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JNK/SAPK activity contributes to TRAIL-induced apoptosis

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Abstract

We report here that JNK/SAPKs are activated by TRAIL in parallel to induction of apoptosis in human T and B cell lines. Death signaling as well as JNK/SAPK activation by TRAIL in these cells is FADD- and caspase-dependent since dominant-negative FADD or the caspase inhibitor zVAD prevented both, apoptosis and JNK/SAPK activity. JNK/SAPK activity in response to triggering of CD95 by an agonistic antibody (αAPO-1) was also diminished by dominant-negative FADD or zVAD. Correspondingly, a cell line resistant to αAPO-1-induced death exhibited crossresistance to TRAIL-induced apoptosis and did not upregulate JNK/SAPK activity in response to TRAIL or αAPO-1. Inhibition of JNK/SAPK activity, by stably transfecting cells with a dominant-negative JNKK-MKK4 construct, reduced apoptosis in response to TRAIL or αAPO-1. Therefore, activation of JNK/SAPKs by TRAIL or αAPO-1 occurs downstream of FADD and caspases and contributes to apoptosis in human lymphoid cell lines.

Keywords: apoptosis; TRAIL (APO2-L); CD95-L (APO1-L/Fas-L); JNKK-MKK4

Abbreviations: JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; JNKK-MKK4, c-Jun N-terminal kinase, MAP kinase kinase; DILs, Death-inducing ligands; αAPO-1, agonistic CD95 receptor antibody; TRAIL-R1, TRAIL Receptor 1; TRAIL-R2, TRAIL Receptor 2; TNF, Tumor Necrosis Factor; TNF-R1, Tumor Necrosis Factor Receptor 1; SD, Standard Deviations

Introduction

TRAIL (also known as APO-2 ligand) is a member of the tumor necrosis factor (TNF) family of death inducing ligands (DILs) and leads to apoptosis in a wide variety of cells.[1,2] The effects of TRAIL are mediated by five distinct cell surface receptors and two of them possess an intact intracellular 'death domain'.[3–7] Among members of the TNF receptor superfamily this intracellular 'death domain' is significantly conserved and required to transduce the apoptosis-inducing signal through interaction with the 'death domains' of adapter proteins.[4,5,8–10] FADD is a major adapter molecule which binds either directly or indirectly to the death domains of CD95 receptor (CD95), TNF receptor 1 (TNF-R1) or TRAIL receptor 1 (TRAIL-R1) and TRAIL receptor 2 (TRAIL-R2) to transduce the apoptotic signal by activation of caspase 8 (FLICE) followed by sequential activation of other caspases.[5,8,9,11–14] The emerging model from these molecular studies is that death receptors via adapter proteins like FADD directly engage and activate apoptotic ICE family proteases. However, this model fails to explain how diverse physiologic signals like sphingosine-1-phosphate,[15] activation of protein kinase C by phorbol esters[16] or Bcl-2[17,18] may modulate death receptor-mediated apoptosis.

Cross-linking of CD95 and TNF-R1 can also activate the Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway.[19–24] The significance of JNK/SAPK activation has been unclear. One hypothesis is that activation of the JNK/SAPK pathway contributes to CD95- and TNF-mediated apoptosis[22,25,26] whereas others[23,24] suggested that JNK/SAPK activation is not required for TNF-R1-induced apoptosis. Recently, a novel signaling protein, DAXX was identified that binds specifically to the CD95 death domain. Overexpression of DAXX enhanced CD95-mediated apoptosis by activation of the JNK/SAPK pathway in a FADD independent manner.[26] Correspondingly, activation of JNK/SAPKs by TNF-R1 is described to be mediated through a TRAF2 pathway which also acts independently of FADD.[23,24]

Signaling events downstream of TRAIL receptors are largely unknown. We found an involvement of JNK/SAPKs in apoptosis signaling downstream of FADD and caspases following ligation of TRAIL and αAPO-1 in human lymphoid cell lines.

Results

TRAIL induces JNK/SAPK activity downstream of caspases

To examine whether JNK/SAPKs are activated during TRAIL-induced apoptosis we analyzed JNK/SAPK activity in human T (JURKAT) and B (BJAB) cell lines following treatment with recombinant TRAIL protein. Crosslinking of TRAIL leads to a rapid induction of JNK/SAPKs within 2 h which parallels the onset of apoptosis (Figure 1A,B). TRAIL-

control supernatant from Pichia pastoris transfected with empty vector induced neither significant JNK/SAPK activity nor apoptosis. Next we measured TRAIL-induced JNK/SAPK activity in the presence of zVAD, an inhibitory peptide substrate for caspases which also blocks the receptor proximal caspase FLICE.[27] Pre-incubation of JURKAT cells with zVAD completely prevented TRAIL-induced JNK/SAPK activity and cell death. In contrast, αAPO-1-mediated activity of JNK/SAPKs was not totally prevented by zVAD (Figure 1D). This might be due to a cross-talk between the FADD- and DAXX-dependent signaling pathways initiated by triggering of CD95.[26] TRAIL-induced JNK/SAPK activity and apoptosis was also not detectable in JURKAT-derived JAPO cells. This cell line exhibits cross-resistance towards TRAIL and αAPO-1-mediated apoptosis and does neither cleave caspases nor activate JNK/SAPKs although CD95 receptor is expressed (Figure 1A and C and data not shown). This suggests that TRAIL induces JNK/SAPK activity downstream of FLICE/caspases in lymphoid cell lines similar as shown for CD95 signaling (Figure 1D).[19] Therefore, a common element of the CD95 and TRAIL pathway might be defective in JAPO cells which is necessary for JNK/SAPK activation.

TRAIL-induced apoptosis and JNK/SAPK activity depends on intact FADD signaling

To further dissect the TRAIL-induced pathway leading to JNK/SAPK activation we determined the effect of FADD signaling located upstream of FLICE/caspases and used a B cell line in which FADD signaling was blocked by stable transfection of a FADD dominant-negative construct (BJAB-FADD-DN cells). In BJAB-FADD-DN cells apoptosis and activation of JNK/SAPKs induced by treatment with TRAIL was totally inhibited compared to the vector control line (Figure 2A and B). The same result was obtained after ligation of CD95 in mutant and control BJAB cells (Figure 2C and D). Thus, death signaling downstream of TRAIL and CD95 receptors may use common elements in the following sequence: death-receptors→FADD→caspases→JNK/SAPKs.

TRAIL-induced apoptosis is attenuated in cells stably transfected with a JNKK-MKK4 dominant-negative construct

To examine more specifically whether enhanced JNK/SAPK activity might contribute to TRAIL-induced apoptosis we

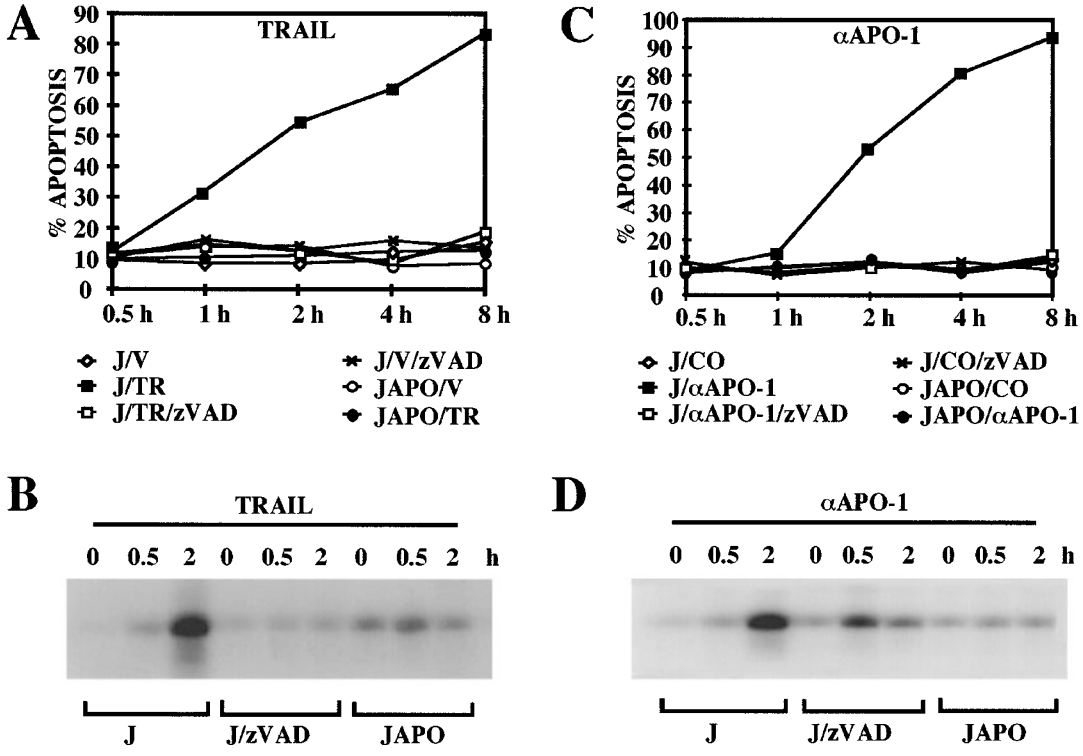

Figure 1 TRAIL-induced JNK/SAPK activity occurs downstream of caspases. (**A**) JURKAT (J) or αAPO-1 resistant JURKAT cells JAPO were incubated with 100 ng/ml recombinant TRAIL protein (J/TR; JAPO/TR) or with control supernatant from Pichia pastoris transfected with empty vector (J/V; JAPO/V). Likewise, JURKAT cells were pre-incubated with 50 μM zVAD and subsequently treated with TRAIL or control supernatant (J/TR/zVAD; J/V/zVAD). 0.5, 1, 2, 4 or 8 h later early apoptotic changes were measured by annexin staining using flow cytometry. (**B**) Cells were treated in a similar way as described in **A** and at the time points indicated nuclei-free supernatant was normalized for protein content and immunoprecipitated with anti-JNK/SAPK antibody-conjugated sepharose beads. GST-cJun-1-166 fusion protein was added to the immuno complexes and incubated in kinase buffer in the presence of [γ-^{32}P]ATP. The phosphorylated fusion protein was resolved by 10% SDS–PAGE and visualized by autoradiography. (**C** and **D**) Cells were treated and analyzed as described in (**A** and **B**) except that cells were treated with αAPO-1 agonistic antibody (αAPO-1, 2 μg/ml) instead of TRAIL. The results depicted are the mean of three separate experiments ± S.D.

inhibited JNK/SAPK activity by transfecting JURKAT cells with a dominant-negative JNKK-MKK4 construct which partially inhibits JNK/SAPK activity.[28] JNKK-MKK4 has been shown to be a direct activator for JNK/SAPKs.[29] Cotransfection with the gene for the GFP marker protein enabled separate forward side scatter FACScan analysis of transfected cells. However, using this treatment, cell death induced by stimulation with TRAIL or αAPO-1 for 8 and 24 h, respectively, was not significantly influenced compared to empty vector transfected control cells (Figure 3A). The same result was found using CEM leukemic T cells. However, detection of cell death by gating on GFP-cotransfected cells is less sensitive than measuring apoptosis. Furthermore, an enhancement of TRAIL and/or CD95-induced apoptosis could not be recorded in transfectants which died already. Therefore we selected stably transfected cells by continuous culture in G418. This treatment strongly diminished activity of JNK-SAPKs compared to empty vector transfected cells as examined by a kinase assay (Figure 3B). In control experiments we detected expression of α-Tubulin and the amount of phosphorylated ERK protein by Western blot. Whereas α-Tubulin was found to be equal expressed in both cell lines phosphorylation of ERKs in cells with a repressed JNK/SAPK activity was induced. This is in line with the recent finding that a balance between JNK/SAPK and ERK affects the apoptotic outcome since activation of the JNK/SAPK pathway in combination with a repressed growth factor activated ERK-cascade was required for apoptosis.[30] Correspondingly, apoptosis induced by stimulation with TRAIL or αAPO-1 for 4 and 8 h, respectively, was strongly diminished in cells with a repressed JNK/SAPK activity compared to empty vector transfected control cells as detected by annexin-staining and flow cytometry (Figure 3C). The same result was found in HeLa cells stably transfected with JNKK-MKK4 dominant-negative or empty vector. Therefore, activation of JNK/SAPKs contributes to apoptosis signaling downstream of death receptors.

Discussion

In the present study we demonstrate that JNK/SAPKs are activated in response to treatment of leukemic T cell lines with TRAIL in a similar kinetic as found for cross-linking of CD95. Both, TRAIL- and CD95-induced JNK/SAPK activity was inhibited by preventing signaling of FADD or caspases. Furthermore, inhibition of JNK/SAPKs in cells stably transfected with dominant-negative JNKK-MKK4 diminished TRAIL or αAPO-1-mediated apoptosis. Therefore, activation of JNK/SAPKs may contribute to apoptosis signaling downstream of death receptors. However, death signaling in lymphoid cell lines seems to differ from the pathway initiated in HeLa, 293 or MCF7 cells since in these systems a dichotomy upstream of FADD leading to JNK/SAPK activity was described. Thus, crosslinking of CD95 or TNF-R1 in

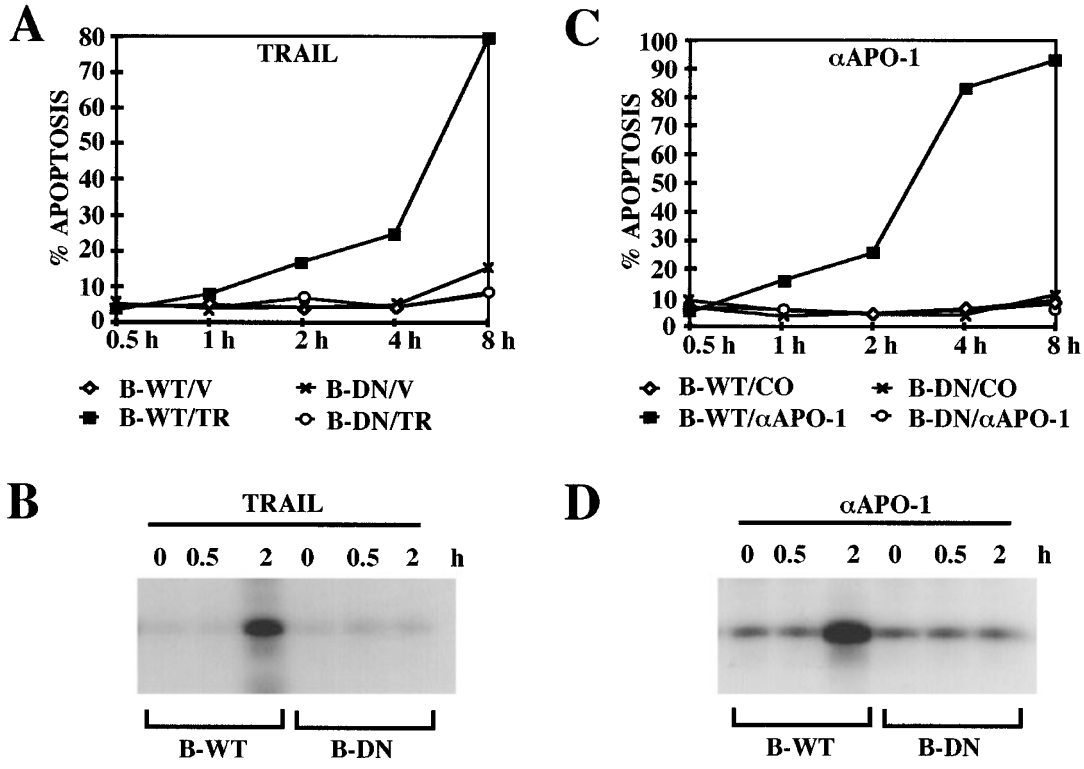

Figure 2 TRAIL-induced apoptosis and JNK/SAPK activity depends on intact FADD signaling in human B cells. Human B lymphoma BJAB cells stably transfected with pcDNA3 carrying the cDNA for inactive FADD-WT (B-WT) or a pcDNA3-FADD-DN construct (B-DN) were treated with TRAIL or αAPO-1 and analyzed as described in Figure 1

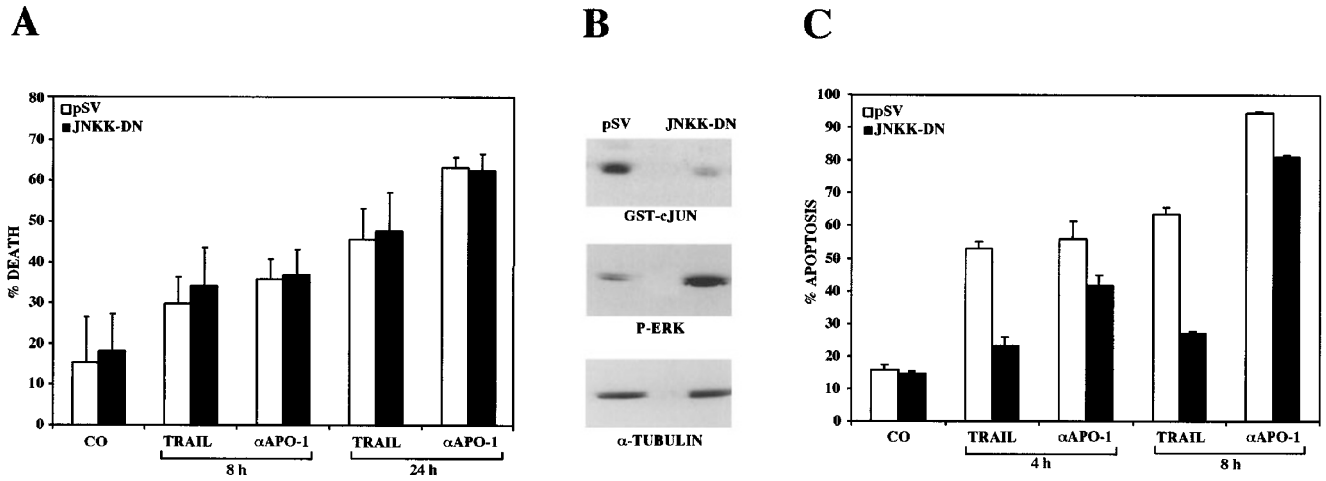

Figure 3 TRAIL-induced apoptosis in transiently and stably transfected cells harboring dominant-negative JNKK-MKK4. (**A**) JURKAT cells were transfected with empty vector (pSV, white bars) or vector containing the cDNA for JNKK-MKK4 dominant-negative (JNKK-DN, black bars) and cotransfected with pEGFP as described in Materials and Methods. Seventy-two hours after transfection cells were stimulated with recombinant TRAIL protein (100 ng/ml) or αAPO-1 (1 μg/ml). Eight or 24 h later cell death was measured by forward side scatter analysis by gating at GFP-positive cells using a FACScan cytometer. The percentage of GFP-positive cells 72 h after transfection was 26% (pSV) and 23% (JNKK-DN). (**B**) JNK/SAPK activity of untreated JURKAT cells stably transfected with empty vector (pSV) or vector containing the cDNA for JNKK-MKK4-dominant-negative (JNKK-DN) was examined by kinase assay as described in Figure 1B and D and Materials and Methods. In addition, the amount of phosphorylated ERK or the expression of α-Tubulin proteins was examined by Western blot using extracts from the same cells. (**C**) JURKAT cells stably transfected with empty vector (pSV, white bars) or vector containing the cDNA for JNKK-MKK4 dominant-negative (JNKK-DN, black bars) were stimulated with recombinant TRAIL protein (100 ng/ml) or αAPO-1 (1 μg/ml). After 4 or 8 h apoptosis was analyzed by annexin-staining and flow cytometry. The results depicted are the mean of three separate experiments performed in duplicates ± S.D.

HeLa, 293 or MCF7 cells seems to engage predominantly the death domain adapter proteins DAXX and TRAF2, respectively which define a pathway distinct from FADD/FLICE/caspases to activate JNK/SAPKs.[23,24,26,31] DAXX-signaling might also contribute to CD95-mediated JNK/SAPK activity in lymphoid cells since we observed that inhibition of caspases prevented not in all cases αAPO-1-mediated activity of these kinases. In contrast, TRAIL-induced JNK/SAPK activity was totally blocked by zVAD, dominant-negative FADD and in αAPO-1-resistant JAPO cells suggesting that FADD is a critical element for activation of JNK/SAPKs by TRAIL. However, the contribution of the individual receptors for TRAIL in induction of JNK/SAPK activity remains to be further highlighted.

The precise role of JNK/SAPK activation following direct triggering of death receptors is unclear. Our data correspond to the recent findings that JNK/SAPK activation contributes to CD95-mediated apoptosis since counteracting activation of JNK/SAPKs by sphingosine-1-phosphate inhibits CD95-induced cell death in some cells[15] and activation of JNK/SAPKs by overexpression of DAXX induces apoptosis.[26] Furthermore, activity of ASK-1, an apoptosis signal-regulating kinase of the JNK/SAPK pathway which can be activated by DAXX is both sufficient to mediate apoptosis and required for TNF-α-induced cell death.[32,33]

Paradoxically, JNK/SAPK activation in response to various stimuli was also demonstrated to be dispensable for apoptosis or even to prevent this process.[23,24,34,] Thus, the JNK/SAPK pathway might directly or indirectly counteract the expression of survival factors, such as NF-κB[24] or Bcl-2.[35] Correspondingly, activation of the JNK/SAPK pathway in combination with a repressed growth factor activated ERK-cascade has been shown to be required for apoptosis and a balance between JNK/SAPK and ERK has been found to be critical in affecting the apoptotic outcome.[30] In addition, JNK/SAPK activity may also occur independently of death receptor triggering as described in response to cellular stress.[36] In contrast to direct triggering of death receptors inhibition of caspases by zVAD did not prevent but even superinduced activity of JNK/SAPKs in response to cellular stress in lymphoid cells.[37]

Thus, the co-operation of multiple signaling pathways as well as cell type-specific variations allows fine-tuned, targeted regulation and creates multiple checkpoints for the modulation of death signals.

Materials and Methods

Reagents and cell lines

zVAD (Enzyme Systems Products, Dublin, USA) stock was dissolved in dimethylsulfoxide and stored in aliquots at −20°C. Final concentrations of the solvent in medium were 0.1%. Anti-CD95-agonistic antibody (αAPO-1) and recombinant TRAIL were prepared as previously described.[14,38] Stock solutions were dissolved in PBS.

The following human cell lines were used: Apoptosis-sensitive JURKAT (acute human T cell leukemia) cells which rapidly die in response to treatment with αAPO-1. An αAPO-1-resistant subclone (JAPO) has been selected by continuous culture of the parental JURKAT cells in increasing doses of αAPO-1 during a period of 1 year

(starting with doses of 10 ng and ending at 10 μg/ml). BJAB is a human B lymphoma cell line stably transfected with pcDNA3 vector carrying the cDNA for inactive FADD-WT (BJAB-WT) or a pcDNA3-FADD-DN construct (BJAB-FADD-DN) as described.[39] All cell lines were grown in RPMI-1640 medium supplemented with 10% FCS (Biochrom, Hamburg, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES and 2 mM L-glutamine (all from Boehringer Ingelheim, Germany). For selection of stably transfected JURKAT cells 25 μg of either pSV empty vector or a JNKK-MKK4 dominant-negative expression construct were transfected by electroporation (975 μF, 220 V). Transfected cells were resuspended in 10 ml fresh medium and living cells were selected by continuous culture in G418 (Calbiochem, California, USA).

Measurement of apoptosis

Early apoptotic changes were identified by staining of cells with fluoresceinthiocyanate (FITC)-conjugated annexin V (Bender Med Systems, Vienna, Austria) and analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) as described.[36]

JNK/SAPK assay

JNK/SAPKs were immunoprecipitated with polyclonal antibodies raised against JNK1 (C-17, Santa Cruz, USA) and JNK2 (FL-18, Santa Cruz, USA) and activity was detected using GST-Jun 1/166 as substrate as described.[28,36]

Western blot

Expression of α-Tubulin and the amount of phosphorylated ERK proteins was detected by Western blotting as described.[36] α-Tubulin was stained with a mouse monoclonal antibody obtained from Calbiochem (California, USA). Phosphorylated ERK was detected using a specific mouse monoclonal antibody (Santa Cruz, California, USA). Bound antibodies were detected by anti-mouse/horseradish peroxidase conjugates (Santa Cruz, California, USA). Enhanced chemiluminescence system (Amersham, Braunschweig, Germany) was used for detection.

Transient transfections

1×10^8 JURKAT cells were pelleted, resuspended in 200 μl PBS and transfected with 25 μg expression construct and 5 μg GFP construct by electroporation (975 μF, 220 V). After transfection the cells were separated by Ficoll-gradient centrifugation and resuspended in fresh medium at 5×10^5/ml. Twenty-four hours later living cells were Ficoll-separated again and resuspended in fresh medium at 5×10^5/ml. Twelve hours or 24 h later cells were stimulated.

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