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Distinct Differences between TNF Receptor 1- and TNF Receptor 2-mediated Activation of NFκB

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Tumor necrosis factor (TNF) signaling is mediated via two distinct receptors, TNFR2 and TNFR1, which shows partially overlapping signaling mechanisms and biological roles. In the present study, TNFR2 and TNFR1 signal transduction mechanisms involved in activation of NFκB and CMV promoter-enhancer were compared with respect to their susceptibility towards inhibitors of intracellular signaling. For this, we used SW480 cells, where we have shown that TNF-signaling can occur independently through each of the two receptors. The TNFR1 response was inhibited by D609, bromophenacyl bromide (BPB), nordihydroguararetic acid (NDGA), and by sodium salicylate, while TNFR2-mediated activation of NFκB and CMV promoter-enhancer was resistant to these compounds. The signaling mechanisms known to be affected by these inhibitors include phospholipases as well as redox- and pH-sensitive intracellular components. Our results imply that TNFR2 signaling involved in NFκB activation proceeds independently of these inhibitor-sensitive signaling components, indicating distinct signaling pathways not shared with TNFR1.

Keywords: NFκB, Signal Transduction, TNFR1 receptor, TNFR2 receptor

Abbreviations: β-gal, β-galactosidase; BPB, bromophenacyl bromide; CMV IE, cytomegalovirus immediate early; FADD, Fas-associated death domain protein; IKK IκB-kinase complex; NDGA, nordihydroguayaretic acid; NFκB, nuclear factor κB; OD, optical density; sPLA2, secretory phospholipase A2; PLC, phospholipase C; PMA, phorbol myristate acetate; RIP, receptor-interacting protein; TNF, tumor necrosis factor; TNFR tumor necrosis factor receptor; TRAF, tumor necrosis factor receptor associated factor; TRADD, TNF receptor- associated death domain protein.

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Introduction

The cytokine tumor necrosis factor (TNF) is a central mediator of inflammation, immune response and antiviral defence (Old, 1988). TNF is a strong activator of transcription factor NFκB which is involved in transcriptional regulation of genes coding for immunoglobulin κ light chain, MHC antigens, cytokines, cytokine receptors and adhesion molecules, as well as in the activation of human immunodeficiency virus and cytomegalovirus (CMV) (Baeuerle and Henkel, 1994). NFκB which is constitutively present in the cytoplasm, bound to NFκB inhibitor, IκB (Beg *et al*., 1993), is activated posttranslationally by cellular signals that lead to proteasomal degradation of IκB (Palombella *et al*., 1994) after its phosphorylation by the IκB-kinase (IKK) complex (Karin and Ben-Neriah, 2000). Elucidation of TNF activated signal transduction pathways leading to NFκB activation is a major challenge in trying to understand how TNF mediates cellular events involved in inflammation and immune responses.

TNF binds and signals through two distinct receptors, TNFR1 (CD120a) and TNFR2 (CD120b) which are both present on most cells (Tartaglia *et al*., 1991). TNFR1 and TNFR2 are members of the TNF receptor superfamily characterized by cysteine-rich pseudorepeats in their extracellular regions (Beutler and van Huffel, 1994; Smith *et al*., 1994). Activation of TNFR1 appears to be sufficient to induce most common TNF reponses like activation of NFκB, cytotoxicity and proliferation (Tartaglia *et al*., 1991; Schutze *et al*., 1992; Laegreid *et al*., 1994). However, it has been demonstrated that in some cell types, TNFR2 can independently mediate cellular responses like activation of NFκB (Laegreid *et al*., 1994; Mukhopadhyay *et al*., 2001), proliferation (Grell *et al*., 1998) and cell death (Heller *et al*., 1992; Medvedev *et al*., 1994; Grell *et al*., 1999; Baxter *et al*., 1999). A number of studies even indicate that signaling through both receptors may be important for TNF responses like regulation of adhesion molecule expression, and cell death

(Shalaby *et al*., 1990; Abe *et al*., 1993; Declercq *et al*., 1998; Grell *et al*., 1999). Furthermore, TNFR1 and TNFR2 play distinct and specific roles in discriminating between the effects of TNF and the TNF related ligand lymphotoxin-α (Medvedev *et al*., 1996a; Medvedev *et al*., 1996b). The diverse involvement of the two receptors in the large repertoire of cellular TNF responses, brings attention to the molecular mechanisms underlying intracellular events induced by each of TNFR1 and TNFR2, including cross-talks between these events.

TNF induced NFκB activation has been found to involve a number of intracellular proteins associated with the non-homologous intracellular domains of TNFR1 and TNFR2. TNF receptor associated protein 2 (TRAF2), whose overexpression has been shown to activate NFκB (Rothe *et al*., 1995b) binds directly to TNFR2 (Rothe *et al*., 1994). TRAF1 binds to TRAF2 (Rothe *et al*., 1994) and this heterocomplex in turn leads to recruitment of inhibitors of apoptotic protein (cIAP1) and/or cIAP2 (Rothe *et al*., 1995a). It is now thought that mostly TRAF2 interacts with TNFR2 directly, with TRAF1 interacting indirectly and TRAF3 also able to associate. TRAF2 is recruited to TNFR1 indirectly throught a specific interaction with the protein TNF receptor-associated death domain protein (TRADD) (Hsu *et al*., 1995) that directly binds to TNFR1 throught its own death domain sequence (Hsu *et al*., 1995). TRADD recruits the downstream signaling adaptor molecules Fas-associated death domain protein (FADD) and receptor-interacting protein (RIP). RIP, originally identified as a Fas-associating molecule (Stanger *et al*., 1995), interacts with both TNFR1/TNFR2 (Hsu *et al*., 1996; Liu *et al*., 1996).

TNFR1-mediated NFκB activation has been reported to involve phosphatidylcholine specific phospholipase C (PC-PLC), sphingomyelinase (SMase), phospholipase A2 (Schutze *et al*., 1992; Yang *et al*., 1993; Thommesen *et al*., 1998) atypical PKCs (Anthonsen *et al*., 2001) and protein kinase C -ζ (PKC-ζ) activated by ceramide (Lozano *et al*., 1994; Folgueira *et al*., 1996). TNFR2-mediated signaling is less studied even though a TNFR2 specific signaling component, the tyrosine kinase Etk/Bmx, has recently been identified to be involved in TNF-induced angiogenesis (Pan *et al*., 2002). Activation of TNFR2 in some cells has been shown to be proliferative, but it is also known to have a function in regulating TNF-induced apoptosis (reviewed in (MacEwan, 2002). Furthermore, TNFR2 signaling seems to play an important independent role in chronic inflammatory conditions like Crohns disease (Holtmann *et al*., 2002). Opposite roles of TNFR1 and TNFR2 is reported. In retinal ischemia TNFR1 auguments neuronal death, while TNFR2 promotes neuroprotection, suggesting distinct biological roles (Fontaine *et al*., 2002).

Our previous studies on TNFR2- and TNFR1-mediated activation of NFκB and CMV immediate early enhancer have indicated the excistence of both overlapping and independent signaling mechanisms (Laegreid *et al*., 1994; Laegreid *et al*., 1995). In the present study we compared the effect of commonly used inhibitors on TNFR2 and TNFR1 mediated responses in SW480 cells. We found that TNFR1 mediated activation of transcription factor NFκB and CMV promoter-enhancer is inhibited by a number of compounds to which the TNFR2 response was insensitive. The results indicate that TNFR2 mediated NFκB activation proceeds via signaling mechanisms distinctly different from those involved in TNFR1 induced NFκB activation.

Materials and Methods

Cells and reagents SW480/β-gal cells (generously provided by Dr. Gerald Ranges, Miles Inc., West Haven, CT, USA) contain a beta galactosidase (β-gal) reportergene under the control of the cytomegalovirus immediate early promoter-enhancer (CMV IE) (Galloway *et al*., 1992). SW480/β-gal cells were grown in RPMI1640 (Gibco Laboratories, Paisley, Scotland), supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS (HyClone, Logan, USA) and 40 μg/ml garamycin. Human recombinant TNF, with specific activity 7.6×10^7 U/mg protein, was generously supplied by Dr. Refaat Shalaby, Genentech Inc. (South San Francisco, USA). TNFR2 antiserum (p75 AS) was generated by multiple injections of a rabbit with recombinant soluble TNFR2 (Laegreid *et al*., 1994). The generation and purification of the mAb htr-9 and utr-4 against TNFR1 and TNFR2 respectively, has been reported (Brockhaus *et al*., 1990). Htr-9 and utr-4 was generously provided by Dr. M Brockhaus , Hoffmann La-Roche Ltd. (Basel, Switzerland). Biotinylation of htr-9 and utr-4 was performed as described (Updyke and Nicolson, 1984). All mAbs were purified on a Sepharose goat anti-mouse IgG column (Zymed Laboratories Inc., South San Francisco, USA). Affinity purified antibodies against NFκB proteins p50, p65, p49, rel C and rel B, as well as antibodies against ISGF-3 p84/p91 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA).

4-Bromophenacyl bromide (BPB) (32 mM), nordihydroguaiaretic acid (NDGA) (0,1 M), and PMA (1 mg/ml) (Sigma Chemical Company, St.Louis, USA), were dissolved in 96% ethanol. Tricycloedecan-9-yl-xanthogenate (D609) (10 mg/ml) (Kamyia Biomedical Company, Thousand Oaks, CA, USA) and sodium salicylate (1 M) (Sigma) was dissolved in medium without serum, benzamidine (Sigma) in 50% ethanol at 0.5 M, phenylmethylsulfonyl fluoride (PMSF) (Sigma) in isopropanol at 0.1 M, and pyrrolidine dithiocarbamate (PDTC) (Sigma) in PBS at 0,2 M. BPB and PDTC were always prepared fresh before use, while the other reagents were stable for some weeks (D609, PMSF, 4°C) or months (benzamidine, NDGA, –20°C) (PMA, –80 °C).

Stimulation of cells Cells were seeded out in normal growth medium at 1×10^6 cells pr well in 6-well plates (for preparation of nuclear extracts) or at 2×10^4 in 96-well microtiter plates (for reportergene analysis) and cultivated for three days (postconfluent). After pretreatment with inhibitors for the indicated time periods, the stimulating agent was added and incubation was continued in the presence of inhibitors. D609 was found to loose activity in the presence of 10% FCS, therefore all experiments with inhibitors (pretreatment and stimulation) were performed in medium with 2% FCS.

Quantitative band shift assays and supershift analysis Preparation of nuclear extracts, native polyacrylamide gelelectrophoresis with [^{32}P]-labelled NFκB oligonucleotide probe (Promega Corporation, Madison, USA) and quantitation with a PhosphoImager (Molecular Dynamics, Sunnyvale, USA) was carried out as described (Laegreid *et al*., 1995). Radioactivity counts were termed «PhosphoImager units» and were used to compare the relative amounts of radioactivity in bands within the same gel. Bandshift assays with [^{32}P] labeled OCT oligonucleotide (Promega) (5'-TGTCGAATGCA AATCACTAGAA-3') were performed in order to control that a similar amount of nuclear proteins was applied for each sample. All samples were analysed in at least two bandshift assays. For supershift analysis, nuclear extracts were first incubated with [^{32}P] labeled NFκB probe in binding buffer (with poly(dI)(dC)) for 15 min. at room temperature. Then, 2 mg of antibody was added and incubation was continued for 1 h on ice before native polyacrylamide gelelectrophoresis.

Beta-galactosidase assay The β-galactosidase assay was performed essentially as described previously (Galloway *et al*., 1992). Conversion of the substrate clorophenol-red D-galactopiranozide (CPRG, Boehringer Mannheim, Mannheim, Germany) was measured as optical density (OD) at 570 nm.

Flow cytometric analysis of TNFR mAb Cells were labeled with 10 μg/ml of biotinylated htr-9 or biotinylated utr-4 and 0.2 μg / ml streptavidin-phycoerythrin (Becton Dickinson, Mountain View, USA) as previously described (Shalaby *et al*., 1990). Background fluorescence was estimated by adding 0.2 μg/ml streptavidin-phycoerythrin/FITC-goat anti-murine Ig (Becton Dikinson). After washing three times in PBS-A$^+$ buffer, cells were analysed in a FACScan flow cytometer (Becton Dickinson).

Results

TNFR2 mediated activation of CMV promoter-enhancer and NFκB is resistant to D609 and BPB, while the TNFR1- and PMA responses are inhibited TNFR1 and TNFR2 signal transduction mechanisms have mostly been studied separately, in different cell lines. We have shown that SW480 adenocarcinoma cells provide a good model system for the comparison of TNFR2 and TNFR1 signal transduction (Laegreid *et al*., 1994; Laegreid *et al*., 1995; Medvedev *et al*., 1996a; Medvedev *et al*., 1996b), since in this cell line, both receptors are endogenously expressed and can be specifically and selectively activated by agonistic TNFR1 or TNFR2 antibodies (Laegreid *et al*., 1994).

In order to further characterize the differences between TNFR1 and TNFR2 signal transduction pathways, SW480/β-gal cells were stimulated with agonistic TNFR1 mAb htr-9 or with agonistic TNFR2 rabbit antiserum p75 AS in the presence of inhibitors of intracellular signal transduction components. The cellular response was measured as activation of transcription factor NFκB in band shift assays, or as activation of the CMV IE promoter-enhancer in a β-galactosidase reportergene assay.

TNFR1- and TNF-mediated increase in β-galactosidase activity was inhibited in cells stimulated in presence of the xantate D609, an inhibitor of phospholipase C (PLC) (Muller-Decker, 1989; Schutze *et al*., 1992) (Fig. 1A). D609 also suppressed PMA mediated activation of the CMV promoter-enhancer (Fig. 1C). Inhibition of the TNFR1- and PMA-responses is evident at 6.25 mg D609/ml, but is most pronounced at 12.5 and 25 mg D609/ml. Interestingly, the TNFR2 response was not affected by D609 (Fig. 1B), indicating that TNFR2 mediated activation of the CMV promoter-enhancer does not depend on components sensitive to this compound. Bandshift analysis of nuclear extracts from cells treated with TNF or with agonistic TNFR antibodies in the presence of D609, showed that D609 strongly inhibited TNFR1- and TNF-mediated activation of NFκB (Fig. 1D & E). TNFR2 mediated activation of NFκB was unaffected or enhanced by D609 (Fig. 1D & F). Although D609 by itself weakly activated the CMV promoter-enhancer (Fig. 1A), it was not found to induce activation of NFκB (data not shown).

Similar to D609, the alkylating agent 4-bromophenacyl bromide (BPB), which inhibits both type PLA2 and PLC phospholipases (Martin *et al*., 1987), suppressed TNFR1-, TNF- as well as PMA-responses in the SW480 β-gal reportergene assay (Fig. 2A & 2C), while TNFR2 mediated activation of the CMV promoter-enhancer was insensitive to BPB (Fig. 2B).

TNFR2- and PMA-mediated activation of CMV promoter-enhancer and NFκB is resistant to NDGA and sodium salicylate, while the TNFR1-response is inhibited The antioxidant nordihydroguaiaretic acid (NDGA) (Niki, 1987) inhibits a variety of reactions dependent on oxidative processes, like lipoxygenase mediated generation of arachidonic acid metabolites (Miyazawa *et al*., 1985) and cytochrome P-450 catalysed electron transfer (Montag *et al*., 1993). NDGA inhibited TNFR1- and TNF-induced activation of the CMV promoter-enhancer (Fig. 3A), while the TNFR2 response was not affected (Fig. 3B). Similarly, the PMA response was insensitive to NDGA (Fig. 3C). In the present study, NDGA also strongly inhibited TNFR1- and TNF-mediated activation of NFκB. The inhibitiory effect was evident at 2 μM (approx. 30%), but was further increased at 10 mM (50-70% inhibition) (Fig. 3D & E). TNFR2 mediated activation of NFκB was insensitive to NDGA up to 2 mM, while a weak inhibition (less than 20%) was noted at 10 mM. (Fig. 3D & F).

The anti-inflammatory agent sodium salicylate is also known as an antioxidant (Sagone and Husney, 1987) and inhibitor of cyclooxygenases (Vane and Botting, 1995). This compound caused a weak, but highly reproducible suppression of TNFR1- and TNF-mediated CMV promoter-enhancer activation (Fig. 4A), while the TNFR2- and PMA responses were not inhibited (Fig. 4B and 4C).

TNFR1 cell surface expression is not reduced by inhibitor treatment One possible mechanism underlying the observed

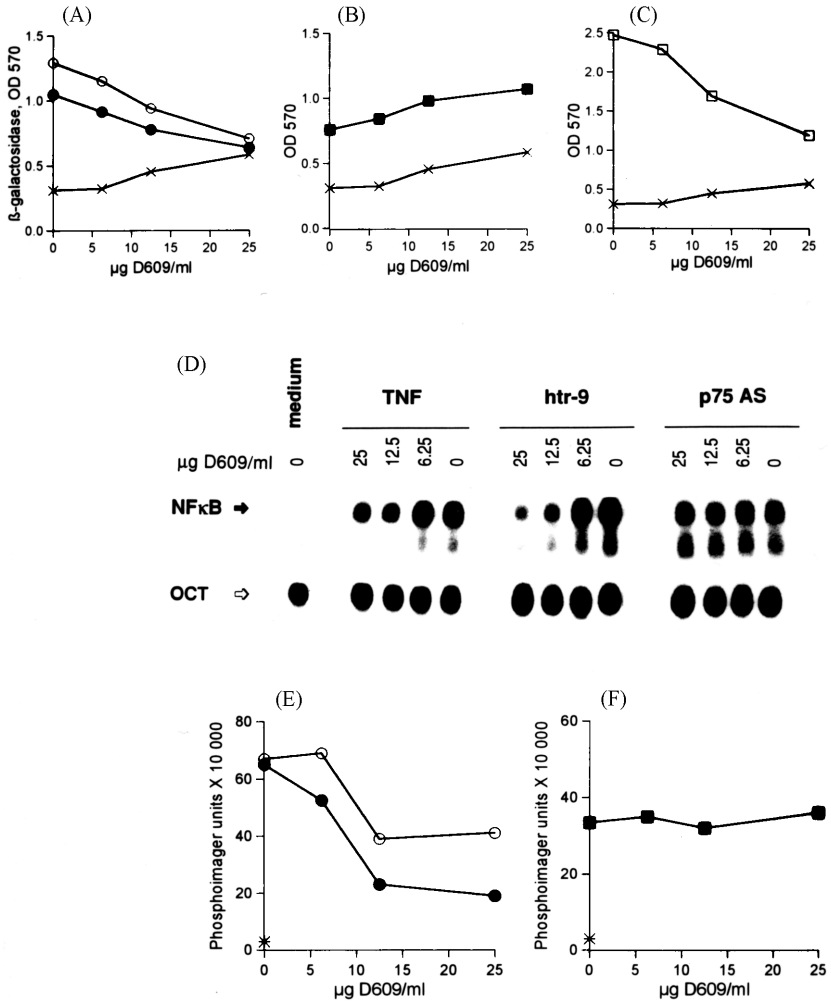

Fig. 1. TNFR1- and TNFR2-mediated activation of the CMV promoter-enhancer and NFκB in the presence of D609. SW480/β-gal cells were pretreated for ½ h with D609 and stimulated with TNF (10 ng/ml; ○), TNFR1 mAb htr-9 (10 µg/ml; ●), TNFR2 antiserum p75AS (diluted 1 : 100; ■) or PMA (5 ng/ml; □), or kept in medium alone (×). Activation of the CMV promoter-enhancer was measured as β-galactosidase activity after stimulation for 4 h with TNF or htr-9 (A), p75 AS (B), or PMA (C). NFκB and OCT bandshift gel analysis (D) was performed after 1h stimulation. Quantitation of specific NFκB bandshifts is shown for TNF (○) or htr-9 (●) treated cells (E) and for p75AS (■) treated cells (F). The results shown are representative of four independent experiments.

selective inhibition of TNFR1 mediated responses could be downregulation of this receptor. Therefore, we performed flow cytometric analyses of cell surface TNFR expression in SW480/β-gal cells treated with inhibitors. We found that TNFR1 expression was not significantly changed in cells treated with D609 (12.5 µg/ml) or NDGA (4 µM) (Fig. 5). NDGA did not change TNFR1 expression even at 10 µM[1]. TNFR1 levels were unaffected by treatment with sodium salicylate (1 h; 10 mM) and upregulated by BPB (1 h; 16 µM)[1]. Thus, it is unlikely that inhibition of the TNFR1 response is due to a decrease in TNFR1 cell surface levels. TNFR2 expression was either unaffected or slightly reduced by all inhibitors analysed (Fig. 5; data not shown). However, even though they caused a weak reduction in TNFR2 cell surface levels, compounds like D609 or NDGA did not inhibit TNFR2 mediated responses (Fig. 1 and 3). Thus, the observed effects of the inhibitors are not a consequence of their effect on TNF receptor surface expression. This suggests that the inhibitors, at the concentrations employed in the present study, act at intracellular sites.

TNFR2 and TNFR1 mediate activation of NFκB complexes with similar subunit composition In order to examine whether the differences in TNFR2 and TNFR1 signaling mechanisms may manifest themselves in the activation of different subsets of rel protein hetero- and homodimers, we performed supershift analyses of TNFR2- and TNFR1-activated proteins binding to the NFκB consensus sequence. Two NFκB specific bandshifts of which the slower migrating complex is most strongly upregulated, are detected in nuclear

[1]data not shown.

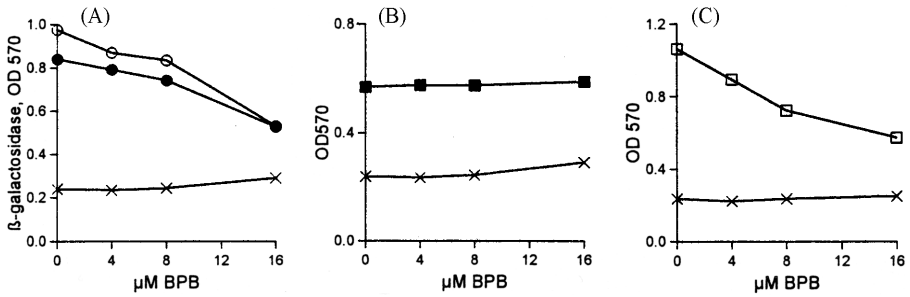

Fig. 2. TNFR1- and TNFR2-mediated activation of the CMV promoter-enhancer in the presence of BPB. SW480/β-gal cells were pretreated for $^1/_2$ h with BPB, followed by stimulation with TNF (○), TNFR1 mAb htr-9 (●), TNFR2 antiserum p75AS (■) or PMA (□), or kept in medium alone (×) and measurement of b-galactosidase activity as indicated in Fig. 1. The results shown are representative of four independent experiments.

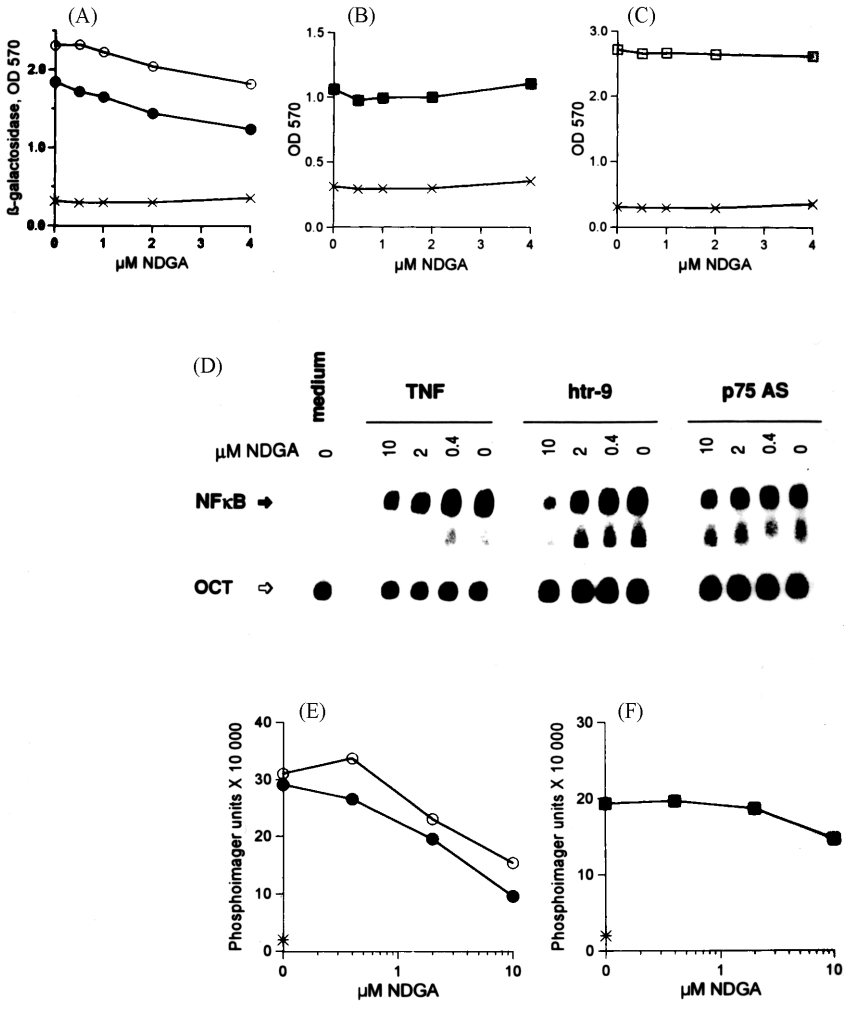

Fig. 3. TNFR1- and TNFR2-mediated activation of the CMV promoter-enhancer and NFκB in the presence of NDGA. SW480/β-gal cells were pretreated for $^1/_2$ h with NDGA, followed by stimulation with TNF (○), TNFR1 mAb htr-9 (●), TNFR2 antiserum p75AS (■) or PMA (□), or kept in medium alone (×) and analysis as indicated in Fig. 1. (A-C) β-galactosidase activity, (D) NFκB and OCT bandshift, (E) PhosphoImager quantitation of specific NFκB bandshifts in TNF (○) or htr-9 (●) treated cells, (F) PhosphoImager quantitation of specific NFκB bandshifts in p75AS (■) treated cells. The results shown are representative of four independent experiments.

extracts from stimulated SW480/β-gal cells (Laegreid *et al.*, 1994). Supershifts of NFκB bandshifts were produced by antisera against NFκB p50, NFκB p65 (relA), NFκB relC and NFκB relB (Fig. 6). The faster migrating complex was

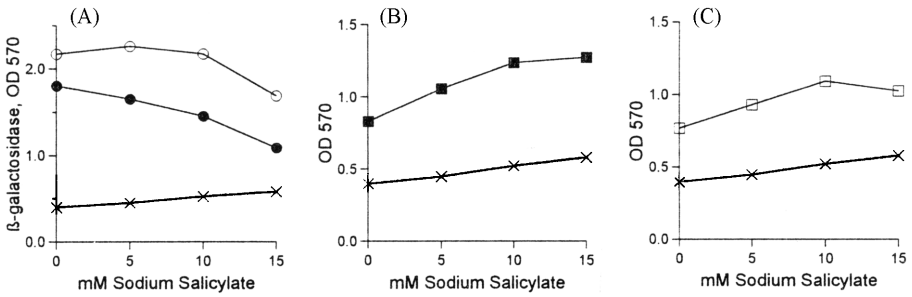

Fig. 4. TNFR1- and TNFR2-mediated activation of the CMV promoter-enhancer in the presence of sodium salicylate. SW480/β-gal cells were pretreated for 1 h with sodium salicylate, followed by stimulation with TNF (○), TNFR1 mAb htr-9 (●), TNFR2 antiserum p75AS (■) or PMA (□), or kept in medium alone (×) and measurement of β-galactosidase activity as indicated in Fig. 1. The results shown are representative of four independent experiments.

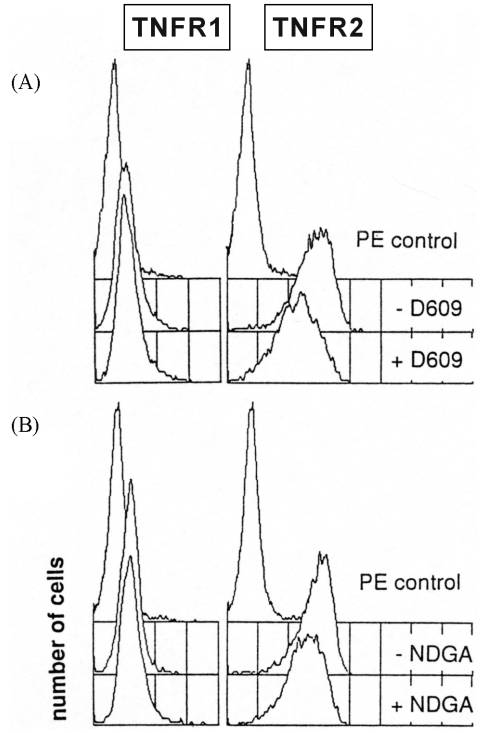

Fig. 5. Flow cytometric analysis of TNFR expression in SW480/β-gal cells cultivated in the presence or absence of 12.5 μg/ml D609 ($^1/_2$ h) (A), or 4 μM NDGA ($^1/_2$ h) (B).

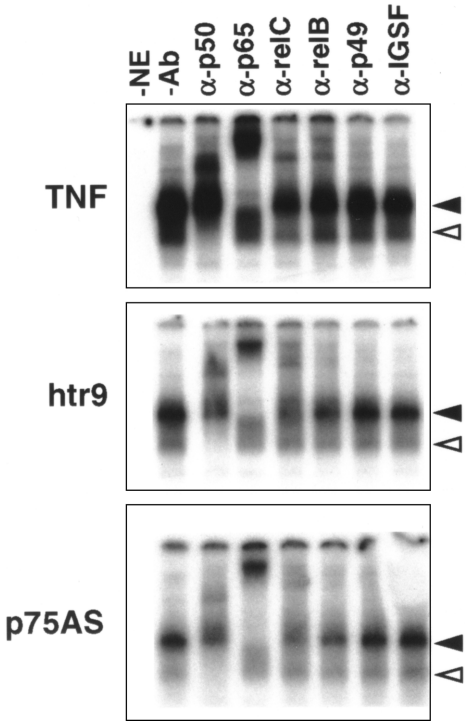

Fig. 6. Supershift analysis of NFκB proteins activated by TNFR1 and TNFR2. Nuclear ectracts from SW480/β-gal cells stimulated for 1h with either TNF (10 ng/ml), TNFR1 mAb htr-9 (10 μg/ml) or TNFR2 antiserum p75AS (dilution 1 : 100) were subjected to supershift analysis as described in Materials and methods. The bandshifts representing specific binding to the NFκB concensus sequence, as identified by competition with unlabeled oligonucleotides (Laegreid *et al*., 1994) are indicated by arrowheads.

recognized only by anti-p50, and thus most likely represents p50/p50 homodimers. Anti-p65 produced a strong supershift of the slower migrating complex, which was also recognized by anti-p50 (Fig. 6), indicating that this complex mainly contains the p50/p65 heterodimer which is the most abundant NFκB factor in many cell types. In addition, the slower migrating complex represents hetero- and/or homodimers containing NFκB p65, rel C and rel B. Antibodies against the interferron-γ activated transcription factor ISGF-3 p84/p91 produced no change in bandshift mobilities (Fig. 7, lane 6), indicating that the rel-supershifts are antigen-specific. No major differences were observed in the supershift patterns of nuclear extracts from cells stimulated via TNFR2 compared to extracts from cells stimulated with agonistic TNFR1 antibodies (Fig. 6). Thus, although the two TNFR receptors employ different signaling pathways, they seem to activate similar subsets of rel protein hetero- and homodimers in SW480/β-gal cells.

Discussion

The cell line SW480/β-gal expresses both TNFR1 and TNFR2 receptor, which both independently activates NFκB and CMV promoter-enhancer. The TNFR2 signaling pathway is less studied and less well understood due to lack of receptor specific agonists. By use of TNFR2 antiserum p75 AS, however, we are able to specifically activate TNFR2 without affecting TNFR1, and hereby study the differences between these signaling pathways (Laegreid *et al*., 1994).

In the present study we show that four different compounds, the phenolic plant lignan NDGA, the xantate D609, the alkylating agent bromophenacyl bromide (BPB) and anti-inflammatory agent sodium salicylate, inhibit TNFR1- but not TNFR2-mediated activation of NFκB and CMV promoter-enhancer. The differences in their chemical structure and activity suggest that these compounds do not affect the same signaling component. This is also confirmed by our finding that the PMA response in SW480/β-gal cells was inhibited by D609 and BPB, but not by NDGA or salicylate. Our observations in the human adenocarcinoma cell line SW480/β-gal are compatible with reports from studies of several other cell types. Thus, TNFR1 mediated NFκB activation was found to be inhibited by NDGA in fibroblasts (Schulze-Osthoff *et al*., 1993), by D609 in Jurkat cells and pre-B cells (Schutze *et al*., 1992; Wiegmann *et al*., 1994) and by sodium salicylate in Jurkat cells, endothelial cells and epithelial cells (Kopp and Ghosh, 1994; Pierce *et al*., 1996; Schwenger *et al*., 1998). The resistance of TNFR2 mediated NFκB activation towards these inhibitors to our knowledge has not been previously reported. The present study indicates that the TNFR1 signaling mechanism leading to NFκB activation involves several distinct intracellular components which do not participate in the TNFR2 pathway.

The inhibitiory effect of D609 may indicate that PLC is involved in the signaling mechanism whereby TNFR1 activates NFκB in SW480/β-gal cells. This hypothesis is supported by the observation that the TNFR1 response is also reduced by BPB which, similarly to D609, can inhibit phospholipase C (PLC) (Martin *et al*., 1987). However, secretory phospholipase A2 (sPLA2) is reported to be more potently inhibited by BPB than are type C phospholipases (Marshall *et al*., 1991). Studies in our laboratory with more selective sPLA2 inhibitors strongly indicate that sPLA2 is involved in TNFR1 mediated NFκB activation in human keratinocytes (Thommesen *et al*., 1998) and recently a functional coupling between sPLA2 and cytosolic (c) PLA2 in TNF-mediated NFκB activation was reported (Anthonsen *et al*., 2001). The effect of BPB in the present study may suggest that sPLA2 play a role in TNFR1 signaling, also in SW480/β-gal cells. BPB also has been reported to inhibit TNF-mediated NFκB activation in 293 embryonic cells (van Puijenbroek *et al*., 1999).

Inhibition of TNFR1 mediated NFκB activation in SW480/β-gal cells by antioxidants like NDGA and salicylate is in accordance with the observation that in some cell lines, redox signaling is involved in NFκB activation (Suzuki *et al*., 1997). Alternatively, NDGA and salicylate, both being weak organic acids, may act through pH-sensitive intracellular components in the TNFR1 signaling pathway, as cytoplasmic acidification is proposed to be an important factor for the inhibitory effect of salicylate (Miyazawa *et al*., 1985).

Salicylate has been reported to inhibit a variety of kinases (Jiang *et al*., 2003), but does not inhibit TNFR2 responses measured in SW480 cells.

We have found that NDGA inhibits TNFR1- but not TNFR2-mediated NFκB activation also in the rhabdomyosarcoma cell line KYM-1 (unpublished results, Andrei Medvedev and Astrid Lægreid), which is another cell type where both TNF receptors can independently activate NFκB (Laegreid *et al*., 1994). Interestingly, Grell *et al* found that TNFR1 mediated cytotoxicity in KYM-1 cells was insensitive to NDGA, while TNFR2 induced KYM-1 cytotoxicity was inhibited (Grell *et al*., 1999). This indicates that the signaling mechanisms involved in the induction of cytotoxicity differ from those involved in activation of NFκB, both for TNFR1 and for TNFR2.

Given the known effects of the inhibitors used, it is possible that this TNFR1-specific pathway involves phospholipases like PLC and/or PLA2 as well as redox- or pH-sensitive intracellular components. This study suggests that TNFR2 contributes with more than a “ligand passing” role, as also shown for NFκB activation in HeLa and HEK 293cells (McFarlane *et al*., 2002). Our study supports the view that TNFR2 independently can activate cellular responses and thus contributes with more than a ligand passing role, as also shown for NFκB activation of HeLa and HEK 293 cells (McFarlane *et al*., 2002) and for activation of the tyrosine kinase Etk/Bmx in angiogenesis (Pan *et al*., 2002). Given the known effects of the inhibitors used, it is possible that the TNFR1 specific pathway involves phospholipases like PLC and/or PLA2 as well as redox- or pH-sensitive intracellular components. One may speculate that the TNFR1 specific pathway is linked to TRADD, since this TNFR-binding protein is not known to associate with TNFR2. It will be of interest to investigate a possible role of Etk/Bmx in TNFR2-mediated NFκB activation and to conduct futher studies to elucidate the biologial role of the TNFR2 signaling pathway.

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References

Abe, Y., Gatanaga, M., Osuka, Y., Kimura, S., Burger, R.,

Granger, G. and Gatanaga, T. (1993) Role of 55- and 75-kDa tumor necrosis factor membrane receptors in the regulation of intercellular adhesion molecules-1 expression by HL-60 human promyelocytic leukemia cells in vitro. *J. Immunol*. **150**, 5070-5079.

Anthonsen, M. W., Solhaug, A. and Johansen, B. (2001) Functional coupling between secretory and cytosolic phospholipase A2 modulates tumor necrosis factor-alpha- and interleukin-1beta-induced NF-kappa B activation. *J. Biol. Chem*. **276**, 30527-30536.

Baeuerle, P. A. and Henkel, T. (1994) Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol*. **12**, 141-179.

Baxter, G. T., Kuo, R. C., Jupp, O. J., Vandenabeele, P. and MacEwan, D. J. (1999) Tumor necrosis factor-alpha mediates both apoptotic cell death and cell proliferation in a human hematopoietic cell line dependent on mitotic activity and receptor subtype expression. *J. Biol. Chem.* **274**, 9539-9547.

Beg, A. A., Finco, T. S., Nantermet, P. V. and Baldwin, A. S. Jr (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol. Cell. Biol*. **13**, 3301-3310.

Beutler, B. and van Huffel, C. (1994) Unraveling function in the TNF ligand and receptor families. *Science* **264**, 667-668.

Brockhaus, M., Schoenfeld, H. J., Schlaeger, E. J., Hunziker, W., Lesslauer, W. and Loetscher, H. (1990) Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc. Natl. Acad. Sci.* **87**, 3127-3131.

Declercq, W., Denecker, G., Fiers, W. and Vandenabeele, P. (1998) Cooperation of both TNF receptors in inducing apoptosis: involvement of the TNF receptor-associated factor binding domain of the TNF receptor 75. *J. Immunol*. **161**, 390-399.

Folgueira, L., McElhinny, J. A., Bren, G. D., MacMorran, W. S., Diaz-Meco, M. T., Moscat, J. and Paya, C. V. (1996) Protein kinase C-zeta mediates NF-kappa B activation in human immunodeficiency virus-infected monocytes. *J. Virol*. **70**, 223-231.

Fontaine, V., Mohand-Said, S., Hanoteau, N., Fuchs, C., Pfizenmaier, K. and Eisel, U. (2002) Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J. Neurosci*. **22**, 216.

Galloway, C. J., Madanat, M. S., Sarr, T., Espevik, T., Dumas, M. L., Mitra, G. and Ranges, G. E. (1992) Anti-tumor necrosis factor receptor and tumor necrosis factor agonist activity by an anti-idiotypic antibody. *Eur. J. Immunol*. **22**, 3045-3048.

Grell, M., Becke, F. M., Wajant, H., Mannel, D. N. and Scheurich, P. (1998) TNF receptor type 2 mediates thymocyte proliferation independently of TNF receptor type 1. Eur. *J. Immunol*. **28**, 257-263.

Grell, M., Zimmermann, G., Gottfried, E., Chen, C. M., Grunwald, U., Huang, D. C., Wu Lee, Y. H., Durkop, H., Engelmann, H., Scheurich, P., Wajant, H. and Strasser, A. (1999) Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. *EMBO J*. **18**, 3034-3043.

Heller, R. A., Song, K., Fan, N. and Chang, D. J. (1992) The p70 tumor necrosis factor receptor mediates cytotoxicity. *Cell*. **70**, 47-56.

Holtmann, M. H., Schuchmann, M., Zeller, G., Galle, P. R. and Neurath, M. F. (2002) The emerging distinct role of TNF-receptor 2 (p80) signaling in chronic inflammatory disorders. *Arch. Immunol. Ther. Exp*. **50**, 279-288.

Hsu, H., Huang, J., Shu, H. B., Baichwal, V. and Goeddel, D. V. (1996) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity*, **4**, 387-396.

Hsu, H., Xiong, J. and Goeddel, D. V. (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* **81**, 495-504.

Jiang, G., Dallas-Yang, Q., Liu, F., Moller, D. E. and Zhang, B. B. (2003) Salicylic acid reverses phorbol 12-myristate-13-acetate (PMA)- and tumor necrosis factor alpha (TNFalpha)-induced insulin receptor substrate 1 (IRS1) serine 307 phosphorylation and insulin resistance in human embryonic kidney 293 (HEK293) cells. *J. Biol. Chem*. **278**, 180-186.

Karin, M. and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu. Rev. Immunol*. **18**, 621-663.

Kopp, E. and Ghosh, S. (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265**, 956-959.

Laegreid, A., Medvedev, A., Nonstad, U., Bombara, M. P., Ranges, G., Sundan, A. and Espevik, T. (1994) Tumor necrosis factor receptor p75 mediates cell-specific activation of nuclear factor kappa B and induction of human cytomegalovirus enhancer. *J. Biol. Chem*. **269**, 7785-7791.

Laegreid, A., Thommesen, L., Jahr, T. G., Sundan, A. and Espevik, T. (1995) Tumor necrosis factor induces lipopolysaccharide tolerance in a human adenocarcinoma cell line mainly through the TNF p55 receptor. *J. Biol. Chem*. **270**, 25418-25425.

Liu, Z. G., Hsu, H., Goeddel, D. V. and Karin, M. (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell*. **87**, 565-576.

Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L. and Moscat, J. (1994) Protein kinase C zeta isoform is critical for kappa B-dependent promoter activation by sphingomyelinase. *J. Biol. Chem*. **269**, 19200-19202.

MacEwan, D. J. (2002) TNF receptor subtype signalling: differences and cellular consequences. *Cell. Signal*. **14**, 477-492.

Marshall, L. A., Bauer, J., Sung, M. L. and Chang, J. Y. (1991) Evaluation of antirheumatic drugs for their effect in vitro on purified human synovial fluid phospholipase A2. *J. Rheumatol*. **18**, 59-65.

Martin, T. W., Wysolmerski, R. B. and Lagunoff, D. (1987) Phosphatidylcholine metabolism in endothelial cells: evidence for phospholipase A and a novel Ca^{2+}-independent phospholipase C. *Biochim. Biophys. Acta.* **917**, 296-307.

McFarlane, S. M., Pashmi, G., Connell, M. C., Littlejohn, A. F., Tucker, S. J., Vandenabeele, P. and MacEwan, D. J. (2002) Differential activation of nuclear factor-kappaB by tumour necrosis factor receptor subtypes. TNFR1 predominates whereas TNFR2 activates transcription poorly. *FEBS Lett*. **515**, 119-126.

Medvedev, A. E., Espevik, T., Ranges, G. and Sundan, A. (1996) Distinct roles of the two tumor necrosis factor (TNF) receptors in modulating TNF and lymphotoxin alpha effects. *J. Biol. Chem*. **271**, 9778-9784.

Medvedev, A. E., Laegreid, A., Sundan, A. and Espevik, T. (1996b) A non-competitive P55 TNF receptor antibody enhances the specific activity of lymphotoxin-alpha. *Scand. J. Immunol*. **43**, 439-448.

Medvedev, A. E., Sundan, A. and Espevik, T. (1994) Involvement of the tumor necrosis factor receptor p75 in mediating cytotoxicity and gene regulating activities. *Eur. J. Immunol*. **24**, 2842-2849.

Miyazawa, K., Iimori, Y., Makino, M., Mikami, T. and Miyasaka, K. (1985) Effects of some non-steroidal anti-inflammatory drugs and other agents on cyclooxygenase and lipoxygenase activities in some enzyme preparations. *J. Pharmacol*. **38**, 199-205.

Montag, A. G., Oka, T., Baek, K. H., Choi, C. S., Jay, G. and Agarwal, K. (1993) Tumors in hepatobiliary tract and pancreatic islet tissues of transgenic mice harboring gastrin simian virus 40 large tumor antigen fusion gene. *Proc. Natl. Acad. Sci. USA* **90**, 6696-6700.

Mukhopadhyay, A., Suttles, J., Stout, R. D. and Aggarwal, B. B. (2001) Genetic deletion of the tumor necrosis factor receptor p60 or p80 abrogates ligand-mediated activation of nuclear factor-kappa B and of mitogen-activated protein kinases in macrophages. *J. Biol. Chem*. **276**, 31906-31912.

Muller-Decker, K. (1989) Interruption of TPA-induced signals by an antiviral and antitumoral xanthate compound: inhibition of a phospholipase C-type reaction. *Biochem. Biophys. Res. Commun*. **162**, 198-205.

Niki, E. (1987) Antioxidants in relation to lipid peroxidation. *Chem. Phys. Lipids*, **44**, 227-253.

Old, L. J. (1988) Tumor necrosis factor. *Sci. Am.* **258**, 59-60.

Palombella, V. J., Rando, O. J., Goldberg, A. L. and Maniatis, T. (1994) The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell*. **78**, 773-785.

Pan, S., An, P., Zhang, R., He, X., Yin, G. and Min, W. (2002) Etk/Bmx as a tumor necrosis factor receptor type 2-specific kinase: role in endothelial cell migration and angiogenesis. *Mol. Cell. Biol*. **22**, 7512-7523.

Pierce, J. W., Read, M. A., Ding, H., Luscinskas, F. W. and Collins, T. (1996) Salicylates inhibit I kappa B-alpha phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration. *J. Immunol.* **156**, 3961-3969.

Rothe, M., Pan, M. G., Henzel, W. J., Ayres, T. M. and Goeddel, D. V. (1995a) The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell*. **83**, 1243-1252.

Rothe, M., Sarma, V., Dixit, V. M. and Goeddel, D. V. (1995b) TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* **269**, 1424-1427.

Rothe, M., Wong, S. C., Henzel, W. J. and Goeddel, D. V. (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell*. **78**, 681-692.

Sagone, A. L. Jr and Husney, R. M. (1987) Oxidation of salicylates by stimulated granulocytes: evidence that these drugs act as free radical scavengers in biological systems. *J. Immunol*. **138**, 2177-2183.

Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G. and Fiers, W. (1993) Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J*. **12**, 3095-3104.

Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Kronke, M. (1992) TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. *Cell*. **71**, 765-776.

Schwenger, P., Alpert, D., Skolnik, E. Y. and Vilcek, J. (1998) Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced IkappaB alpha phosphorylation and degradation. *Mol. Cell. Biol.* **18**, 78-84.

Shalaby, M. R., Sundan, A., Loetscher, H., Brockhaus, M., Lesslauer, W. and Espevik, T. (1990) Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. *J. Exp. Med*. **172**, 1517-1520.

Smith, C. A., Farrah, T. and Goodwin, R. G. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell*. **76**, 959-962.

Stanger, B. Z., Leder, P., Lee, T. H., Kim, E. and Seed, B. (1995) RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell*. **81**, 513-523.

Suzuki, Y. J., Forman, H. J. and Sevanian, A. (1997) Oxidants as stimulators of signal transduction. *Free Radic. Biol. Med*. **22**, 269-285.

Tartaglia, L. A., Weber, R. F., Figari, I. S., Reynolds, C., Palladino, M. A. Jr and Goeddel, D. V. (1991) The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc. Natl. Acad. Sci. USA* **88**, 9292-9296.

Thommesen, L., Sjursen, W., Gasvik, K., Hanssen, W., Brekke, O. L., Skattebol, L., Holmeide, A. K., Espevik, T., Johansen, B. and Laegreid, A. (1998) Selective inhibitors of cytosolic or secretory phospholipase A2 block TNF-induced activation of transcription factor nuclear factor-kappa B and expression of ICAM-1. *J. Immunol.* **161**, 3421-3430.

Updyke, T. V. and Nicolson, G. L. (1984) Immunoaffinity isolation of membrane antigens with biotinylated monoclonal antibodies and immobilized streptavidin matrices. *J. Immunol. Methods* **73**, 83-95.

van Puijenbroek, A. A., Wissink, S., van der Saag, P. T. and Peppelenbosch, M. P. (1999) Phospholipase A2 inhibitors and leukotriene synthesis inhibitors block TNF-induced NF-kappaB activation. *Cytokine* **11**, 104-110.

Vane, J. R. and Botting, R. M. (1995) New insights into the mode of action of anti-inflammatory drugs. *Inflamm. Res.* **44**, 1-10.

Wiegmann, K., Schutze, S., Machleidt, T., Witte, D. and Kronke, M. (1994) Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell*. **78**, 1005-1015.

Yang, Z., Costanzo, M., Golde, D. W. and Kolesnick, R. N. (1993) Tumor necrosis factor activation of the sphingomyelin pathway signals nuclear factor kappa B translocation in intact HL-60 cells. *J. Biol. Chem*. **268**, 20520-20523.