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Ligand-induced internalization of TNF receptor 2 mediated by a di-leucin motif is dispensable for activation of the NFκB pathway

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ABSTRACT

Endocytosis is an important mechanism to regulate tumor necrosis factor (TNF) signaling. In contrast to TNF receptor 1 (TNFR1; CD120a), the relevance of receptor internalization for signaling as well as the fate and route of internalized TNF receptor 2 (TNFR2; CD120b) is poorly understood. To analyze the dynamics of TNFR2 signaling and turnover at the plasma membrane we established a human TNFR2 expressing mouse embryonic fibroblast cell line in a TNFR1$^{-/-}$/TNFR2$^{-/-}$ background. TNF stimulation resulted in a decrease of constitutive TNFR2 ectodomain shedding. We hypothesized that reduced ectodomain release is a result of TNF/TNFR2 complex internalization. Indeed, we could demonstrate that TNFR2 was internalized together with its ligand and cytoplasmic binding partners. Upon endocytosis the TNFR2 signaling complex colocalized with late endosome/lysosome marker Rab7 and entered the lysosomal degradation pathway. Furthermore, we identified a di-leucin motif in the cytoplasmic part of TNFR2 suggesting clathrin-dependent internalization of TNFR2. Internalization defective TNFR2 mutants are capable to signal, i.e. activate NFκB, demonstrating that the di-leucin motif dependent internalization is dispensable for this response. We therefore propose that receptor internalization primarily serves as a negative feed-back to limit TNF responses via TNFR2.

1. Introduction

Tumor necrosis factor (TNF, also called TNFα) is an important inflammatory mediator with pleiotropic effects on virtually every organ system [1]. At the cellular level TNF regulates diverse functions, such as cytokine release, proliferation, differentiation and apoptosis. TNF is synthesized as a type II transmembrane protein (mTNF) forming stable homotrimers in the plasma membrane. Subsequent proteolytic cleavage of the ectodomain by TNF alpha converting enzyme (TACE/ADAM 17) results in the release of soluble TNF trimers (sTNF). TNF exerts its function through the stimulation of its two receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Whereas TNFR1 can be equally activated by sTNF and mTNF, only mTNF is capable to fully activate TNFR2 [2,3].

The intracellular tail of TNFR1 contains a death domain, which recruits the adaptor protein TRADD upon activation [4]. Binding of TRADD can initiate two signaling cascades. On the one hand, TRADD may interact with RIP1 and TRAF2 [5,6] leading, via several steps involving ubiquitination of adaptor proteins and recruitment of the IKK complex, to the activation of the transcription factor NFκB [7]. On the other hand, TRADD may recruit FADD [6] and procaspase 8 [8] leading to the formation of the death inducing signaling complex (DISC) resulting in the induction of apoptosis.

In contrast to TNFR1, TNFR2 does not contain a death domain and therefore cannot directly induce apoptosis. Nevertheless, TNFR2 has been described to potentially exert pro-apoptotic functions in the context of limiting an immune response after injury or during resolving inflammations [9]. Other experimental models have shown that the pro-apoptotic function of TNFR2 depends on the crosstalk with TNFR1 [10]. Upon stimulation of TNFR2 TRAF2 is directly recruited to TNFR2 [11,12], which is essential for activation of the NFκB-pathway, promoting cell

Abbreviations: ADAM, a disintegrin and metalloproteinase; BSA, bovine serum albumin; CHO-TNF$_{\Delta[1-12]}$, Chinese Hamster Ovary cells transfected with TNF$_{\Delta[1-12]}$; DKO MEF, mouse embryonic fibroblasts from TNFR1$^{-/-}$/TNFR2$^{-/-}$ mice; EEA1, early endosomal antigen 1; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; FADD, Fas-associated protein with death domain; HRP, horseradish peroxidase; huTNFR2, human TNF receptor 2; IκBα, nuclear factor of kappa-light polypeptide gene enhancer in B cell inhibitor, alpha; IKK, IκBα-kinase; MEF, mouse embryonic fibroblasts; MMP, matrix metalloproteinase; mTNF, membrane-bound TNF; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PBA, PBS supplemented with BSA and sodium azide; PFA, paraformaldehyde; PI3K, phosphatidyl inositol 3-kinase; PKB, protein kinase B; R2 MEF, mouse embryonic fibroblasts of TNFR1$^{-/-}$/TNFR2$^{-/-}$ mice transfected with human TNFR2; RIP, receptor interacting protein; sTNF, soluble TNF; TACE, TNF alpha converting enzyme; TNF, tumor necrosis factor; TNF-A546, TNF labeled with Alexa546; TNFR, TNF receptor; TRADD, TNF receptor 1-associated death domain protein; TRAF2, TNF receptor associated factor 2.

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proliferation and cell survival [13]. Another molecule involved in TNFR2 signaling is the protein kinase PKB/Akt, which is activated dependent on phosphatidylinositol 3-kinase (PI3K) [14]. This appears of particular relevance in neuroprotection and regeneration. Primary neurons, e.g., are spared from glutamate induced excitotoxicity *in vitro* [15]. In addition, TNFR2 signaling promotes neuronal survival and oligodendrocyte regeneration in *in vivo* models of ischemic and neurotoxic insults [16,17], respectively.

TNF signaling is tightly regulated by processing and internalization of its receptors. Both TNFR1 and TNFR2 can be processed by TACE/ADAM17, which causes the shedding of the soluble ectodomains [18,19]. Interestingly, the type of cellular response induced by TNF is spatially controlled: Whereas binding of TNF to TNFR1 at the plasma membrane results in the recruitment of signal complex I and activation of the canonical NFκB-pathway, internalization of the TNF/TNFR1 complex causes a switch in the molecular composition of the signaling complex, constituting signal complex II to induce the pro-apoptotic pathway [20]. In contrast to the wealth of molecular details known about TNFR1 signal pathways, little is known about the respective mechanisms of TNFR2 signaling. Moreover, although it has been described that TNFR2 can be internalized after binding to TNF [21], the relevance of internalization for signal transduction and the fate of the internalized receptor is largely unknown.

To shed light into TNFR2 signaling, we have established a cell model suited to study TNFR2 function and signaling mechanisms by biochemical and microscopic methods. For this, embryonic fibroblasts from TNFR1$^{-/-}$/TNFR2$^{-/-}$ mice were stably transfected with human TNFR2 expression constructs. Due to the absence of mouse TNF receptors, this cell model allows to selectively characterize TNFR2 behavior and ligand-induced signaling pathways. Using TRAF2 recruitment and NFκB activation as paradigms of TNF signaling, we have analyzed the dynamics of TNFR2 complex formation and signal transduction in relation to receptor shedding and internalization. Whereas internalization is dispensable for TNFR2 activation, our data suggest that both internalization and shedding serve to down regulate cellular responsiveness to TNF.

2. Materials and methods

2.1. Materials

The TNFR2 specific antibody 80M2 has been described [22]. The antibodies 5N, specific for human TNF, HP8002, specific for the mouse TNFR1, HP8003, specific for the mouse TNFR2, HP9003, MR2-1 and MR2-1 coupled to FITC, specific for the human TNFR2, were from Hbt (Uden, The Netherlands). Antibodies against NFκB p65, IκBα, early endosomal antigen 1 (EEA1), Rab7, clathrin heavy chain, α-tubulin and β-actin were from Cell Signaling Technologies (Danvers, MA). The antibody AP1040 against mouse TRAF2 was from Calbiochem (Darmstadt, Germany). Antibodies coupled to fluorescein isothiocyanate (FITC) and Alexa488 or Alexa546 were from Hbt and Invitrogen (Karlsruhe, Germany), respectively. Antibodies coupled to horseradish peroxidase (HRP) were from Jackson ImmunoResearch (Suffolk, UK). GM6001 was purchased from Calbiochem and DAPI was from Sigma (Taufkirchen, Germany). All other chemicals were of analytical grade.

2.2. Generation of stable cell lines and cell culture

pEFpuroTNFR2[L319A/L320A] expression constructs coding for the LL/AA TNFR2 mutant were generated by site-directed mutagenesis of pEFpuroTNFR2 plasmid containing wild type TNFR2. To generate cell lines expressing human TNFR2 muteins, Simian Virus 40 large T antigen immortalized mouse embryonic fibroblasts from TNFR1$^{-/-}$/TNFR2$^{-/-}$ mice, generously provided by Daniela Männel (University of Regensburg, Regensburg, Germany), were transfected with the plasmids pEFpuroTNFR2 [23] (R2 MEF) or pEFpuroTNFR2[L319A/L320A] (LL/AA MEF) using lipofectamine 2000 (Invitrogen) according to the manufacturers recommendations. Stably transfected cells were selected with 5 μg/ml puromycin (PAA, Cölbe, Germany). After two weeks huTNFR2-positive cells were sorted by flow cytometry following the methods of [23].

Chinese Hamster Ovary (CHO) cells expressing $TNF_{[\Delta 1-12]}$ (CHO-$TNF_{[\Delta 1-12]}$) have been described elsewhere [2]. Mouse fibroblasts and CHO cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 5% (v/v) heat-inactivated FCS (PAN Biotech, Aidenbach, Germany) and 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

2.3. TNF stimulation

To analyze the TNFR2 pathway cells were always incubated for 30 min with the monoclonal mouse antibody 80M2 (1 μg/ml) to preactivate the TNFR2 on the cell surface thereby mimicking receptor activation by the membrane form of TNF, if soluble recombinant TNF is added. For this purpose the cells were incubated for different time intervals with a soluble, human TNFR2 specific TNF-mutein ($CysHisTNF_{143N/145R}$) [23,24] always referred to as TNF. At time point zero the experiment was stopped either by cell lysis or fixation.

2.4. SDS-PAGE and Western blotting

Cells were lysed with homogenization buffer (10 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 1.5 mM KCl, 1% NP-40, 0.2 mM PMSF, 20 mM ß-glycerophosphate and 100 μM Na_3VO_4) at 4 °C for 30 min. Lysates were centrifuged (2 min at 9600 *g*) and protein concentration of supernatants were determined using the BCA method (Pierce, Bonn, Germany). 20 μg total protein were denatured in Laemmli buffer and resolved by SDS-PAGE. For Western blot analyses the proteins were transferred onto nitrocellulose membranes. Non-specific protein binding was blocked with 5% skimmed milk powder solution in PBS/0.1% Tween20 and the membranes were incubated overnight at 4 °C using antibodies specific for the protein of interest. After incubation with appropriate HRP-conjugated secondary antibodies for 90 min at room temperature the signals were detected by enhanced chemiluminescence (Super Signal, Pierce).

2.5. Immunoprecipitation

Approximately 5×10^6 R2 MEF were seeded in T75 cell culture flask (Greiner Bio-One, Frickenhausen) and cultivated overnight. Cells were stimulated as indicated and homogenized with lysis buffer (10 mM TRIS pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 0.5% NP-40, 100 μM Na_3VO_4, 0.2 mM PMSF, 20 mM ß-glycero-phosphate, Roche complete protease inhibitor cocktail) for 30 min on ice. The lysate was centrifuged (2 min; 9600 *g*; 4 °C) and the supernatant was incubated with 5 μg TNFR2 specific antibodies MR2-1 (Hbt) for 2 h at 4 °C. Immunocomplexes were precipitated with Protein G agarose (Pierce) for 2 h at 4 °C. Precipitates were washed four times with washing buffer (10 mM TRIS pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM EGTA) and bound proteins were eluted by boiling 5 min at 95 °C in Laemmli buffer. Eluted proteins were separated by SDS-PAGE.

2.6. Flow cytometry

5×10^5 cells/well were seeded in 6-well plates (Greiner Bio-One), cultivated overnight and harvested with trypsin/EDTA. Cells were incubated with antibodies specific for mouse TNFR1 (HP8002; 0.5 μg/ml) and mouse TNFR2 (HP8003; 0.5 μg/ml) or human TNFR2 (MR2-1; 2 μg/ml) in PBS containing 0.05% BSA and 0.02% sodium azide (PBA) for 1 h at 4 °C. Cells were washed three times with PBA and incubated for 45 min with FITC-labeled anti-mouse secondary antibodies. Before analysis by flow cytometry (Cytomics FC500, Beckmann-Coulter,