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The Met-196 → Arg Variation of Human Tumor Necrosis Factor Receptor 2 (TNFR2) Affects TNF-α-induced Apoptosis by Impaired NF-κB Signaling and Target Gene Expression*

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Tumor necrosis factor-α (TNF-α)-induced signaling is pivotally involved in the pathogenesis of chronic inflammatory diseases. A polymorphism in the TNF receptor 2 (*TNFR2*) gene resulting in a juxtamembrane inversion from methionine ($TNFR2^{196MET}$) to arginine ($TNFR2^{196ARG}$) has been genetically associated with an increased risk for systemic lupus erythematosus and familial rheumatoid arthritis. Albeit the mutation does not affect the TNF binding kinetics of TNFR2, the present study provides evidence that the mutation results in a significantly lower capability to induce TNFR2-mediated NF-κB activation. Pretriggering of TNFR2 with a receptor-specific mutein leads to an enhancement of TNFR1-induced apoptosis, which is further increased in cells carrying the $TNFR2^{196ARG}$ variant. A diminished induction of NF-κB-dependent target genes conveying either anti-apoptotic or pro-inflammatory functions, such as cIAP1, TRAF1, IL-6, or IL-8 is observed. The mutated form $TNFR2^{196ARG}$ shows a reduction of inducible TRAF2 recruitment upon TNF-α stimulation. The findings suggest a common molecular mechanism for the involvement of the $TNFR2^{196ARG}$ variant in the etiopathogenesis of different chronic inflammatory disorders.

Tumor necrosis factor-α (TNF-α)[1] is a pleiotropic cytokine which plays important roles in innate and adaptive immune responses (1, 2). TNF-α mediates a dual role triggering cellular survival or induction of apoptosis depending on the complex environment (3). TNF-α binds to two distinct receptors, the 55-kDa TNF receptor 1 (TNFR1; CD120a) and the 75-kDa TNFR2 (CD120b), which are members of the TNFR superfamily. This family consists of approx. 30 receptors including Fas/Apo-1/CD95, CD27, CD30, LT-βR, OX-40, and TRAIL-R1 to R4, which share homologies within their extracellular domains containing one to six copies of highly conserved cysteine-rich amino acid motifs (4, 5). The group may be further divided by their conserved intracellular adaptor domains (*e.g.* death domain, DD) mediating intracellular signals via homotypic/heterotypic protein-protein interactions (6, 7). TNFR1, CD95/Fas/Apo1, DR3, DR4 (TRAILR1), and DR5 (TRAILR2) contain DDs and therefore belong to the subgroup of death receptors, being able to recruit other DD proteins by homophilic association (8, 9). On the other hand, TNFR2 is a member of the non-DD receptor subgroup, such as CD30, CD40, RANK, 4–1BB, and OX40, which lacks the ability to directly activate the apoptotic machinery via DD adaptors (1).

Despite the lack of a DD, TNFR2 can induce cell type-specific receptor-interacting protein (RIP)-dependent cell death (10–12) indicating distinct molecular signaling mechanisms for TNFR2-induced apoptosis. It has been reported that TNFR2-mediated cell death is based on the induction of endogenous TNF-α and subsequent triggering of TNFR1 (11, 13). In addition, it is now widely accepted that engagement of TNFR2 may result in cIAP1-dependent depletion of TRAF2 leading to an enhancement of TNFR1-mediated apoptosis without the involvement of endogenous TNF-α (14–17).

Whereas soluble TNF-α does bind to both TNF receptors and is a strong activator of TNFR1, TNFR2 has been reported to become fully activated only upon stimulation with membrane-bound TNF-α (18, 19). Initially, TNFR2 was believed to play a minor role in the TNF system, only displaying modulating or supportive functions, for instance by collecting and handing over the ligand to TNFR1, a process designated as "ligand passing" (6). However, the use of TNFR2- or TNFR1-specific agonistic antibodies and receptor-specific ligands, TNF-α muteins (19–21), led to the elucidation of differential signaling pathways engaged by the two receptor subtypes. The major adaptor protein is thought to be TRAF2, which is responsible for the activation of NF-κB signaling (via the IκB (IKK) complex) as well as engagement of the JNK pathway (21–24).

The TNFR2 gene is located on chromosome 1p36.2 and is

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[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; TNFR, TNF receptor; sTNF, soluble TNF-α; DD, death domain; JNK, c-Jun NH_2-terminal kinase; CRD, cysteine-rich domain; MEF, mouse embryonic fibroblast; FACS, fluorescence-activated cell sorting; CHX, cycloheximide; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; EGFP, enhanced green fluorescent protein; PARP, poly(ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase.

organized in 10 exons and 9 introns (25). In addition to non-coding single nucleotide polymorphisms in exons 4, 9, and 10, a further single nucleotide polymorphism (T/G) was recently described in exon 6 at nucleotide 676 of the TNFR2 mRNA (GenBank™ accession number NM_001066) resulting in an amino acid exchange in the fourth extracellular cysteine-rich domain (CRD4) from methionine ($TNFR2^{196MET}$) to arginine ($TNFR2^{196ARG}$) at position 196 (GenBank™ accession number NP_001057) (26) (Fig. 1*A*). An association of this variant with an increased susceptibility for chronic inflammatory disorders, such as systemic lupus erythematodes (27, 28), human T cell lymphotrophic virus type I-associated myelopathy (29), periodontitis, familial rheumatoid arthritis (30, 31), and ulcerative colitis (32), has been reported.

In the present work, we examined the mechanism by which this pathophysiologically important variant $TNFR2^{196ARG}$ may influence inflammatory signaling pathways. Stably transfected HeLa cell populations and immortalized fibroblasts from *TNFR/TNFR2*−/− double knock-out mice were used as model systems. The differential NF-κB activation, target gene induction, and apoptotic properties downstream of the $TNFR2^{196ARG}$ risk variant suggest a common molecular mechanism which might contribute to the etiopathogenesis of the associated inflammatory diseases.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human epithelial HeLa S3 cells (ACC 161) were obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Immortalized mouse embryonic fibroblasts (MEFs) from $TNFR1^{-/-}TNFR2^{-/-}$ double-deficient mice have been described previously (19). MEF and HeLa cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FCS and penicillin/streptomycin (each at 50 μg/ml) and grown in 5% CO_2 and 37 °C.

pcDNA3.1-TNFR2 Constructs and Mutagenesis—Expression plasmids for TNFR2 were generated by inserting cDNA coding for wild-type TNFR2 ($TNFR2^{196MET}$) into HindIII and XhoI sites of pcDNA3.1 (Invitrogen). For expression of the $TNFR2^{196ARG}$ variant, the codon was mutated from ATG to AGG using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmid for the expression of fusion protein EGFP-TRAF2 has been described before (17).

Antibodies and Reagents—Human recombinant TNFα was acquired from R&D Systems (Minneapolis, MN). G418 (Geneticin) for selection of transfected cells was from PAA Laboratories (Somerset, UK). Mouse monoclonal antibody specific for TNFR2 (ab8161) was purchased from Abcam (Cambridge, MA). Monoclonal antibody mouse-anti-TNFR2 MAB726 for immunoprecipitation was purchased from R&D Systems. Alexa Fluor® 488 chicken anti-mouse IgG, goat-anti-rabbit IgG, and goat-anti-mouse IgG conjugated with Cy3 were from Molecular Probes (Eugene, OR). Rabbit polyclonal IgG anti-TNFR2 was obtained from Stressgen (San Diego, CA). Rabbit polyclonal IgG anti-TRAF2 was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse-anti-β-Actin antibody was purchased from Sigma (Deisenhofen, Germany). All other primary antibodies were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences (Buckinghamshire, UK). The monoclonal mouse antibody 80M2 for cross-linking of TNFR2 has been described elsewhere (33).

Generation of HeLa Cells Stably Expressing TNFR2 Variants—HeLa cells (1×10^6) were transfected with 2 μg of pcDNA3.1 constructs for TNFR2 variants with FuGENE 6 (Roche Applied Science, Basel, Switzerland) according to the manunfacturer's protocol. Transfected cells were selected by 4 weeks of cultivation in medium supplemented with 800 μg/ml G418 and three rounds of fluorescence-activated cell sorting (FACS) (FACStar+, BD Biosciences) using mouse monoclonal antibody 80M2 and fluorescein isothiocyanate-labeled goat anti-mouse IgG plus IgM as secondary antibody (Dianova, Germany). Cells stably expressing TNFR2 variants were propagated at 250 μg/ml G418. HeLa stably transfected with pcDNA3.1 without insert were used as control cells (mock).

Binding Experiments—To determine the effect of the amino acid substitution Met-196 → Arg on binding kinetics of TNFR2 under physiological conditions, association/dissociation studies were performed at 37 °C using stably transfected $TNFR1/TNFR2^{-/-}$-MEF expressing only the respective TNFR2 variant. Recombinant human ^{125}iodotyrosyl-TNF (^{125}I-TNF) was purchased from Amersham Biosciences (Uppsala, Sweden). For the investigation of TNF-α association kinetics, the radiolabeled ligand (20 ng/ml) was preincubated at 37 °C in 50 μl of RPMI 1640 containing 2% bovine serum albumin, 0.1% sodium azide and 20 mM Hepes (binding buffer). The binding reaction was started by addition of 50 μl of cell suspension (4×10^5 cells/50 μl of binding buffer), followed by incubation for 10 s to 10 min at 37 °C. After separating the cells from the binding medium by centrifugation through a mineral oil layer ($14{,}000 \times g$, 15 s), the tips of the tubes were cut off, and cell-bound radioactivity was determined in a gamma counter (LKB Wallac, Sollentuna, Sweden). For dissociation experiments, 4×10^5 cells/50 μl were preincubated at 4 °C for 2 h in the presence of 20 ng/ml ^{125}I-TNF-α in binding buffer. Subsequently, the cells were suspended in 50 μl of prewarmed (37 °C) binding buffer containing 100-fold molar excess of unlabeled TNF-α on a mineral oil layer. Incubation was performed at 37 °C for 10 s to 10 min, followed by separation of the cells by centrifugation and counting of cell-bound radioactivity as described above. Unspecific binding was determined in parallel by incubating cells in the presence of 10 ng/ml ^{125}I-TNF-α and 200-fold molar excess of unlabeled TNF-α at 4 °C for 2 h and was subtracted from total binding. All experiments were performed in triplicates.

Recombinant Expression of Receptor-specific TNF-α Muteins—Plasmids for recombinant expression of TNF-α muteins TNF32W/86T (R1-TNF) and CysTNF143N/145R (Cys-R2TNF) have been described (19–21). Recombinant proteins were expressed in *Escherichia coli* strain M15 (pREP4) (Qiagen, Hilden, Germany) and isolated by nickel-nitrilotriacetic acid chelate columns, and the remaining lipopolysaccharide was removed using AffinityPak Detoxi-Gel (Pierce). Protein concentration was determined by DC protein assay (Bio-Rad).

Cell Death Assay—Cell viability was determined by crystal violet uptake (15, 34). In brief, stably transfected HeLa cells (1.5×10^4 cells/well) were grown overnight in a 96-well plate. Cells were stimulated with freshly prepared serial dilutions of various TNF-α-like agents in the presence of 2.5 μg/ml cycloheximide (CHX) with or without prestimulation with Cys-R2TNF for 6 h. Where indicated, mouse antibody 80M2 was used for cross-linking of TNFR2 at 2 μg/ml for 30 min before stimulation. After 24 h cells were washed three times in PBS and stained for 15 min (20% methanol, 0.5% crystal violet). Cells were washed three times with water and air-dried followed by dissolving of the dye with methanol. Optical density at 550 nm was measured using an enzyme-linked immunosorbent assays plate reader (Milenia Kinetic, DPC Biermann GmbH, Bad Nauheim, Germany).

SDS-PAGE and Immunoblotting—Cells (5×10^5 cells/well) were grown in 6-well plates overnight, followed by stimulation as indicated. Western blots were performed as described (35). Lysed cells were cleared from debris by centrifugation and supernatants were collected. 15 μg of protein extract was separated by denaturing SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking, membranes were probed with specific primary antibodies, washed, and incubated with horseradish peroxidase-conjugated IgG as secondary antibody. Proteins were visualized by chemiluminescence (ECL, Amersham Biosciences). To determine even transfer and equal loading, membranes were stripped and reprobed with an antibody specific for β-actin (Sigma).

Luciferase Assay—For quantification of NF-κB activity, transient transfection was used. Cells were seeded in a 96-well plate at a density of 1.5×10^4 (HeLa) or 8×10^3 (MEF) per well. The next day, cells were transfected with 35 ng of pcDNA3.1$TNFR2^{196MET}$, pcDNA3.1$TNFR2^{196ARG}$, or pcDNA3.1 empty vector (mock-control) together with 12 ng of 3× NF-κB-luciferase reporter plasmid (Clontech) and 3 ng of pRL-TK *Renilla* reporter plasmid (Promega, Madison, WI) for normalization of transfection efficiency and cell viability. After 18 h (HeLa) or 36 h (MEF) of incubation, medium was exchanged by medium containing cross-linking antibody 80M2 (2 μg/ml) where indicated or pure medium. Cells were incubated for 30 min, followed by addition of freshly prepared serial dilutions of indicated agents. After another 18-h incubation (6 h for MEF), cells were washed in PBS and lysed in 1× passive lysis buffer (Promega). Dual luciferase assay was performed using a dual luciferase reporter assay system (Promega) and a MicroLumatPlus luminometer (Berthold, Bundoora, Australia).

Target Gene Expression—Stable HeLa transfectants (5×10^5 cells/well) were grown in 6-well plate overnight. The following day cells were stimulated with freshly prepared dilutions of TNFR2-specific Cys-R2TNF ranging from 100 to 1 ng/ml or left untreated for 18 h. After washing, cells were harvested by centrifugation, and total RNA was isolated using RNeasy kit (Quiagen) following the manufacturer's instructions. Reverse transcription was performed by Advantage-RT-for-

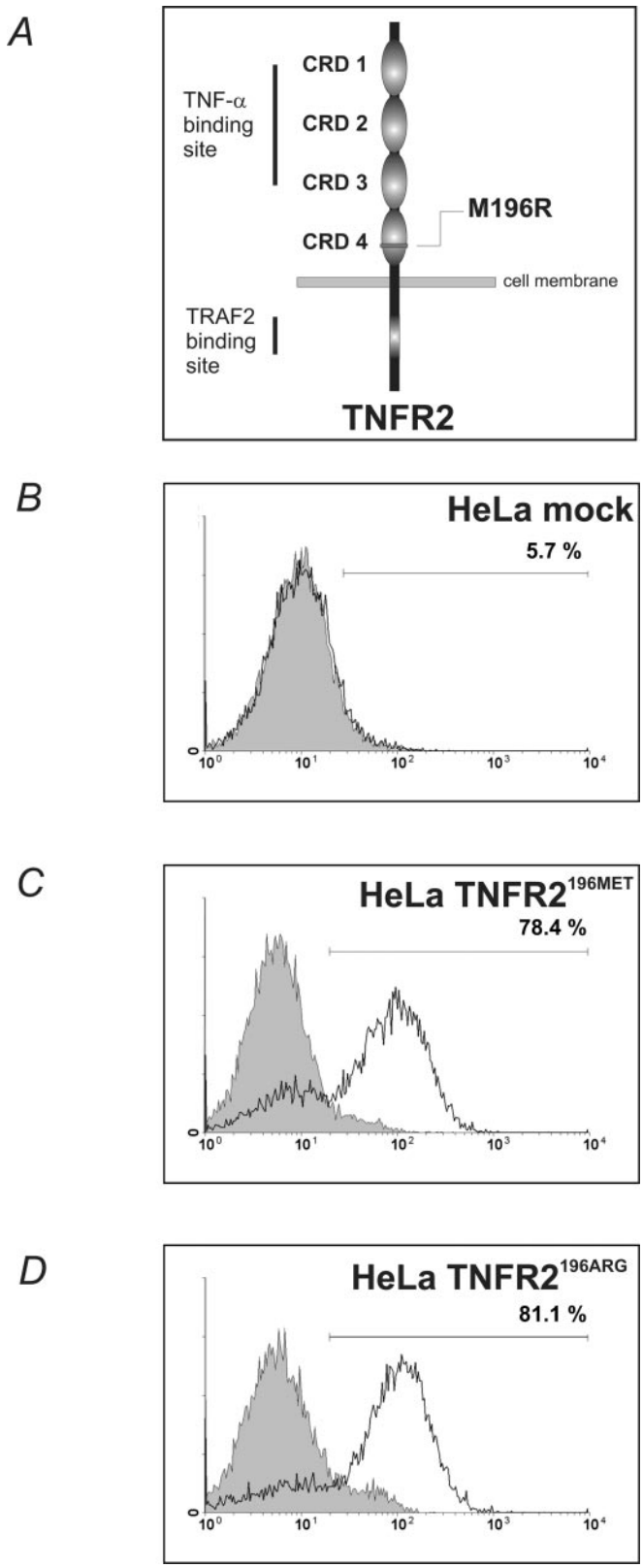

FIG. 1. **Expression of TNFR2^{196MET} and TNFR2^{196ARG} in stably transfected HeLa cells.** *A*, schematic representation of TNFR2 indicating the position of the M196R mutation within the fourth CRD. Note that CRD4 is not directly involved in TNF-α binding. *B–D*, cells were transfected with pcDNA3.1 plasmids encoding for full-length TNFR2^{196MET} and TNFR2^{196ARG} (or empty pcDNA3.1 vector as control). Selection was carried out for a period of 3–4 weeks with selective antibiotic G418 at 800 μg/ml and three rounds of FACS-assisted cell sorting. For detection of TNFR2 on the cell surface, monoclonal antibody mouse-anti-TNFR2 (R&D Systems) and Alexa Fluor® 488-conjugated anti-mouse IgG were used.

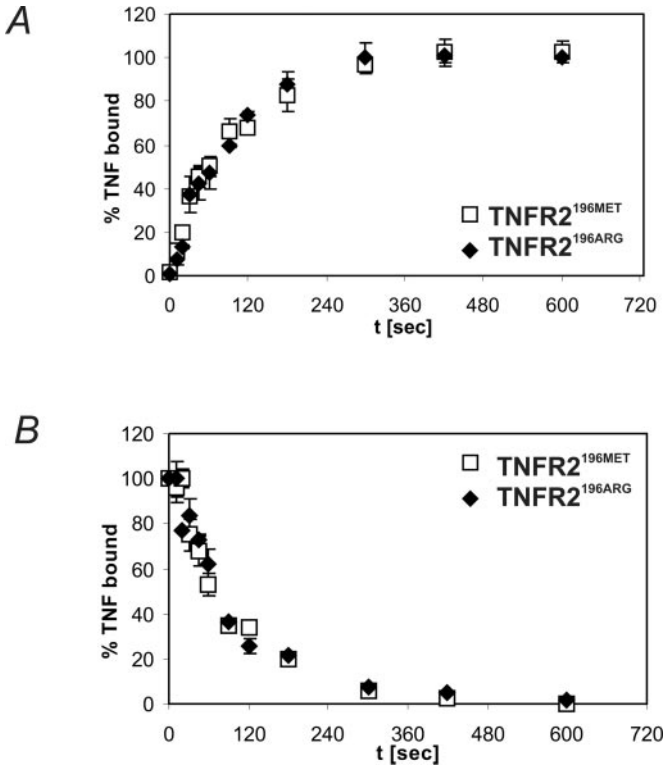

FIG. 2. **Binding parameters of wild type (TNFR2^{196MET}) and mutated TNFR2 (TNFR2^{196ARG}) at 37 °C.** *A*, TNFR2^{196MET} and TNFR2^{196ARG} MEF cells were incubated with 20 ng/ml ^{125}I-TNF at 37 °C, and cell-bound radioactivity was determined at the indicated time points. *B*, TNFR2^{196MET} and TNFR2^{196ARG} MEF cells were preincubated with 20 ng/ml ^{125}I-TNF at 4 °C. Dissociation of the radiolabeled ligand was measured at 37 °C in the presence of 100-fold excess of unlabeled TNF-α. Nonspecific binding determined in the presence of a 200-fold excess of unlabeled TNF-α was less than 5% of total binding and has been subtracted. All experiments were performed in triplicate (mean ± S.E.).

PCR (Clontech), and expression of TNF-α target genes was assayed by using sequence specific primers and standard PCR procedures.

The following primer pairs were used: β-actin, 5′-GAT GGT GGG CAT GGG TCA G-3′ (forward) and 5′-CTTAATGTCACGCACGATT-TCC-3′ (reverse); BCL-X_L, 5′-GCG TGG AAA GCG TAG ACA A-3′ (forward) and 5′-TCA TTT CCG ACT GAA GAG TGA G-3′ (reverse); cIAP1, 5′-GCC TGA TGC TGG ATA ACT GG-3′ (forward) and 5′-GCT CTT GCC AAT TCT GAT GG-3′ (reverse); cIAP2, 5′-GCC TGA TGC TGG ATA ACT GG-3′ (forward) and 5′-GGC GAC AGA AAA GTC AAT GG-3′ (reverse); IL-6, 5′-GAA CTC CTT CTC CAC AAG CGC-3′ (forward) and 5′-AGG CAA GTC TCC TCA TTG AAT CC-3′ (reverse); IL-8, 5′-ACC GGA AGG AAC CAT CTC ACT-3′ (forward) and 5′-AAA CTT CTC CAC AAC CCT CTG C-3′ (reverse); TNFR1, 5′-CTG TAC CAA GTG CCA CAA AGG A-3′ (forward) and 5′-GCT GCA ATT GAA GCA CTG GA-3′ (reverse); TRAF1, 5′-GAG GTG GAC TCT CAC CAA ATG AGA AGA-3′ (forward) and 5′-CCC TTT GGG GTT ATA CAT TGC TCA GTG G-3′ (reverse); TRAF3, 5′-AAG CAG ACA GCA TGA AGA GCA-3′ (forward) and 5′-GCT TGA ATG CAT CTC CCA AAT G-3′. Resulting amplicons were resolved on 2% agarose gels stained with ethidium bromide and visualized through a UV light digital imaging system. Images were evaluated using densitometrical software (SigmaGel, Jandel Scientific, San Rafael, CA). For each mRNA, the number of cycles directly above detection level (linear phase) was determined and used for evaluation.

ELISA—Stably transfected HeLa cells were seeded in a 24-well plate at a density of 8×10^4 cells/well. The following day cells were stimulated with freshly prepared dilutions of Cys-R2TNF as described above or left untreated. After 24 h of incubation, supernatants were collected, cleared by centrifugation, and assayed for IL-6, IL-8, and GM-CSF release using enzyme-linked immunosorbent assay kits (BIOSOURCE) and an ELISA plate reader. Normalization for cell number was performed using a modified crystal violet uptake protocol.

Fluorescence Microscopy—HeLa cells were seeded on sterile cover slips at 2×10^5 cells/well in 6-well plates and grown overnight. The next

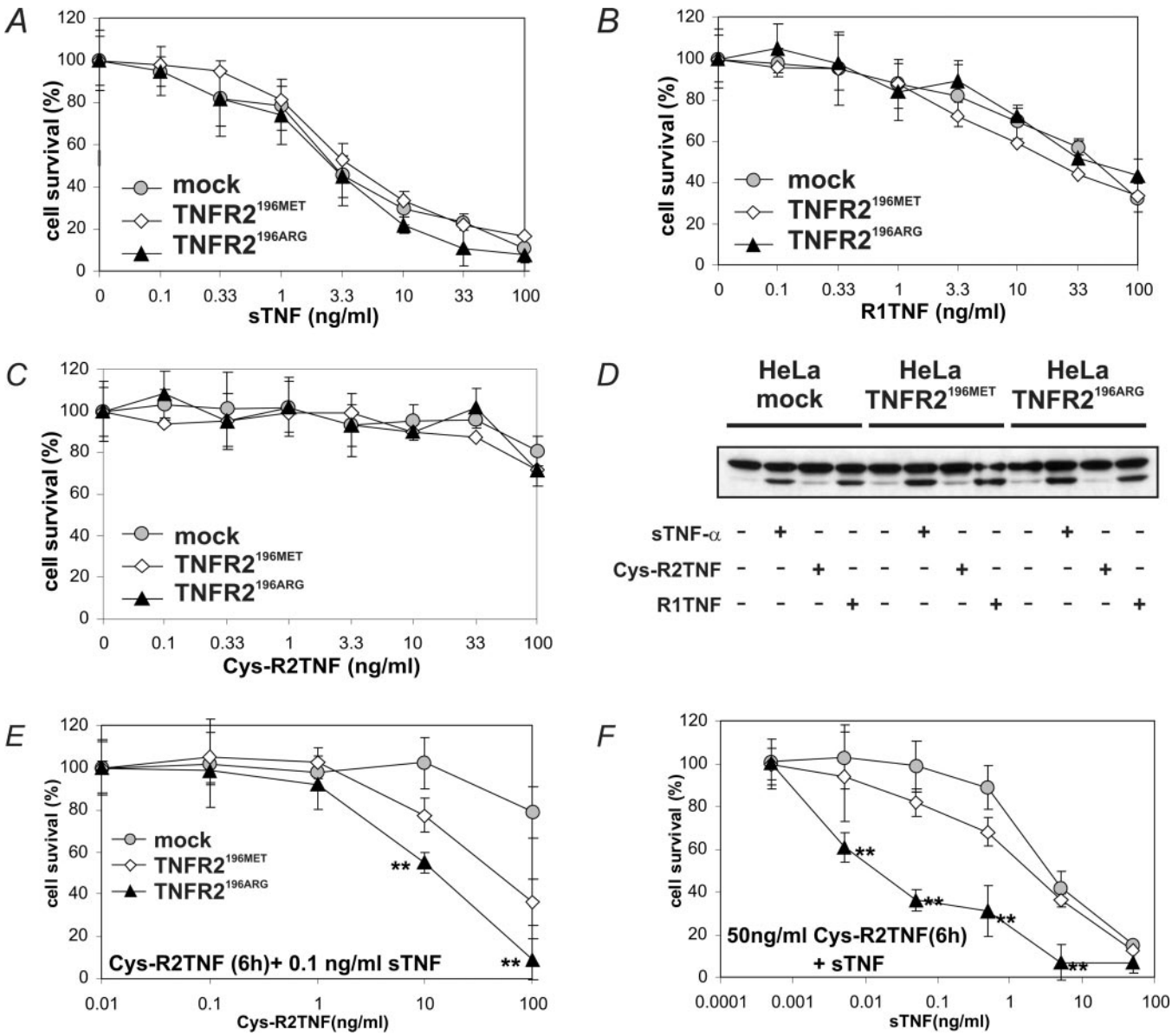

FIG. 3. **Cytotoxic effects of soluble TNF-α and TNFR-specific muteins; prestimulation of wild-type (TNFR2^{196MET}) and mutated TNFR2 (TNFR2^{196ARG}) differentially affects TNFR1-mediated cell death in HeLa cells.** Stably transfected cells were left untreated or stimulated for 24 h with various concentrations of sTNF (*A*), TNFR1-specific mutein R1TNF (*B*), or TNFR2-specific Cys-R2TNF (*C*). All experiments were performed in the presence of 2.5 μg/ml CHX. After incubation, the number of viable cells was determined by crystal violet staining as described under "Experimental Procedures." All measurements were made at least in triplicate in three independent experiments. Results are expressed as mean ± S.E. *D*, PARP cleavage as a hallmark of apoptotic cleavage events was investigated by Western blot. Representative results out of four independent experiments are shown. Reduction in cell viability and PARP cleavage was only detectable in sTNF and R1TNF treated cells. Exclusive stimulation of TNFR2 or expression of wild-type and mutant form of TNFR2 did not affect cell death. *E*, stably transfected HeLa cells were left untreated or stimulated for 24 h with 0.1 ng/ml sTNF after 6 h pretreatment with various concentrations of Cys-R2TNF. *F*, cells were left untreated or stimulated for 24 h with various concentrations of sTNF after prestimulation with 50 ng/ml Cys-R2TNF for 6 h. All experiments were performed in the presence of CHX (2.5 μg/ml). After incubation, the number of surviving cells was determined by crystal violet staining. All measurements were done at least in triplicate in three independent experiments. Results are expressed as mean ± S.E. TNFR2^{196MET} and TNFR2^{196ARG} cells differ significantly in their ability to mediate TNFR1/TNFR2 cross-talk. **, $p < 0.01$.

day cells were transiently transfected with 170 ng/well of pEGFP-TRAF2 and 333 ng/well of either pcDNA3.1TNFR2^{196MET} or pcDNA3.1TNFR2^{196ARG} using FuGENE 6 (Roche Applied Science). After 24 h the medium was replaced by medium containing cross-linking antibody 80M2 (2 μg/ml) or pure medium, followed by 30 min of incubation. Cells were stimulated with 10 or 100 ng/ml Cys-R2TNF for 10 min at 37 °C, washed twice in ice-cold PBS, fixed for 20 min at room temperature with 4% paraformaldehyde, and stained with 80M2 in PBS + bovine serum albumin (0.1%) for 30 min and goat-anti-mouse IgG-Cy3 (Molecular Probes) conjugate in the dark. Omitting the primary antibody and transfection with an empty EGFP-construct served as negative control. Slips were transferred to glass slides and examined using a TCS-SP1 confocal laser-scanning microscope (Leica Microsystems, Bensheim, Germany).

Coimmunoprecipitation—TNFR2^{196MET}-, TNFR2^{196ARG}-, or mock-transfected HeLa cells were seeded in 10-ml dishes at 4×10^6 cells/dish and grown overnight. The next day, cells were stimulated with 10 or 100 ng/ml Cys-R2TNF for 10 min, washed with ice-cold PBS, scraped off the plates, and pelleted by centrifugation. Cells were lysed in 300 μl of buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 30 mM NaF, and a mixture protease and phosphatase inhibitors (protease inhibitor mixture and PICII, Sigma) by vortexing three times for 45 s. After preclearing of lysate by incubation with protein A/G-agarose (Santa Cruz Biotechnology) and centrifugation, TNFR2 complexes were precipitated using 2 μg monoclonal mouse anti-TNFR2 IgG (Abcam) for 2 h and protein A/G-agarose for 1 h. Precipitates were washed three times, denatured by boiling in 2× Laemmli buffer, and separated by SDS-PAGE. After transfer onto nitrilotriacetic acid membranes, samples were probed

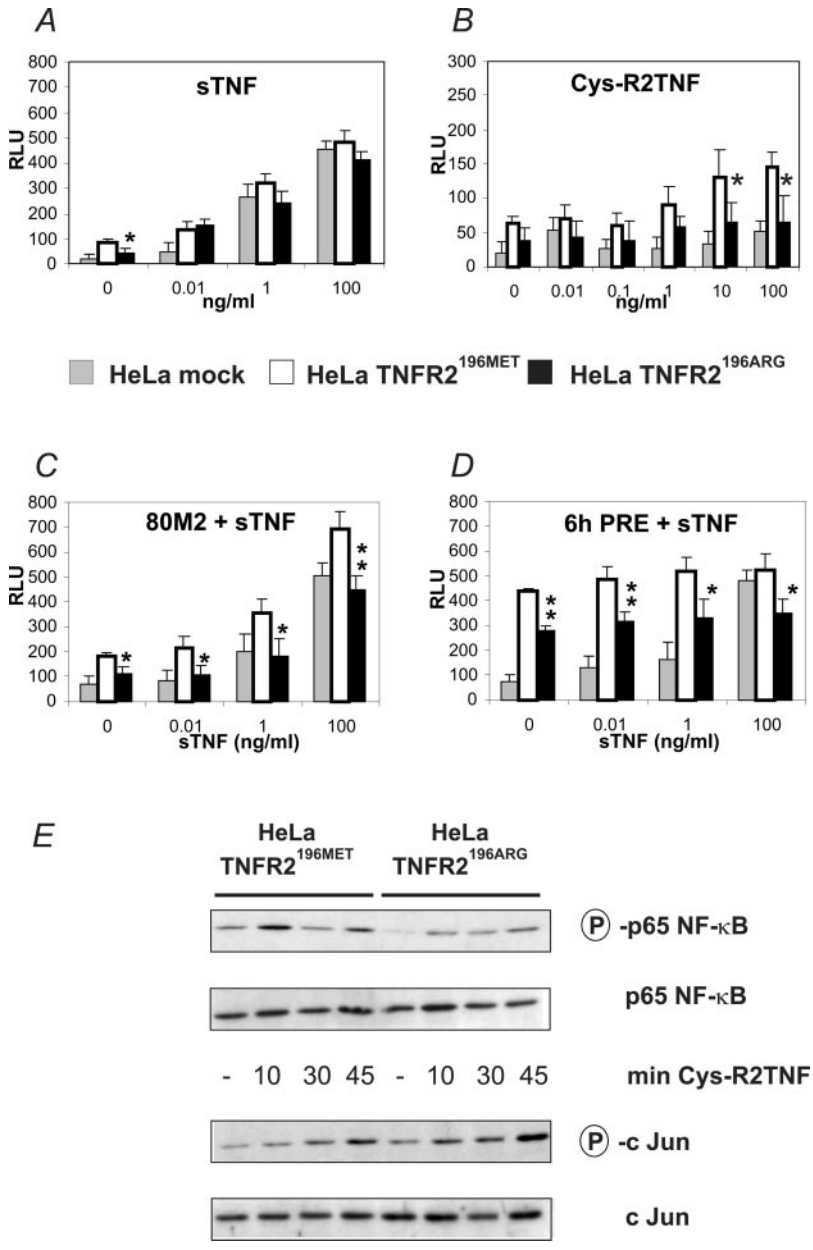

FIG. 4. **Differential induction of NF-κB activity via TNFR2^{196MET} and TNFR2^{196ARG}; cross-talk with TNFR1 signals.** *A*, HeLa cells (15,000/well) were left untreated or stimulated for 18 h with various concentrations of sTNF (*A*) or with a TNFR2-specific Cys-R2TNF (*B*). Cross-linking of TNFR2 with the monoclonal anti-TNFR2 antibody 80M2 enhances sTNF-α-induced NF-κB activity in the wild-type TNFR2^{196MET} cells. This effect is abrogated in the TNFR2^{196ARG} cells (*C*). After 6-h pretreatment with Cys-R2TNF (333 ng/ml), the luciferase activity was significantly lower in the TNFR2^{196ARG} cells (*D*). Experiments were performed using a NF-κB luciferase reporter plasmid as described. Results are expressed as relative luciferase units. Normalization regarding transfection efficiency was performed by concomitant measurement of *Renilla* luciferase activity. All stimulations were done in triplicate in ≥3 independent experiments (mean ± S.E.; *, $p < 0.05$; **, $p < 0.01$). *E*, phosphorylation of NF-κB on serine 536 and c-Jun on serine 63/73 upon TNFR2 triggering (100 ng/ml CysR2-TNF) was determined by Western blots. Note a diminished phosphorylation of NF-κB in the cells stably expressing mutant TNFR2^{196ARG} *versus* wild-type TNFR2^{196MET} (representative blot of four independent experiments).

for precipitated TNFR2 using rabbit anti-TNFR2 antibody and, after stripping, for co-precipitated TRAF2 using rabbit anti-TRAF2 IgG (Santa Cruz Biotechnology).

RESULTS

Expression of TNFR2 Variants in HeLa Cells—To investigate the effect of TNFR2 variants on TNF-α-mediated signaling events and TNF-α-induced apoptosis, we stably transfected HeLa cells with plasmids carrying the full-length cDNA for either the wild type TNFR2^{196MET} or the mutant TNFR2^{196ARG} or with empty vector (mock-transfection). HeLa cells do not express endogenous TNFR2 at detectable levels (36–38) and only express moderate amounts of TNFR1 (1,000–3,000 receptors/cell) (39). As shown in the FACS analysis in Fig. 1, stably transfected cells expressed nearly identical levels of TNFR2 (approximately 50,000 receptors/cell; data not shown), while expression was undetectable in parental and mock-transfected HeLa. The percentage of positive cells was 78.4% for TNFR2^{196MET} and 81.1% for TNFR2^{196ARG} cells. To rule out an influence of the mutation on antibody affinity, different antibodies were used which gave nearly identical results. Thus, different expression levels of the two receptor variants can be ruled out to cause the observed effects.

Physiological Binding Characteristics of TNFR2^{196MET} and TNFR2^{196ARG} Variants—The determination of binding kinetics under physiological conditions (37 °C) showed no differences between the two variants of TNFR2 in TNFR1/2 double-deficient MEF cells reconstituted with either TNFR2^{196MET} or TNFR2^{196ARG} (Fig. 2). Association of radioactively labeled TNF (^{125}I-TNF) at 10 ng/ml appeared with a $t_{½}$ of 60–65 s for both variants, dissociation half-life time was determined with $t_{½}$ of 68–75 s. Both binding parameters are in good accordance with previous results for wild-type TNFR2 (19, 36).

Differential Induction of Cell Death by TNFR2^{196MET} and TNFR2^{196ARG} Variants; Influences of Soluble TNF-α, Cross-linking, and Receptor-specific Muteins—In a first approach, we studied the induction of cell death by different stimuli in our cell system. As described earlier, HeLa cells do not undergo apoptosis by TNF-α alone, but are sensitive to TNF-α in the presence of low doses of the protein synthesis inhibitor CHX (2.5 μg/ml) (40). To molecularly dissect TNFR signaling, recep-

tor specific TNF muteins were used: (i) CysTNF143N/145R (CysR2TNF) for a specific activation of TNFR2, which carries an additional cysteine residue thus mimicking a stimulation via membrane bound TNF-α and TNF32W/86T (R1TNF) for exclusive stimulation of TNFR1 (19). The data in Fig. 3 show that, in contrast to TNFR1, exclusive activation of TNFR2 alone or in combination with the cross-linking antibody 80M2, which enhances the stability of TNF-α/TNFR2 complexes (18), was not able to induce cell death in any of our cell lines (Fig. 3*C*; data for 80M2 not shown). This result is consistent with the inability of TNFR2 to recruit DD-containing adaptor proteins and to engage the caspase cascade leading to the extrinsic type of apoptosis. In contrast to this soluble TNF-α (sTNF) and a TNFR1-specific mutein cause a reduction in cell viability in a dose-dependent manner, as shown in Fig. 3, *A* and *B*. Stimulation with either TNFR1-specific R1TNF (Fig. 3*B*) or sTNF (Fig. 3*A*) did not result in differential induction of cell death in the three cell lines. The cytotoxic effects observed in the viability assays were paralleled by cleavage of poly(ADP-ribose) polymerase (PARP), one of the hallmarks of apoptotic cell death. Only stimuli triggering TNFR1 (sTNF, R1-TNF) were able to induce PARP processing efficiently, while R2-TNF alone did not result in the generation of the 85-kDa cleavage product (Fig. 3*D*).

Subsequently, we studied the induction of apoptosis under conditions where TNFR2 is exclusively triggered before TNFR1 is activated. This kind of experimental setup has been used to clarify the molecular mechanisms underlying the molecular cross-talk of TNFR2-dependent enhancement of TNFR1-mediated cytotoxic effects (15, 17). In fact, our data show a dose-dependent enhancement of TNFR1-dependent apoptosis induction in TNFR2-positive cells with a significantly stronger effect on $TNFR2^{196ARG}$ cells. The cytotoxic effect of sTNF could in part (>85%) be blocked by the pan-caspase-inhibitor Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) indicating that caspases are crucially involved (data not shown).

With a fixed concentration of death-inducing sTNF (0.1 ng/ml), the amount of TNFR2-specific mutein sufficient to induce apoptosis enhancement was significantly lower in TNFR2-positive HeLa cells when compared with mock-transfected cells. The $TNFR2^{196ARG}$ variant showed an even higher sensitivity (Fig. 3*E*). *Vice versa*, by prestimulation with 50 ng/ml Cys-R2TNF for 6 h, the death-inducing concentration of sTNF was significantly lower in $TNFR2^{196ARG}$ cells than in $TNFR2^{196MET}$- or mock-HeLa cells (Fig. 3*F*).

The results demonstrate that the presence of the $TNFR2^{196ARG}$ variant leads to a higher sensitivity for apoptotic TNFR1 signaling than the wild-type $TNFR2^{196ARG}$.

Signal Transduction Pathways Engaged by TNFR2 Triggering; Evidence for an Impaired NF-κB Response in the $TNFR2^{196ARG}$ Cells—To clarify quantitative differences of NF-κB signaling between the TNFR2 variants, NF-κB reporter gene assays were carried out. As shown in Fig. 4*A*, NF-κB activation was not different among the three different cell lines when stimulation was performed with sTNF alone. No differences were also observed when triggering TNFR1 with R1-TNF (data not shown). Exclusive triggering of TNFR2 by Cys-R2TNF led to a pronounced NF-κB activation in $TNFR2^{196MET}$ cells, whereas the $TNFR2^{196ARG}$ cells exhibited a significantly lower luciferase activity (Fig. 4*B*). Cross-linking of TNFR2 with 80M2 positively affected NF-κB signaling in $TNFR2^{196MET}$ cells treated with sTNF, while pretreatment of $TNFR2^{196ARG}$ cells did not result in an increase of NF-κB activation (Fig. 4*C*). To further investigate a cross-talk between the two TNFRs cells were stimulated with a TNFR2 receptor specific mutein (Cys-R2TNF) 6 h before triggering TNFR1 signaling. This pre-

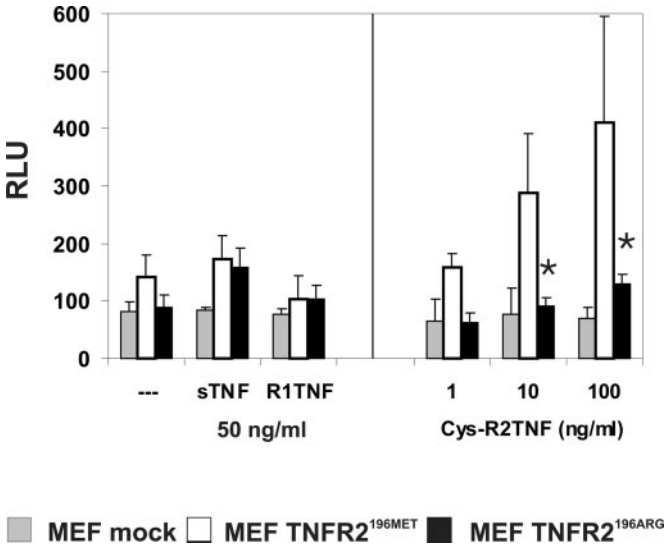

FIG. 5. **Differential induction of NF-κB activity via $TNFR2^{196MET}$ and $TNFR2^{196ARG}$ in TNFR1/TNFR2 double-deficient mouse fibroblasts.** TNFR1/$TNFR2^{-/-}$ mouse embryonic fibroblasts transiently transfected with the human TNFR2 variants were stimulated for 6 h with either sTNF (50 ng/ml), R1TNF (50 ng/ml), or Cys-R2TNF (1–100 ng/ml). NF-κB activity was assessed by dual luciferase assay as described. Normalization regarding transfection efficiency was performed by concomitant measurement of *Renilla* luciferase activity. All stimulations were done in triplicate in $n = 4$ independent experiments. Results are expressed in mean relative light units ± S.E.; *, $p < 0.05$. Upon TNFR2 triggering, cells transfected with the mutated $TNFR2^{196ARG}$ variant showed a significantly lower activation of NF-κB than cells tranfected with wild-type $TNFR2^{196MET}$. The observed effect was independent from a prior TNFR2-cross-linking with 80M2 (data not shown).

treatment results in a weak activation of NF-κB via TNFR2. However, it causes an inability of prestimulated wildtype TNFR2 positive cells to adequately react on subsequent TNFR1-stimulation resulting in a diminished activation of the NF-κB pathway when compared with cells transfected with an empty control vector. Interestingly, the pretreatment of $TNFR2^{196ARG}$ cells also led to a similar negative TNFR1/TNFR2 cross-talk, the cells only significantly differed in their CysR2TNF-induced NF-κB activation level with a lower luciferase levels in the mutated $TNFR2^{196ARG}$ cells under the same conditions (Fig. 4*D*).

These findings were reflected in different NF-κB phosphorylation patterns upon stimulation with the TNFR2-specific TNF-α mutein (Cys-R2TNF). Whereas mock-transfected (and parental) HeLa cells did not show any response (data not shown), a time-dependent phosphorylation of p65NFκB (Fig. 4*E*) could be demonstrated in $TNFR2^{196MET}$ as well as $TNFR2^{196ARG}$ cells. A significantly altered NF-κB phosphorylation on serine 536 was detectable in $TNFR2^{196ARG}$ cells when compared with $TNFR2^{196MET}$ cells in the Western blot analysis. No activation of p38 MAPK and ERK1/2 was seen (data not shown); however, there was a clear time-dependent phosphorylation of c-Jun, which did not differ among the $TNFR2^{196MET}$ and $TNFR2^{196ARG}$ cells indicating that in both our cell lines JNKs are similarly activated upon TNFR2-triggering.

To make sure that our findings were independent from the cell type and to investigate TNFR2 signaling in the absence of endogenous TNFR1, NF-κB reporter gene assays were also performed in TNFR1/TNFR2-double-deficient mouse fibroblasts (MEF). In cells transiently transfected with TNFR2 constructs, only TNFR2-specific stimuli (Cys-R2TNF) are capable of significantly triggering NF-κB activation (Fig. 5). Dose-response correlations demonstrate a higher sensitivity of the $TNFR2^{196MET}$-transfected cells, which confirms the above find-

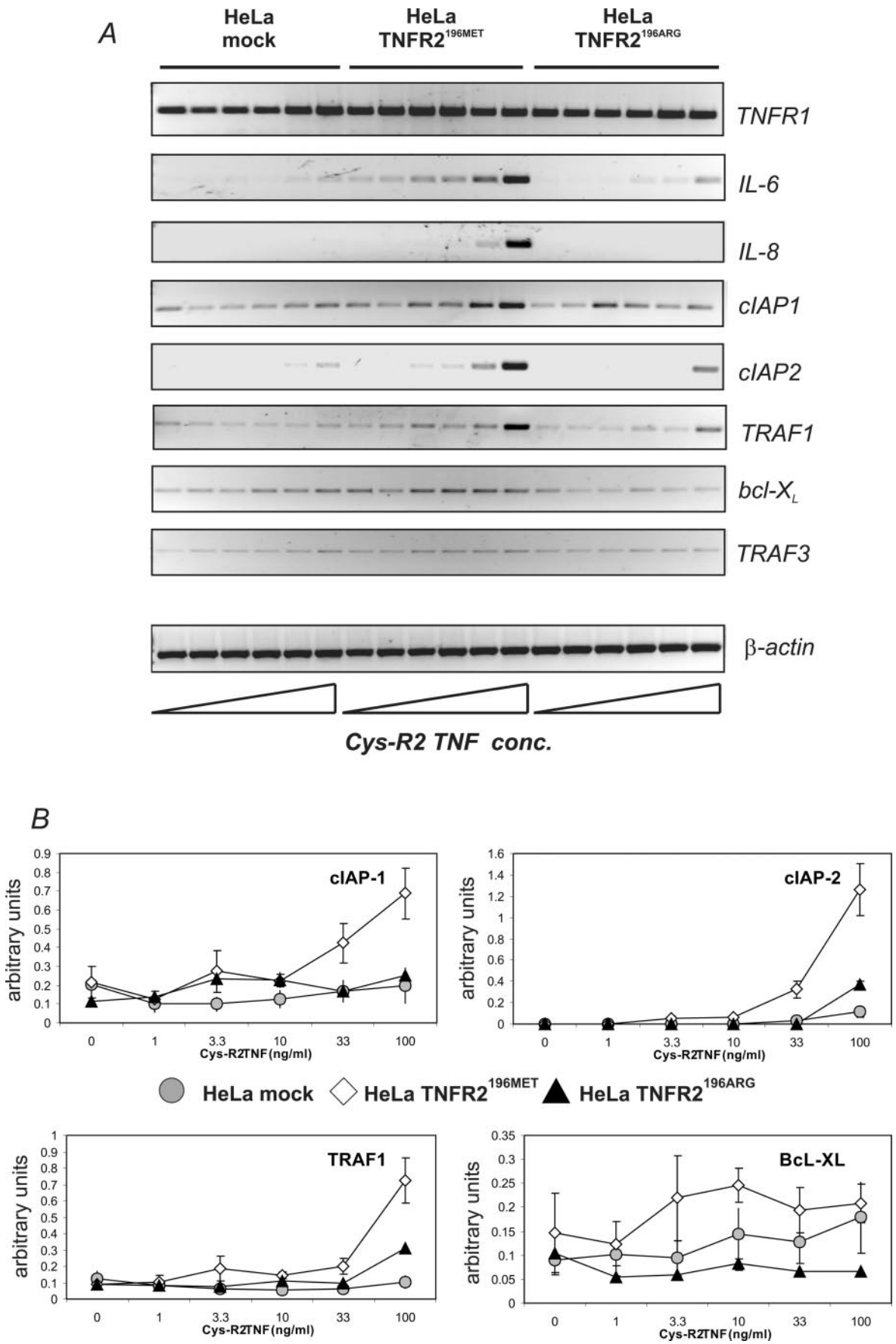

FIG. 6. **Expression of TNF-α target genes.** Stably transfected HeLa cells were stimulated for 24 h with rising concentrations of Cys-R2TNF (0, 1, 3, 10, 33, and 100 ng/ml). After isolation of total RNA, reverse transcription was performed and TNF target gene expression was assayed by semiquantitative RT-PCR; representative results of five independent experiments are shown (*A*). Densitometric analysis was performed to analyze TNFR2-dependent gene expression. Optical densities were normalized for β-actin (mean ± S.E. from five independent experiments) (*B*).

ings derived from stable HeLa cells in the absence of endogenous TNFRs.

Analysis of NF-κB-dependent Target Gene Expression—To investigate whether the differential activation of the NF-κB pathway is also represented by an altered induction of target genes, we investigated the expression profile of known NF-κB dependent anti-apoptotic and pro-inflammatory genes, *e.g.* c-IAP1, c-IAP2, bcl-2, bcl-X_L, MnSOD, c-$FLIP_L$, GM-CSF, IL-6, and IL-8. As expected, an induction of the tested NF-κB target genes upon TNFR2-specific stimulation was only detectable in TNFR2-positive cells. However, the induction of target genes was significantly lower in $TNFR2^{196ARG}$ cells in comparison to $TNFR2^{196MET}$ cells (Fig. 6). It is important to note that we did not find differences in expression of TNFR1 among the three cell lines nor did we see different expression of TNFR2 variants (Figs. 1 and 6). For IL-6 and IL-8, the findings were confirmed at the protein level by ELISA (Fig. 7).

Differential Interaction of TNFR2 Variants and TRAF2; Fluorescence Microscopy and Immunoprecipitation Studies—To gain insight into the differential properties of the TNFR2 variants to recruit the downstream adaptor protein TRAF2, co-localization studies of TNFR2 with co-transfected EGFP-TRAF2 were performed by confocal laser scanning microscopy. The two variants of the TNFR2 showed different recruitment behavior. As shown in Fig. 8*A*, stimulation of $TNFR2^{196MET}$ with 10 or 100 ng/ml Cys-R2TNF resulted in a recruitment of cytosolic EGFP-TRAF2 to the membrane receptor complexes. Pretreatment of the cells with 80M2 enhanced this effect at low concentrations of mutein (data not shown). In contrast to this, $TNFR2^{196ARG}$ showed a significant lower ability to associate with TRAF2 fusion proteins upon stimulation, which is depicted by a lack of stimulus-dependent co-localization of TRAF2 to membrane TNFR2 complexes.

To confirm these results, native TNFR2 was immunoprecipitated and the amount of complexed TRAF2 was investigated by Western blot. Again, an impaired ability of the $TNFR2^{196ARG}$ variant to recruit the adaptor protein TRAF2 could be demonstrated. After 10 min of stimulation with 10 or 100 ng/ml Cys-R2TNF, the amount of TRAF2 associated to TNFR2 is significantly reduced in $TNFR2^{196ARG}$cells when compared with $TNFR2^{196MET}$ expressing cells. As TNFR2 protein was present at comparable levels in both precipitates, the altered TRAF2 signal does not reflect an altered affinity of the TNFR2 antibody against the two variants (Fig. 8*B*). Precipitates of HeLa cells transfected with an empty control vector were used as specificity controls and showed no TRAF2 signal. These data suggest that the limited TNFR2-mediated activation of NF-κB in $TNFR2^{196ARG}$ expressing cells is manifested at the level of RISC (receptor-induced signaling complex) formation.

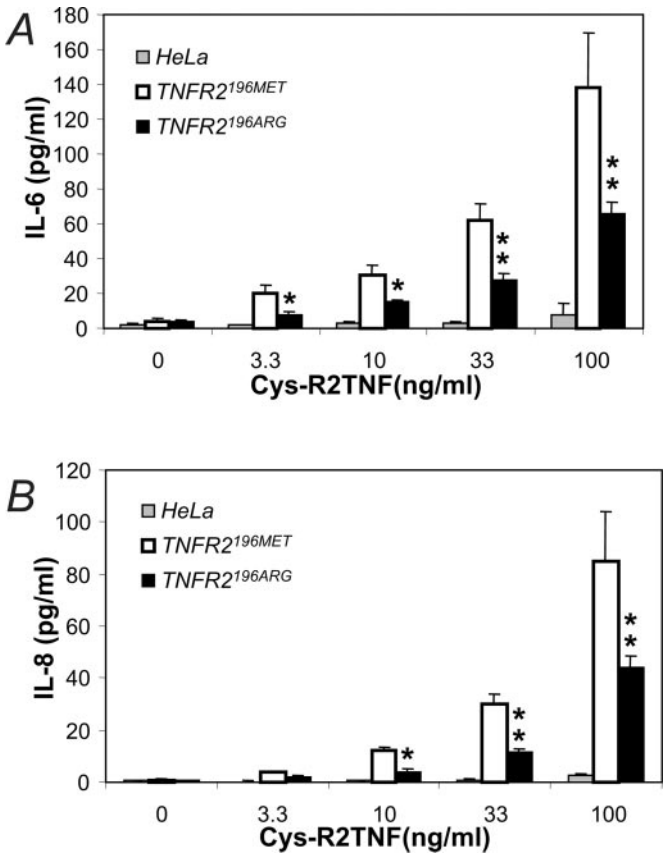

FIG. 7. **TNFR2-dependent cytokine secretion; IL-6 and IL-8 ELISA.** Stably transfected HeLa (8×10^4 cells/well) were stimulated with Cys-R2TNF or left untreated for 24 h. Supernatants were collected and assayed for IL-6 (*A*) and IL-8 (*B*) release using enzyme-linked immunosorbent assay kits (BIOSOURCE) and an ELISA plate reader. For normalization regarding cell number a modified crystal violet uptake protocol was used ($n = 3$ independent experiments in duplicate; *, $p < 0.05$; **, $p < 0.01$).

DISCUSSION

We have investigated the functional impact of a single nucleotide polymorphism in exon 6 of the TNFR2 gene. This polymorphism results in an amino acid exchange from methionine to arginine at position 196 in the protein sequence (26) and has been genetically associated with an increased susceptibility for the development of chronic inflammatory disorders, *e.g.* systemic lupus erythrematosus and familial rheumatoid arthritis, and other non-inflammatory diseases such as polycystic ovary syndrome (42) or narcolepsy (43, 44). The juxtamembrane amino acid residue on position 196 in the CRD4 of the extracellular part of the receptor is in close proximity to proline at position 211, which has been found crucial for the shedding process of TNFR2 (45). Nevertheless neither release of soluble receptors from the cell surface nor physical binding parameters appear to be influenced by the substitution (Ref. 39 and Fig. 2).

The salient finding of the present study is the observation that the $TNFR2^{196ARG}$ risk variant has a significantly lower capability to induce direct NF-κB signaling via TNFR2 (Fig. 4). The diminished capability of the mutated $TNFR2^{196ARG}$ to induce NF-κB activation is paralleled by a diminished induction of NF-κB-dependent target genes conveying either anti-apoptotic or pro-inflammatory functions, such as cIAP1, cIAP2, TRAF1, IL-6, and IL-8 (Figs. 5 and 6). It has been demonstrated that TNFR2-induced signaling not only involves the NF-κB pathway but may also result in an activation of p38 MAPK, ERK1/2, and JNK/SAPK (46). Our results suggest that the $TNFR2^{196ARG}$ mutation does not affect JNK/SAPK and other MAPK signaling events (Fig. 4*E* and data not shown). However, it must be noted that TNFR2-mediated MAPK activation has been only explicitly described in macrophages (46). Albeit our results in HeLa and MEF cells are suggestive for an involvement of MAPKs in signaling events downstream of TNFR2 also in other cell types, further systematic studies will be required to exclude lineage-specific molecular differences. No direct effect on apoptosis can be detected by means of selective TNFR2 stimulation (Fig. 2, *C* and *D*). TNFR1-mediated apoptosis is not influenced by the pure presence of wild-type or mutant TNFR2 (Fig. 3*A*, B and D), whereas prestimulation of TNFR2 induced increased levels of apoptosis downstream of a subsequent TNFR1 stimulation by soluble TNF-α (Fig. 3, *E* and *F*). This finding is in accordance to previous observations of TNFR1/TNFR2 receptor cross-talk (15, 19, 47). The selective prestimulation of TNFR2 through a TNFR2-specific TNF-α mutein leads to an abrogation of NF-κB activation via TNFR1 upon a second TNF-α pulse (Fig. 3*D*). Interestingly, the

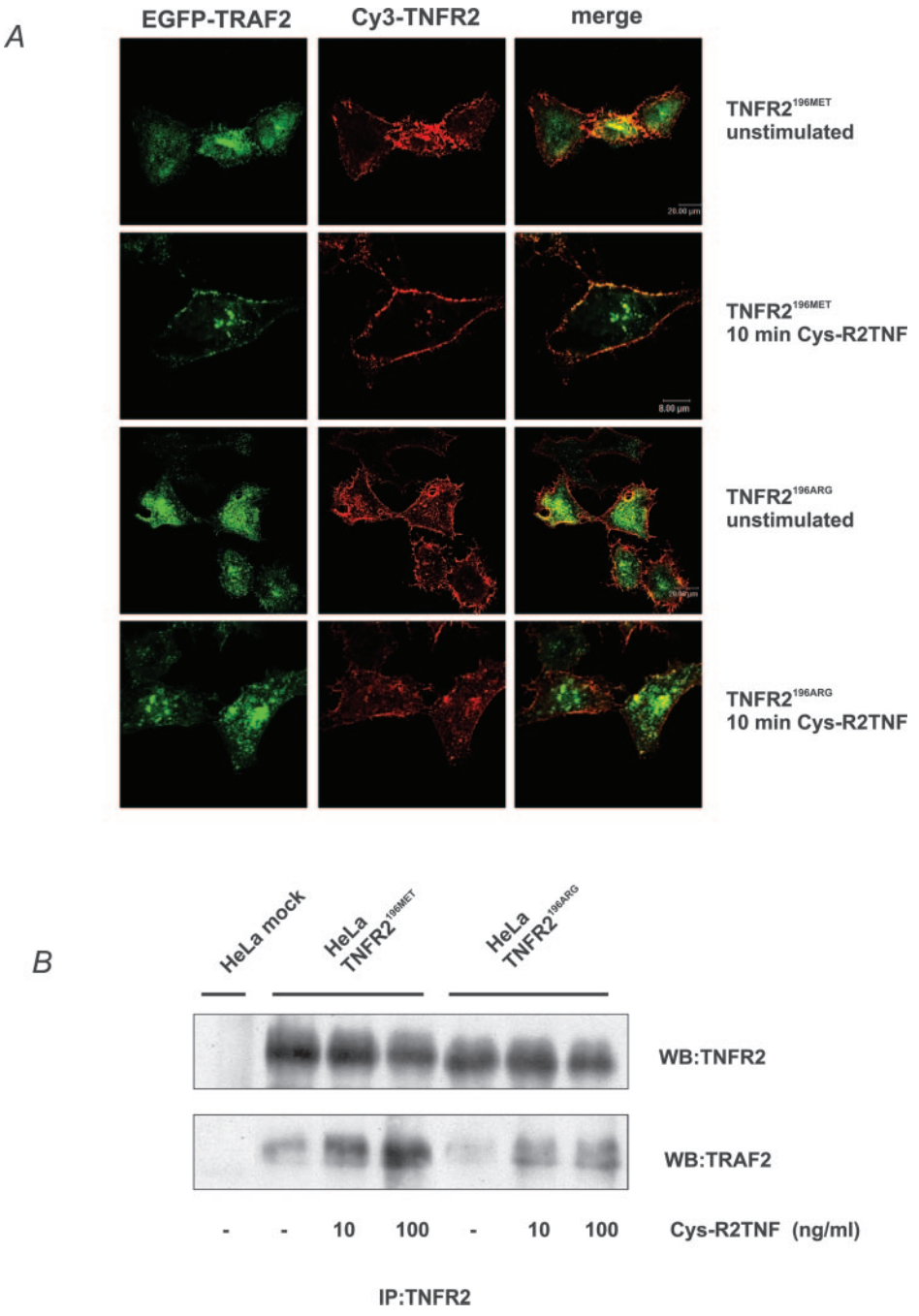

FIG. 8. **The stimulus-induced recruitment of TRAF2 to TNFR2 is diminished in cells carrying the mutated TNFR2^{196ARG}.** *A*, co-localization of TNFR2 variants and GFP-TRAF2 after stimulation with Cys-R2TNF. Transiently transfected HeLa cells were stimulated as indicated for 10 min, washed, and fixed. TNFR2 was visualized with anti-TNFR2 IgG and Cy3-coniugated secondary antibody. Localization of TRAF2 and TNFR2 was detemined by confocal laser scanning microscopy. Note that a strong co-localization is only observed in the wildtype TNFR2^{196MET} cells. *B*, HeLa cells stably overexpressing the two TNFR2 variants were stimulated with 10 or 100 ng/ml Cys-R2TNF for 10 min, washed, pelleted, and lysed. TNFR2 complexes were precipitated using monoclonal mouse anti-TNFR2 IgG and protein A/G-agarose. Precipitates were separated by SDS-PAGE, and samples were probed for precipitated TNFR2 and for co-precipitated TRAF2 by Western blotting. The recruitment of cytosolic TRAF2 is weaker in the mutated TNFR2^{196ARG} cells.

wild-type TNFR2^{196MET} and the variant TNFR2^{196ARG} do not differ in their capability to inhibit TNFR1-mediated NF-κB activation.

It has been proposed that (pre)stimulation of TNFR2 enhances pro-apoptotic signaling of TNFR1 via a stochiometric competition for newly synthesized TRAF2-bound antiapoptotic factors, such as cIAP1, cIAP2, and TRAF1 (17). We demonstrate a diminished recruitment of TRAF2 to the mutated TNFR2^{196ARG} variant upon TNFR2 triggering, which cannot be explained by a differential binding property for TNF-α between variant and wildtype TNFR2 (Fig. 2 and Ref. 39). A diminished recruitment of cytosolic TRAF2 to TNFR2 could also lead to an inhibition of apoptosis downstream of TNFR1 via a higher availability of TRAF2 for TNFR1-induced NF-κB activation. However, we find a clear enhancement of TNFR1-mediated cell death via wild type TNFR2, which is further enhanced in the TNFR2^{196ARG} variant (Fig. 3, *E* and *F*). By virtue of a lack of an adequate TRAF2 recruitment, the TNFR2 mutation may have two different functions contributing to an increased TNFR1 induced cell death: (i) upon TNFR2 prestimulation, the altered TRAF2 recruitment to the TNFR2^{196ARG} variant may lead to a deficient expression of NF-κB-dependent anti-apoptotic factors including cIAP1, cIAP2, A20, and TRAF1. As a result, an impaired formation of TRAF2/TRAF1/cIAP1/cIAP2 complexes would fail to inhibit caspase-8 activation and lead to an enhanced TNFR1-induced apoptotic pathway. An involvement of the CHX-sensitive NF-κB target gene cFLIP (40) seems unlikely, as it is not differentially regulated via a direct TNFR2 stimulus.[2] Interestingly, we demonstrate a

[2] A. Till, P. Rosenstiel, A. Krippner-Heidenreich, S. Mascheretti-Croucher, P. J. P. Croucher, H. Schäfer, P. Scheurich, D. Seegert, and S. Schreiber, unpublished data.

TRAF2-independent TNFR2-induced JNK/c-Jun activation, as it is not altered in the mutated $TNFR2^{196ARG}$ cells (Fig. 3*E*). Together with a diminished NF-κB activation, this signaling cascade could further contribute to an enhancement of TNFR1-mediated apoptosis. (ii) The diminished expression level of Bcl-X_L in the $TNFR2^{196ARG}$ cells upon TNFR2 triggering could also play a key role, as TNFR2-induced Bcl-X_L may serve to counteract the mitochondrial apoptotic amplification loop. The impaired NF-κB activation may also affect basal autocrine loops of pro-inflammatory cytokines, which are capable of exhibiting anti-apoptotic roles, as shown for NF-κB dependent autocrine IL-1β release in pancreatic cancer cells (48).

An exact structural model explaining the mechanism of the differential TRAF2 recruitment to the two TNFR2 variants still remains to be elucidated. A direct influence of the extracellular amino acid exchange on the conformation of the intracellular TRAF2 binding motif seems rather unlikely. With the mutation neither affecting receptor affinity nor receptor shedding, the effect has to be temporospatially located between the binding of TNF-α and the recruitment of cytosolic TRAF2, *e.g.* at the level of cluster formation (19).

There is growing evidence for an involvement of TNFR2 as a major factor in the pathophysiology of human disease. In intestinal inflammation, TNFR2 is up-regulated on lamina propria and peripheral blood T cells of patients suffering from Crohn's disease (49). Furthermore, an important role for TNFR2 in the maintenance of intestinal barrier integrity and differentiation of intestinal epithelial cells has been suggested (50, 51). $TNFR2^{-/-}$ mice show normal T cell development but decreased sensitivity to activation induced cell death indicating that TNFR2 may enhance the toxic functions of TNFR1 or other death receptors (52, 53). Moreover, $TNFR2^{-/-}$ mice are less susceptible to develop chronic inflammatory disorders resembling human diseases, such as inflammatory bowel disease and polyarthritis (54–56). In contrast in experimental allergic encephalomyelitis, a model of human multiple sclerosis, TNFR2 deficiency leads to an enhancement of demyelination and severity of symptoms (41).

Our results point to a specific deficiency of the $TNFR2^{196ARG}$ variant in the recruitment of TRAF2 (Fig. 8) and induction of the NF-κB pathway via TNFR1/TNFR2 cross-talk. It is important to note that activation of TNFR2 alone conveys a much weaker NF-κB response when compared with TNFR1-driven signaling. Despite the fact that under most physiological conditions TNFR1 signaling overrules signals by TNFR2, a significant influence of TNFR2 might be elicited by a cross-talk of the two receptors. The altered induction of apoptosis and the NF-κB pathway may represent a common modifying mechanism, which could serve as an explanation for the association of the $TNFR2^{196ARG}$ variant with an increased susceptibility for chronic inflammatory and proliferative disorders.

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REFERENCES

1. Aggarwal, B. B. (2003) *Nat. Rev. Immunol.* **3,** 745–756
2. Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003) *Cell Death Differ.* **10,** 45–65
3. Ashkenazi, A., and Dixit, V. M. (1998) *Science* **281,** 1305–1308
4. Idriss, H. T., and Naismith, J. H. (2000) *Microsc. Res. Tech.* **50,** 184–195
5. Smith, C. A., Farrah, T., and Goodwin, R. G. (1994) *Cell* **76,** 959–962
6. Tartaglia, L. A., Pennica, D., and Goeddel, D. V. (1993) *J. Biol. Chem.* **268,** 18542–18548
7. Baker, S. J., and Reddy, E. P. (1998) *Oncogene* **17,** 3261–3270
8. Song, H. Y., and Donner, D. B. (1995) *Biochem. J.* **309,** 825–829
9. Boldin, M. P., Mett, I. L., Varfolomeev, E. E., Chumakov, I., Shemer-Avni, Y., Camonis, J. H., and Wallach, D. (1995) *J. Biol. Chem.* **270,** 387–391
10. Dembic, Z., Loetscher, H., Gubler, U., Pan, Y. C., Lahm, H. W., Gentz, R., Brockhaus, M., and Lesslauer, W. (1990) *Cytokine* **2,** 231–237
11. 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) *EMBO J.* **18,** 3034–3043
12. Zheng, L., Fisher, G., Miller, R. E., Peschon, J., Lynch, D. H., and Lenardo, M. J. (1995) *Nature* **377,** 348–351
13. Vercammen, D., Vandenabeele, P., Declercq, W., Van de Craen, M., Grooten, J., and Fiers, W. (1995) *Cytokine* **7,** 463–470
14. Weiss, T., Grell, M., Hessabi, B., Bourteele, S., Muller, G., Scheurich, P., and Wajant, H. (1997) *J. Immunol.* **158,** 2398–2404
15. Weiss, T., Grell, M., Siemienski, K., Muhlenbeck, F., Durkop, H., Pfizenmaier, K., Scheurich, P., and Wajant, H. (1998) *J. Immunol.* **161,** 3136–3142
16. Li, X., Yang, Y., and Ashwell, J. D. (2002) *Nature* **416,** 345–347
17. Fotin-Mleczek, M., Henkler, F., Samel, D., Reichwein, M., Hausser, A., Parmryd, I., Scheurich, P., Schmid, J. A., and Wajant, H. (2002) *J. Cell Sci.* **115,** 2757–2770
18. Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995) *Cell* **83,** 793–802
19. Krippner-Heidenreich, A., Tubing, F., Bryde, S., Willi, S., Zimmermann, G., and Scheurich, P. (2002) *J. Biol. Chem.* **277,** 44155–44163
20. Loetscher, H., Stueber, D., Banner, D., Mackay, F., and Lesslauer, W. (1993) *J. Biol. Chem.* **268,** 26350–26357
21. Haridas, V., Darnay, B. G., Natarajan, K., Heller, R., and Aggarwal, B. B. (1998) *J. Immunol.* **160,** 3152–3162
22. Reinhard, C., Shamoon, B., Shyamala, V., and Williams, L. T. (1997) *EMBO J.* **16,** 1080–1092
23. Helms, M. J., Mohamed, A. A., and MacEwan, D. J. (2001) *FEBS Lett.* **505,** 68–74
24. Jupp, O. J., McFarlane, S. M., Anderson, H. M., Littlejohn, A. F., Mohamed, A. A., MacKay, R. H., Vandenabeele, P., and MacEwan, D. J. (2001) *Biochem. J.* **359,** 525–535
25. Beltinger, C. P., White, P. S., Maris, J. M., Sulman, E. P., Jensen, S. J., LePaslier, D., Stallard, B. J., Goeddel, D. V., de Sauvage, F. J., and Brodeur, G. M. (1996) *Genomics* **35,** 94–100
26. Pantelidis, P., Lympany, P. A., Foley, P. J., Fanning, G. C., Welsh, K. I., and du Bois, R. M. (1999) *Tissue Antigens* **54,** 585–591
27. Komata, T., Tsuchiya, N., Matsushita, M., Hagiwara, K., and Tokunaga, K. (1999) *Tissue Antigens* **53,** 527–533
28. Tsuchiya, N., Komata, T., Matsushita, M., Ohashi, J., and Tokunaga, K. (2000) *Genes Immun.* **1,** 501–503
29. Nishimura, M., Maeda, M., Matsuoka, M., Mine, H., Saji, H., Matsui, M., Kuroda, Y., Kawakami, H., and Uchiyama, T. (2000) *Hum. Immunol.* **61,** 1262–1269
30. Barton, A., John, S., Ollier, W. E., Silman, A., and Worthington, J. (2001) *Arthritis Rheum.* **44,** 61–65
31. Dieude, P., Petit, E., Cailleau-Moindrault, S., Osorio, J., Pierlot, C., Martinez, M., Faure, S., Alibert, O., Lasbleiz, S., De Toma, C., Bardin, T., Prum, B., and Cornelis, F. (2002) *Arthritis Rheum* **46,** 2039–2044
32. Pierik, M., Vermeire, S., Steen, K. V., Joossens, S., Claessens, G., Vlietinck, R., and Rutgeerts, P. (2004) *Aliment Pharmacol. Ther.* **20,** 303–310
33. Grell, M., Scheurich, P., Meager, A., and Pfizenmaier, K. (1993) *Lymphokine Cytokine Res.* **12,** 143–148
34. Aggarwal, B. B., Moffat, B., and Harkins, R. N. (1984) *J. Biol. Chem.* **259,** 686–691
35. Rosenstiel, P., Fantini, M., Brautigam, K., Kuhbacher, T., Waetzig, G. H., Seegert, D., and Schreiber, S. (2003) *Gastroenterology* **124,** 1001–1009
36. Grell, M., Wajant, H., Zimmermann, G., and Scheurich, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 570–575
37. Thoma, B., Grell, M., Pfizenmaier, K., and Scheurich, P. (1990) *J. Exp. Med.* **172,** 1019–1023
38. Brockhaus, M., Schoenfeld, H. J., Schlaeger, E. J., Hunziker, W., Lesslauer, W., and Loetscher, H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3127–3131
39. Morita, C., Horiuchi, T., Tsukamoto, H., Hatta, N., Kikuchi, Y., Arinobu, Y., Otsuka, T., Sawabe, T., Harashima, S., Nagasawa, K., and Niho, Y. (2001) *Arthritis Rheum.* **44,** 2819–2827
40. Kreuz, S., Siegmund, D., Scheurich, P., and Wajant, H. (2001) *Mol. Cell. Biol.* **21,** 3964–3973
41. Eugster, H. P., Frei, K., Bachmann, R., Bluethmann, H., Lassmann, H., and Fontana, A. (1999) *Eur. J. Immunol.* **29,** 626–632
42. Peral, B., San Millan, J. L., Castello, R., Moghetti, P., and Escobar-Morreale, H. F. (2002) *J. Clin. Endocrinol. Metab.* **87,** 3977–3983
43. Hohjoh, H., Terada, N., Kawashima, M., Honda, Y., and Tokunaga, K. (2000) *Tissue Antigens* **56,** 446–448
44. Hohjoh, H., Terada, N., Nakayama, T., Kawashima, M., Miyagawa, T., Honda, Y., and Tokunaga, K. (2001) *Tissue Antigens* **57,** 230–235
45. Herman, C., and Chernajovsky, Y. (1998) *J. Immunol.* **160,** 2478–2487
46. Mukhopadhyay, A., Suttles, J., Stout, R. D., and Aggarwal, B. B. (2001) *J. Biol. Chem.* **276,** 31906–31912
47. Weingartner, M., Siegmund, D., Schlecht, U., Fotin-Mleczek, M., Scheurich, P., and Wajant, H. (2002) *J. Biol. Chem.* **277,** 34853–34859
48. Arlt, A., Vorndamm, J., Muerkoster, S., Yu, H., Schmidt, W. E., Folsch, U. R., and Schafer, H. (2002) *Cancer Res.* **62,** 910–916
49. Holtmann, M. H., Douni, E., Schutz, M., Zeller, G., Mudter, J., Lehr, H. A., Gerspach, J., Scheurich, P., Galle, P. R., Kollias, G., and Neurath, M. F. (2002) *Eur. J. Immunol.* **32,** 3142–3151
50. Mizoguchi, E., Mizoguchi, A., Takedatsu, H., Cario, E., de Jong, Y. P., Ooi, C. J., Xavier, R. J., Terhorst, C., Podolsky, D. K., and Bhan, A. K. (2002) *Gastroenterology* **122,** 134–144

51. Corredor, J., Yan, F., Shen, C. C., Tong, W., John, S. K., Wilson, G., Whitehead, R., and Polk, D. B. (2003) *Am. J. Physiol.* **284,** C953–C961
52. Erickson, S. L., de Sauvage, F. J., Kikly, K., Carver-Moore, K., Pitts-Meek, S., Gillett, N., Sheehan, K. C., Schreiber, R. D., Goeddel, D. V., and Moore, M. W. (1994) *Nature* **372,** 560–563
53. Teh, H. S., Seebaran, A., and Teh, S. J. (2000) *J. Immunol.* **165,** 4814–4821
54. Kontoyiannis, D., Pasparakis, M., Pizarro, T. T., Cominelli, F., and Kollias, G. (1999) *Immunity* **10,** 387–398
55. Alexopoulou, L., Pasparakis, M., and Kollias, G. (1997) *Eur. J. Immunol.* **27,** 2588–2592
56. Peschon, J. J., Torrance, D. S., Stocking, K. L., Glaccum, M. B., Otten, C., Willis, C. R., Charrier, K., Morrissey, P. J., Ware, C. B., and Mohler, K. M. (1998) *J. Immunol.* **160,** 943–952