discussion

In this study, we have observed that both endogenous and exogenous TNFR1 and TNFR2 receptor subtypes stimulate various aspects of NF-κB activation processes. It was previously known that both receptor isoforms are capable of activating NF-κB [1], however, this is the first report to quantitate the relative contribution of each TNFR. The ability of TNFR1 to activate NF-κB appears to account for the vast majority of TNF-stimulated NF-κB transcription factor activation (presumably through TRADD/FADD interaction). However, we consistently observe that TNFR1-mediated activation does not account for all TNF-stimulated NF-κB activation, suggesting a contributory role for TNFR2. This suggests that a simple 'ligand passing' role for TNFR2 (merely presenting TNF ligand to TNFR1 [7]) is not the role for TNFR2 in NF-κB activation. Indeed, TNFR2 directly binds the TRAF-2 intracellular adapter protein (a known stimulator of NF-κB), which also binds indirectly to TNFR1. Another adapter protein which binds both TNFRs is RIP (-1, -2 and -3), 57–74 kDa proteins which contain kinase domains [23]. RIP is capable of transducing both JNK and NF-κB activation by both TNFRs and is thought to be a possible switch in TNFR2-mediated cell death-enhancing or proliferative NF-κB actions, at least in T cells [27]. Another signalling protein thought to activate NF-κB is NIK which phosphorylates IKKs, resulting in activation of IκBα and IκBβ, however, the architecture of TNFR1 and TNFR2 activation of this extracellular signal-regulated kinase is unclear. Moreover, transgenic mice lacking NIK were still capable of TNF-induced NF-κB activation, suggesting more complex adaptive processes in TNFR-induced NF-κB gene transcription [11].

Despite the precise mechanism of TNFR1 and TNFR2 activation of NF-κB being not fully understood, it is clear from our studies here that the majority of soluble TNF-mediated NF-κB activation occurs through TNFR1, with TNFR2 playing a minor role. This does not take into account findings which uncovered that soluble TNF efficiently activates TNFR1, but poorly activates TNFR2 [38,44]. The membrane-bound form of TNF (as is presented to cells in vivo) efficiently activates both TNFR1 and TNFR2. Therefore, the contribution of each TNFR towards NF-κB activation in vivo may be more reliant on TNFR2. Our use here of agonistic antisera to efficiently stimulate TNFR2 provides us with a fairer assessment of the relative functional contribution of TNFR1 and TNFR2. Given that these stimuli may lead to fully active JNK activity by both TNFRs, it is clear that stimulation of NF-κB is predominately through TNFR1. TNFR2 is capable of NF-κB activation (as has been shown by others) but its actions, even in cells expressing large amounts of TNFR2, is over-exaggerated when compared to TNFR1-mediated NF-κB activation.

Partly due to the inability of soluble TNFs to efficiently activate TNFR2, the exact role of TNFR2 in TNF signalling is not fully understood. Some workers claim that endogenously expressed TNFR2 stimulates NF-κB activity [29,31], whereas others claim it to be an artefact of exogenous over-expression of the receptor isotype [4]. Perhaps experimental circumstances do not favour investigations of TNFR2-mediated NF-κB activation, with many researchers opting for over-expression for their studies. For example, over-expression studies in HEK293 cells led to TNFR2-induced NF-κB activation, even in the absence of ligand [45,46]. We did not observe the same agonist-independent TNFR2-induced NF-κB activation in HEK293 cells here. How the exogenous receptor compares to other natural cell types which are not genetically engineered for over-expression is a valid consideration. Moreover, the cell type has been an important factor in assessing the TNFR-induction of NF-κB, with many cells

lacking the necessary signalling machinery to allow observable TNFR stimulation of NF-κB [4,31].

The findings here, shed new light on the relative contribution each of the TNFR subtypes plays in cells expressing TNFR1 and TNFR2. As TNFR1 levels remain relatively constant, with a wide regulation of TNFR2 protein expression in cells experiencing different stimuli or pathological conditions, the contribution each of the TNFR subtypes has towards a TNF-stimulated NF-κB response may alter. Thus, the role of the two TNFRs in TNF-stimulated NF-κB-mediated pro-inflammatory and anti-apoptotic cellular actions may need to be addressed for each cell type under varying cellular conditions. But, it is clear that even given a large excess of TNFR2 over TNFR1 expression in a cell, the vast majority of the TNF-induced NF-κB signal will be mediated by TNFR1 rather than TNFR2.

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