

**Reverse signaling of
Tumor Necrosis Factor.
Novel functions of the signaling
molecule CKIP-1**

Summary of PhD thesis

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Background

Transmembrane and soluble TNF

Tumor necrosis factor (TNF) is the prototype molecule of the TNF superfamily (TNFSF), exerting a broad spectrum of activities through its membrane bound or soluble form. TNF is a pleiotropic cytokine as it is able to induce differentiation, gene-expression, apoptosis or necrosis depending on the type and actual state of the responding cells. TNF is mainly expressed by immune cells but also by other cell types. The 26 kDa membrane bound TNF (tmTNF) is synthesized as a monomer type II polypeptide and assembles into a functional trimer. Mature, soluble TNF is cleaved from tmTNF by TACE. The synthesis of TNF can be induced by many different stimuli including interferons, cytokines, tumor cells, irradiation and hypoxia. Factors from bacteria, viruses, and parasites can also stimulate the production of TNF.

TNF mediates biological activities through type 1 and 2 TNF receptors (TNFR1 and TNFR2), which are expressed as pre-assembled trimers on the cell surface. While TNFR1 is expressed in all nucleated cells, TNFR2 is mainly expressed in immune cells, endothelial cells and neurons. Binding of sTNF to its receptors triggers a series of intracellular events that result in the activation of transcription factors, such as NF- κ B and c-Jun. TNF-activated complex signaling pathways mediate diverse biological processes, including cell growth, cell death, development, inflammatory or stress responses. Whether stimulation of a cell leads to activation or apoptosis, depends on a complex interplay between the metabolic status and microenvironment of the given cell type. Systemic activities of TNF are associated with the secreted soluble form. Level of circulating TNF in healthy individuals is nearly undetectable, but increases dramatically in pathological cases.

TNF is a key mediator in the local inflammatory immune response as it acts as an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, recruiting in turn macrophages and neutrophils to the site of infection. Up-regulation of TNF is known to play a critical role in pathogenic

disorders like rheumatoid arthritis, psoriasis, ankylosing spondylitis, asthma, septic shock and inflammatory bowel disease.

Transmembrane TNF as a ligand

Similar to soluble TNF, tmTNF exists as a homotrimer of uncleaved monomers, with a molecular weight of 26 kDa. Transmembrane TNF also binds to both subtypes of TNF receptors on target cells, however biological activities are supposed to be mediated mainly through TNFR2. Leading to cell death or activation of the target cells, expression of tmTNF contributes to physiological and pathological responses. Cytotoxic activities of tmTNF have been reported for various tumor cells. Importance of tmTNF in inhibition of intracellular pathogens has been also reported. Furthermore, tmTNF has been shown to initiate T cell and macrophage migration as well as granuloma formation. Direct cell-to-cell contacts through tmTNF trigger activation processes in endothelial cells, B cells, T cells, monocytes and NK cells.

Transmembrane TNF as a receptor

When TNF superfamily ligands and their cognate receptors interact, signaling pathways can be activated in both ligand and receptor bearing cells, leading to activation, differentiation or apoptosis. This bidirectional communication has been reported for a number of TNF superfamily members and considered as a fine-tuning mechanism during the immune response. We and others reported receptor-like properties of the tmTNF. Soluble ectodomain of receptor molecule (e.g. Etanercept) or anti-TNF antibodies (e.g. Infliximab, Adalimumab) can also elicit reverse signaling in tmTNF expressing cells. Therapeutic application of these agents in chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease revealed effects of TNF reverse signaling on different immune cells mostly depending on the cell type, including apoptosis or activation. Moreover, TNF reverse signaling has been implicated in undesirable side-effects of anti-TNF therapies, where secondary infections were observed.

Despite the apparent clinical relevance the molecular basis of TNF reverse signaling is largely unknown. The first reports revealed protein kinase C and MAPK/ERK to be involved in TNF reverse signaling. Interaction of tmTNF with its soluble receptor triggers rapid dephosphorylation of tmTNF and concomitant Ca^{2+} signaling. The MAPK pathway and caspases have been implicated in downstream signaling events, whereas other possible participating molecules remained elusive.

Casein kinase 2-interacting protein-1 (CKIP-1) is a multifunctional protein

Identifying signaling molecules recruited to the mTNF molecule and investigating their role may help to unravel molecular details of TNF reverse signaling. A novel protein that interacts with the intracellular domain of mTNF has been identified (hTIP, human TNF intracellular domain interacting protein) and proved to be identical to human casein kinase 2-interacting protein-1 (CKIP-1). CKIP-1 has been found to interact with a series of proteins involved in cellular functions like differentiation, cell motility and cell death.

2 Aim of the study

Since CKIP-1 has been identified as an interacting partner of the pro-inflammatory tmTNF, we hypothesized that it might have a role in immunity as well. We aimed at elucidating possible functions of the novel protein CKIP-1 in innate immunity.

Our specific aims were:

- I) to investigate the involvement of CKIP- 1 in the pro-inflammatory response of human and mouse mononuclear cells;
- II) to reveal the role of CKIP-1 in TNF reverse signaling in human model cell lines.

Materials and Methods

Cell culture

HEK293 cells (ATCC) were grown in a (1:1) mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 10 % low endotoxin FCS. THP-1 (ATCC) cells were maintained in OptiMEM with 2 % FCS.

Expression vectors

The human CKIP-1 coding sequence was kindly provided by Dr. Chie Kohchi (Hiroshima University, Japan) and subcloned into pBS-SK+ and into vectors pcDNA3, pEGFPC1, pECFPC1 and p3xFlag-Myc-CMV-26. The 252 bp N terminal fragment of tmTNF (TNFN_{term}) was fused to GST, EYFP or mCherry. In promoter activation experiments the luciferase gene was controlled by the TNF-alpha promoter or a promoter sequence containing 5x NF-κB binding sites.

Transient transfections and reporter gene assays

THP-1 and HEK293 cells were transiently transfected by Lipofectamine-2000, or Jet PEI or by Amaxa Nucleofector kit following the manufacturer's protocols. To analyze the effect of CKIP-1 on the transcriptional activity of c-Jun the PathDetectTM Trans-Reporting System was used (Stratagene).

Protein expression, purification and GST-pull down assay

GST fusion proteins were purified from *E. coli* BL21 (DE3) lysates using glutathione-Sepharose beads. CKIP-1 was transcribed and translated *in vitro* using the TNT coupled reticulocyte system (Promega) then incubated with fusion protein or GST immobilized on glutathione-Sepharose beads. ³⁵S-labeled proteins were detected by SDS PAGE followed by autoradiography.

Immunoprecipitations and immunoblotting

Immunoprecipitations were performed using the Pierce direct magnetic IP kit (Thermo Scientific) according to the manufacturer's protocol using monoclonal

anti-Flag M2 (Sigma) or anti-RFP (Abcam) antibodies. Proteins were detected by Western blotting using anti-CKIP-1 (Santa Cruz) or anti-Flag M2 (Sigma) antibody and the appropriate secondary antibody conjugated to HRP (Sigma).

Expression Profile Verification

Reactions were carried out in Absolute QPCR SYBR Green mix (ABGene) according to the manufacturer's instructions on a RotorGene 3000 instrument (Corbett Research). Primer sequences for hCKIP-1 were: CCACTCGAGACAGGGCAAAA and AGCCATTAGGTGTCCCCTTGT.

Flow cytometry

For apoptosis measurements, cells were labeled with Annexin V-Alexa647 (Invitrogen) following the manufacturer's instructions. For surface staining measurements, transfected cells were stained with Infliximab-A647, anti-human B7/CD86-APC, anti-human DC-SIGN-APC or the appropriate isotype controls. Samples were analyzed by flow cytometry (BD FACSAriaTM), where cells with damaged membranes were gated by PI exclusion.

Ca²⁺ measurements

Cells were loaded with 5 μ M Indo 1-AM (Invitrogen). *In situ* treated live cells were monitored by fluorescence microscopy. Ratio of fluorescence emission at 405 nm (Ca²⁺-bound dye) and at 485 nm (Ca²⁺-free dye) was calculated for individual cells with the software Matlab®.

Fluorescence microscopy

Cells were analyzed by a custom designed instrument for large area fluorescence imaging (CytoScout[®]) based on an Axiovert 200 microscope (Zeiss). Images were analyzed by the V++ software. For co-localization analysis fluorescence images were recorded with an Olympus FV10i confocal microscope. The Mander's overlap coefficients were calculated using the JACoP plugin for the ImageJ software.

Results

CKIP-1 is involved in the inflammatory response of THP-1 cells

The effect of LPS on the transcriptional activity of *ckip-1* was followed in human THP-1 monocytes by qRT-PCR method. Level of CKIP-1 mRNA was significantly elevated in cells exposed to LPS and a similar upregulation was found in protein levels when followed by Western blot analysis. Furthermore, CKIP-1 expression was found to facilitate the classical activation process as reflected in the significant increase in CD86 surface expression.

CKIP-1 transactivates the TNF promoter in cooperation with-Jun

Increased levels of CKIP-1 protein had practically no influence on the basal activity of the NF- κ B transcription factors in HEK293 cells. In contrast, in cells transiently co-expressing c-Jun and CKIP-1, a remarkable activation of the TNF promoter was observed.

TNF reverse signaling induces Ca²⁺ oscillations in THP-1 cells

We used Infliximab, a therapeutic TNF antibody to elicit reverse signaling in THP-1 cells that are known to express tmTNF on the cell surface. Treatment with the F(ab)₂ fragment of Infliximab triggered THP-1 cells to specifically respond with Ca²⁺ signaling.

TNF reverse signaling leads to relocalization of CKIP-1 in THP-1 cells

When fusion protein CKIP-1-ECFP was expressed in THP-1 cells and visualized by confocal microscopy, in resting THP-1 cells CKIP-1 was found predominantly at the plasma membrane and also at intracellular regions. In contrast to treatments with LPS or hIgG, Infliximab treatment resulted in a remarkable additional translocation of CKIP-1 from the plasma membrane to intracellular regions. Similarly, a considerable relocalization of CKIP-1 from the plasma membrane to intracellular regions was observed in HEK293 model cells upon co-expression of TNFNterm, a short N terminal peptide of tmTNF. Similarly to Infliximab-treated

THP-1 cells, a considerable relocalization of CKIP-1 from the plasma membrane to intracellular regions was observed upon TNFNterm co-expression in HEK293 model cells.

CKIP-1 interacts with the N terminal fragment of tmTNF

CKIP-1 has been identified as an interacting partner of the N terminal intracellular domain of the pro-inflammatory tmTNF in a yeast two hybrid screen, pointing to a possible interaction of these two proteins. Co-localization analysis revealed that a significant fraction of TNFNterm positive pixels overlap with those of positive for CKIP-1 in HEK293 cells. The interaction of CKIP-1 and TNFNterm was corroborated by co-immunoprecipitation and by *in vitro* pull down assays, as well.

TNF reverse signaling reduces CKIP-1 mediated activation

Since reverse signaling affected the subcellular localization of CKIP-1 we tested whether it influences the CKIP-1 mediated activation processes. Remarkably, the CKIP-1 mediated increase in CD86 surface expression was significantly reduced upon Infliximab treatment. Similarly, overexpression of TNFNterm reduced the co-operative effect of CKIP-1 and c-Jun on TNF promoter activation.

Expression of CKIP-1 prevents cells from TNFNterm/ TNF reverse signaling induced apoptosis

In imaging experiments we observed morphological changes and a decreased proliferation rate of HEK293 cells expressing TNFNterm, while changes appeared to be counteracted in cells co-expressing both TNFNterm and CKIP-1. Indeed, co-expression of CKIP-1 almost completely prevented HEK293 cells from the apoptotic effect of TNFNterm, as measured by flow cytometry. In accordance, overexpression of CKIP-1 prevented THP-1 cells from Infliximab-induced apoptosis. In contrast, CKIP-1 did not appear to interfere with apoptosis induced by etoposide.

Discussion

Receptor-like properties of the transmembrane form of the pro-inflammatory cytokine TNF have been reported by several laboratories. Although TNF reverse signaling has been emerging as an important phenomenon in the immune response, its molecular basis remains elusive. Here we examined the role of CKIP-1, an interacting partner of the intracellular domain of tmTNF in inflammation and TNF reverse signaling. CKIP-1 exhibits diverse functions in different cell types such as recruiting proteins to the plasma membrane mediating regulation of the actin cytoskeleton, repressing cell survival signaling and affecting cell differentiation. Although CKIP-1 has been proposed to control cytokine signaling its function in immunity is still unclear. In our model cells CKIP-1 exerted a positive regulatory role in the activation process of monocytes. CKIP-1 has a fairly complex domain structure with several potential sites for post-translational modifications, indicating that modifications and interactions of the protein might alter its localization and activity. Upon treatment with Infliximab, a therapeutically used anti-TNF agent, a massive translocation of CKIP-1 from the plasma membrane to intracellular compartments was observed in model cells. Interestingly, in parallel with the relocalization of CKIP-1, TNF reverse signaling or expression of TNFN^{term} antagonized the pro-inflammatory effects of CKIP-1. Therefore, TNF reverse signaling appeared to have a negative regulatory role in inflammation, which is supported by the fact that reverse signaling has been shown to induce a temporary LPS resistance in monocytic cells.

TNF reverse signaling has been shown to activate both proapoptotic and antiapoptotic signaling pathways, where fate of the cell depends on the degree of differentiation and crosstalk with other signaling pathways. Nevertheless, critical signaling molecules determining the fate of the cell upon TNF reverse signaling have not been revealed. In our experiments Infliximab treatment induced apoptosis in THP-1 cells and CKIP-1 appeared to be a critical factor determining the fate of monocytes interacting with anti-TNF agents. Surprisingly, CKIP-1 could not only prevent THP-1 cells from TNF reverse signaling induced apoptosis but increased the survival of the CKIP-1 expressing cells upon Infliximab

treatment. Interestingly enough, we showed earlier that the NLS containing TNFNterm could accumulate in the nucleus and regulate the transcription of cytokines. A similar unusual phenomenon is known for other TNFSF members, where upon ligand binding the cleaved intracellular domain of the transmembrane molecule translocates into the nucleus and acts as a transcriptional co-activator. The apparent correlation between the relocalization of CKIP-1 and TNFNterm, as well as the specific interference of CKIP-1 with TNF reverse signaling indicates that CKIP-1 and TNFNterm may act through the same signaling complex. This idea is consistent with the findings of yeast two hybriide, *in vitro* pull down, co-immunoprecipitation and co-localization experiments, and further supported by the proposed scaffold nature of CKIP-1 as well as the mutual influence on cellular activities of CKIP-1 and TNF reverse signaling shown in this work.

Our data suggest that expression level of CKIP-1 could regulate the response of tmTNF expressing immune cells upon TNF reverse signaling. Therefore, CKIP-1 may be a promising target during local or systemic anti-TNF therapies. We propose that further studies on CKIP-1 functions in immunity may contribute to more controlled therapeutic approaches.

Highlights of the study

1. CKIP-1 expression is elevated upon LPS challenge in human monocyte model cells
2. Excess CKIP-1 results in activation of monocyte model cells.
3. TNF reverse signaling triggers translocation of CKIP-1, while CKIP-1-mediated activation is decreased.
4. CKIP-1 expression counteracts TNF reverse signaling induced apoptosis.
5. CKIP-1 is proposed as a candidate for regulating the fate of tmTNF expressing cells upon TNF reverse signaling.

Publications related to the thesis

Juhász K., Zvara Á., Lipp A.M., Nimmervoll B., Sonnleitner A., Balogi Z., Duda E. (2013) Casein kinase 2-interacting protein-1, an inflammatory signaling molecule interferes with TNF reverse signaling in human model cells. *Immunol Lett* (in press)

Juhász K., Buzás. K., Duda E. (2013) Importance of reverse signaling of the TNF superfamily in immune regulation. *Expert Rev Clin Immunol* 9(4) (in press)

Publications not related to the thesis

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