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

PhD thesis
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Table of contents

1 Introduction

1.1 The emerging field of tumor necrosis factor

In 1975 an exciting article has been published reporting of an endotoxin-induced serum factor that caused necrosis of tumors. In this paper Carswell and colleagues named first the necrosis inducing molecule as tumor necrosis factor (TNF) (Carswell *et al.*, 1975). Cloning of the TNF gene in 1984 marked the beginning of its "success story". Over the next decade a whole family of related molecules has been identified with roles in cell death, survival, differentiation and organogenesis. However, the dream of TNF as a powerful anticancer molecule has soon dissolved, when introduction of the recombinant protein was found to induce septic shock. In fact, TNF has been identified as a regulator of inflammation and a key molecule in the cytokine network of the immune system. This has led to the development of TNF antagonists used in the treatment of chronic inflammatory diseases, where TNF is often overproduced, radically improving the quality of life of millions of patients with arthritis, inflammatory bowel disease and psoriasis. Furthermore, therapeutic TNF antagonists have been tested in Phase I and II clinical trials in patients with certain cancers with promising results (Harrison *et al.*, 2007; Madhusudan *et al.*, 2005). Therefore, TNF is considered as one of the most exciting molecules in cancer biology, immunology and medicine of the 21st century.

1.2 The TNF molecule

1.2.1 Transmembrane and soluble TNF

Tumor necrosis factor (TNF) is the prototype molecule of the TNF superfamily, exerting a broad spectrum of activities through its membrane bound (tmTNF) or soluble form (sTNF). TNF is a pleiotropic cytokine as it is able to induce differentiation, gene-expression, apoptosis or necrosis in the immune and

neuroendocrine system depending on the type and actual state of the responding cells. TNF is mainly expressed by immune cells such monocytes, macrophages, dendritic cells, T and B cells, but also by other cell types like keratinocytes, adipocytes, cardiac myocytes, osteoblasts, endothelial and epithelial cells. Furthermore, endogenous TNF expression has been reported in several cancer cells as well (Balkwill *et al.*, 2006).

The 26 kDa transmembrane TNF (tmTNF) is generated from a transcript of 1.7 kb with an unusually long and atypical leader sequence. There is a hydrophobic region in this sequence which acts as a transmembrane domain consisting of 26 amino acid residues. A constitutive palmitoylation at position Cys47 between the transmembrane and the cytoplasmic domain of tmTNF has been also described. The authors reasoned that its function may be similar to the role of palmitoylation of Src family of tyrosine protein kinases and G proteins, where the modification anchors the protein to membranes (Utsumi *et al.*, 2001).
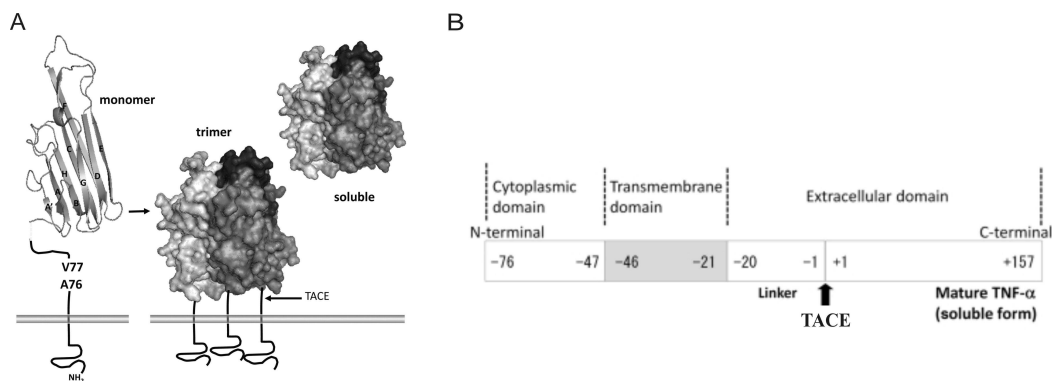
Fig. 1. Schematic structure of the transmembrane TNF
(A) TNF is synthesized as a monomer that folds into a beta-sheet sandwich and assembles into a functional trimer (adapted from Keystone & Ware, 2010) (B) Transmembrane TNF is a type II polypeptide composed of an extracellular domain (177 aa. residues), a transmembrane domain (26 aa. residues, shaded) and an intracellular domain (30 aa. residues). Mature, soluble TNF of 157 amino acid residues is cleaved from tmTNF by TACE (black arrow) (adapted from Horiuchi *et al.*, 2010).

Upon stimulation by e.g. lipopolysaccharide (LPS), newly synthesized TNF rapidly accumulates in the Golgi complex as a type II transmembrane protein (Shurety *et al.*, 2000) and the molecule is then transported to recycling endosomes before delivery to the plasma membrane (Murray *et al.*, 2005). Upon further

stimulation tmTNF can be cleaved by TNF-alpha converting enzyme (TACE) between residues Ala76 and Val77 (Gearing *et al.*, 1994). Soluble TNF is released from the cell surface as a homotrimer in the form of three mature 17 kDa TNF molecules (Black *et al.*, 2002) (Fig.1).

1.2.2 Biological activities of TNF

Since TNF exhibits both beneficial and pathologic effects, its expression requires a rigorous control. Regulation of human TNF expression is fairly complex, involving controls at both transcriptional and post-transcriptional levels. The promoter region of the human TNF gene contains a complex array of potential regulatory elements. Binding sites for multiple transcription factors, including AP-1, Egr-1, CRE, C/EBPb, and AP-2 have been shown to mediate TNF transcription in monocytic cells (Economou *et al.*, 1989; Pope *et al.*, 1994; Wedel *et al.*, 1996; Yao *et al.*, 1997).

The synthesis of TNF can be induced by many different stimuli including interferons, cytokines, tumor cells, irradiation, hypoxia, immune complexes and platelet activating factor. Factors from bacteria, viruses, and parasites can also stimulate production of TNF through Toll-like receptors via nuclear factor-κB (NF-κB) signaling (Kawai & Akira, 2006). Regulation of TNF production occurs mainly post-transcriptionally where 3' UTR sequences modulate translation and degradation of TNF mRNA (Han & Beutler, 1990).

Soluble 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 (Chan *et al.*, 2000). While TNFR1 is expressed in all nucleated cells, TNFR2 is mainly expressed in immune cells, endothelial cells and neurons. TNFR1 is capable of directly inducing apoptosis through its death domain (DD). TNFR2 does not contain DD and can be fully stimulated only by the membrane integrated form of the ligand (Grell *et al.*, 1995). Upon stimulation by soluble TNF, TNFR2 is thought to be a ligand passing receptor that after capturing soluble TNF, rapidly transfers the molecule to TNFR1 (Tartaglia *et al.* 1993).

Binding of sTNF to its receptors triggers a series of intracellular events that result in the activation of transcription factors, such as NF-κB and c-Jun. TNF-activated complex signaling pathways mediate diverse biological processes, including cell growth, cell death, development, inflammatory or stress responses. Notably, binding of TNF to TNFR1 causes upregulation of proapoptotic signals but it also leads to the translocation of NF-κB into the nucleus, protecting the cell from apoptosis. 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. Interestingly, TNF producing T lymphocytes, macrophages and endothelial cells expressing also TNF receptors display TNF responsiveness. Autocrine functions of TNF have been implicated in monocyte mediated cytotoxicity (Smith *et al.*, 1990) and in primary T cell activation (Pimentel-Muiños *et al.*, 1994). Non-immune cells producing both TNF and TNFR can be completely resistant to TNF mediated cytotoxocity (Okamoto *et al.*, 1992) that could be explained by the observation that overexpression of TNF leads to disappearance of TNFR1 and 2 from the plasma membrane (Decoster *et al.*, 1998). Intriguingly, a novel TNFR2 isoform has been described expressed within the cell and not on the cell surface. Activation of this isoform leads to enhanced NF-κB activation providing a possible mechanism for survival of cells producing both TNFR and TNF (Seitz *et al.*, 2001). Juxtacrine activities are also known for TNF such as induction of inflammatory cytokines in neighboring cells or stimulating fibroblasts to express intracellular adhesion molecule 1 (ICAM-1) (Tilders *et al.*, 1994). 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.

1.2.3 TNF and inflammation

The pivotal role in the immune response to bacterial, fungal, viral and parasitic infections accounts for a beneficial function of TNF. It 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. TNF has been called a sentinel cytokine or "the body's fire alarm" as it initiates the defence response to local injury. LPS from the bacterial cell wall is an especially potent stimulus for TNF synthesis. Expressed at high concentrations TNF can lead to chronic inflammation and organ injury. Acute release of very large amounts of TNF during sepsis may result in septic shock with a high mortality rate. Moreover, upregulation 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.

1.3 Transmembrane TNF as a ligand

Since tmTNF can function in cell-to-cell contacts, it is considered to play a critical role in ontogenesis and in local inflammation. Similar to soluble TNF, tmTNF exists as a homotrimer of uncleaved monomers and binds to both subtypes of TNF receptors on target cells, however biological activities are supposed to be mediated mainly through TNFR2 (Grell *et al*., 1995). 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 (Horiuchi *et al*., 2010) and HIV-infected lymphocytes (Lazdins *et al*., 1997). Importance of tmTNF in inhibition of intracellular pathogens has been also reported (Allenbach *et al.*, 2008). In addition, tmTNF has been shown to initiate T cell and macrophage migration, granuloma formation and to be effective against acute *M. tuberculosis* infection (Saunders *et al*., 2005). Direct cell-to-cell contacts through tmTNF trigger activation processes in endothelial cells, B cells, T cells, monocytes and NK cells (Horiuchi *et al*., 2010). Furthermore, short term expression of tmTNF plays a homeostatic role in the heart (Mann *et al.*, 2003) and the liver (Kresse *et al*., 2005).

1.4 Transmembrane TNF as a receptor

In 1994 Smith and colleagues suggested that transmembrane ligands of the TNF superfamily might elicit bidirectional signals, as the molecules are capable of eliciting signaling also in the ligand expressing cell. This was based on the fact that the cytoplasmic parts of the ligands are highly conserved among species (Smith *et al.*, 1994). Indeed, binding of TNF receptors or agonistic antibodies to tmTNF can induce signaling events causing cell activation, cytokine suppression or apoptosis in the tmTNF expressing cells (Eissner *et al.*, 2000; Harashima *et al.*, 2001). Since then the phenomenon of "reverse signaling" has been described for a number of TNF superfamily members and considered as a fine tuning control mechanism in the immune response (Eissner *et al.*, 2004).

1.4.1 Biological activities of TNF reverse signaling

In contrast to the well characterized functions of tmTNF as a ligand, the biological functions of tmTNF as a receptor still remain to be clarified. TNF reverse signaling has been reported to trigger activation in human T cells and NK cells. In addition to its co-stimulatory role and Ca^{2+} signaling inducing capacity in T cells (Higuchi *et al.*, 1997), tmTNF stimulation could induce the production of high amounts of IL-2 and adhesion molecules such as E-selectin (Harashima *et al.*, 2001). Such a positive regulatory effect of TNF reverse signaling has been shown in NK cells leading to increased cytotoxicity (Yu *et al.*, 2009).

TNF reverse signaling has also been described in monocytes, where it served as a silencing mechanism rather than a stimulatory signal. Stimulation of monocytes with various TNF reactive agents, including TNF receptor positive endothelial cells conferred resistance to LPS (Eissner *et al.*, 2000).

In the view of limited experimental data the *in vivo* role of TNF reverse signaling has remained elusive. Based on animal model studies, reverse signaling could have diverse effects on the helper cell and cytolytic functions. Since TNF signaling leads to anergy of monocytic cells *in vitro*, in innate immunity it might

limit the destructive potential of monocytes against target cells. Furthermore, TNF reverse signaling has been proposed to be responsible for resistance to TNFR mediated cell death in cancer cells through continuous activation of NF-κB (Zhang *et al.*, 2008).

1.4.2 TNF reverse signaling in anti-TNF therapies

TNF has been proposed as a mediator in various pathologies including septic shock, cancer, transplantation rejection, multiple sclerosis, diabetes, meningitis, ischemia-reperfusion, ulcerative colitis, rheumatoid arthritis (RA) and Crohn's Disease (CD). Strategies for preventing TNF activity in multiple types of inflammation include neutralization of the cytokine via anti-TNF antibodies, soluble receptors or receptor fusion proteins. Anti-TNF agents (Fig. 2) have been successfully applied to the treatment of CD and RA as well as other chronic inflammatory diseases such as psoriasis, ankylosing spondylitis and Behcet's disease (Tracey *et al.*, 2008). Infliximab, adalimumab and etanercept have been licensed treatments in the USA and Europe for years. Certolizumab pegol and golimumab have been recently approved for clinical use by Food and Drug Administration (FDA).

Emerging data suggest that these drugs have multiple functions and can act not only as antagonists by blocking TNF interactions with TNF receptors. Antibody-based drugs can initiate antibody dependent cytotoxicity as well as inhibit granuloma formation. Moreover, anti-TNF agents are able to induce TNF reverse signaling in tmTNF expressing cells leading to cell activation, cytokine suppression or apoptosis (Horiuchi *et al.*, 2010).

Reverse signaling through tmTNF has been shown to induce cytokine suppression and endotoxin resistance which mechanism may operate also in anti-TNF therapies (Eissner *et al.*, 2004). Since suppressor activities of anti-TNF drugs have been reported to involve the pathways of LPS induction (Kirchner *et al.*, 2004), suppression may occur by exhaustion of common signaling components. Although all TNF antagonist drugs can bind to tmTNF, antibody-based drugs

have been found to be more efficient in cytokine suppression (Nesbitt *et al.*, 2007; Scallon *et al.*, 2002).

Numerous *in vitro* studies have been reported on apoptosis inducing activities of anti-TNF drugs. Infliximab has been found to induce apoptosis in peripheral blood monocytes of healthy individuals and patients with CD (Lügering *et al.*, 2001). It has also been shown that binding of infliximab to activated Jurkat T lymphocytes increases the Bax/Bcl-2 ratio and causes apoptosis. Moreover, infliximab treatment induced a rapid increase in the number of apoptotic lamina propria T lymphocytes in active CD patients (Mitoma *et al.*, 2005). Such a proapoptotic effect of infliximab has also been shown *in vitro* using lamina propria T cells and peripheral blood T cells from CD patients (Di Sabatino *et al.*, 2004). In addition to *in vitro* data sets, infliximab could induce apoptosis of monocytes and T cells in a human-mouse chimeric *in vivo* model. Both infliximab and adalimumab could induce apoptosis in normal blood monocytes, human acute monocytic leukaemia cells (THP-1) and normal blood T cells (Nesbitt *et al.*, 2007; Shen *et al.*, 2005).

Differences between clinical efficiencies of anti-TNF agents may be explained by difference in pharmacokinetics, tissue distributions and functional properties. TNF neutralization by infliximab, adalimumab and etanercept has been a successful treatment strategy for several autoimmune diseases controlling progression and in some cases inducing remission by suppression of cytokine production. However, infectious complications have been reported in anti-TNF treated patients, where the treatment has been associated with an increased risk of infection probably through interfering with innate immunity. Recently, a thorough statistical study proved that anti-TNF therapy is associated with a significant overall risk of serious infections (Galloway *et al.*, 2011). Since increased incidence of reactivation of latent tuberculosis has been reported for clinical anti-TNF therapies (Keane *et al.*, 2001), testing for tuberculosis is now recommended by FDA for patients starting anti-TNF therapies.
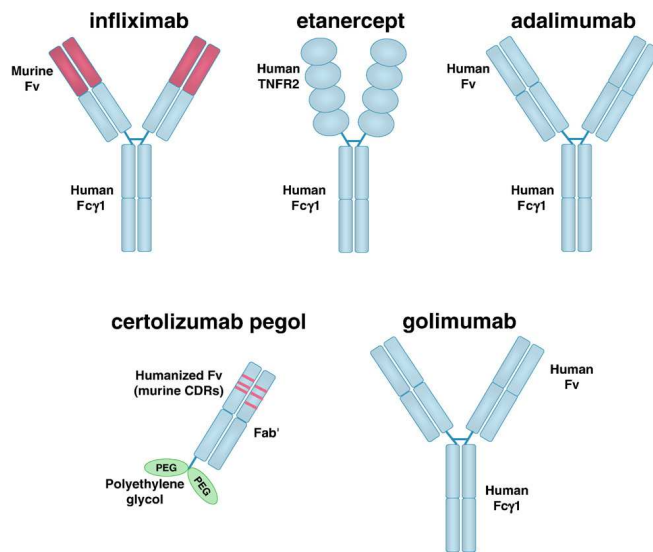
Fig. 2 Schematic structures of clinically used TNF antagonists. Infliximab is a mouse-human chimeric monoclonal anti-TNF antibody of IgG1 isotype. Adalimumab and golimumab are fully human IgG1 monoclonal antibodies. Etanercept is a fusion protein of the extracellular domain of TNF-R2 and the Fc region of IgG1. Certolizumab pegol is a PEGylated F(ab) fragment of a humanized monoclonal anti-TNF antibody (Tracey *et al.*, 2008).

Although a dose-dependent risk of malignancies has been reported in patients with RA treated with anti-TNF antibody (Bongartz *et al.*, 2006), recent meta-analysis of data did not reveal an increased risk (Le Blay *et al.*, 2012). Notably, FDA also investigates the possible association between the use of TNF blockers and the development of lymphoma and other cancers in children and young adults.

1.4.3 Molecular mechanism of TNF reverse signaling

Despite the apparent clinical relevance the molecular basis of TNF reverse signaling is largely unknown. The cytoplasmic domain of the tmTNF is conserved in different species (Watts *et al.*, 1999) and does not possess any enzymatic functions, hence probably acting through adaptor proteins. Our research group reported earlier that the cytoplasmic domain of TNF is serine phosphorylated in LPS-induced tmTNF expressing cells (Pócsik *et al*., 1995). Watts and his colleagues further characterized these sites as a substrate for casein kinase-1 and

demonstrated that interaction of tmTNF with its soluble receptor triggers rapid dephosphorylation of tmTNF by a yet unknown serine/threonine phosphatase and a concomitant Ca^{2+} signaling (Watts *et al*., 1999). However, phospho-amino acids on receptors often provide docking sites for the assembly of signaling complexes. We revealed earlier a functional nuclear localization signal (NLS) sequence in the intracellular domain of tmTNF, further pointing to tmTNF having receptor-like properties (Domonkos *et al*., 2000). Moreover, signal peptide peptidase-like proteases (SPPLs), SPPL2a and SPPL2b have been shown to promote intramembrane proteolysis of tmTNF, leading to IL-12 production in activated human dendritic cells (Friedmann *et al.*, 2006). The cytoplasmic serine residues of tmTNF have been proved essential for infliximab-induced interleukin-10 production, apoptosis and G0/G1 cell cycle arrest, where Infliximab treatment was shown to up-regulate Bax, Bak, and p21 expression (Mitoma *et al.*, 2005). Infliximab had apoptotic effect on circulating monocytes of patients with active CD through activation of caspase-8 and the mitochondrial pathway (Lügering *et al.*, 2001).

Identifying molecules of pathways involved in TNF reverse signaling became of high importance in the last decade. TNF reverse signaling induced the activation of p38, a stress activated member of the mitogen-activated protein kinase (MAPK) family (Kirchner *et al.*, 2004). In mouse macrophages reverse signaling triggered at least two independent pathways that could be distinguished by using Protein Kinase C (PKC) inhibitors. The suppression of the LPS-induced death factor was dependent on PKC, whereas the suppression of LPS-mediated cytokine release was not (Eissner *et al.*, 2000). Interestingly, the activation of TNF reverse signaling rendered macrophages refractory to a subsequent activation of the MAPK pathway by LPS (Kirchner *et al*., 2004). However, pathways activated by TNF reverse signaling leading to cytokine suppression remained to be further explored.

Molecular mechanism of apoptosis caused by TNF reverse signaling upon anti-TNF therapies has been studied more intensively, but the mechanism still remains elusive (Fig. 3). Upregulation of Bax and Bak and posphorylation of c-Jun N-

terminal kinase (JNK) has been shown upon infliximab-induced apoptosis in T cells (Mitoma *et al.*, 2005). Infliximab-induced monocyte apoptosis in CD patients required the activation of caspase-8, -9 and -3, while the authors suggested that apoptotic signaling is rather triggered by binding anti-TNF captured soluble TNF to the TNF receptor (Lügering *et al.*, 2001). Waetzig and his colleagues found activation of MAPK cascades by soluble TNFR1 and anti-TNF antibody in THP-1 monocytes. Intriguingly, they showed a secondary, autocrine loop of transforming growth factor beta (TGF-beta) signaling that leads to apoptosis (Waetzig *et al.*, 2005).
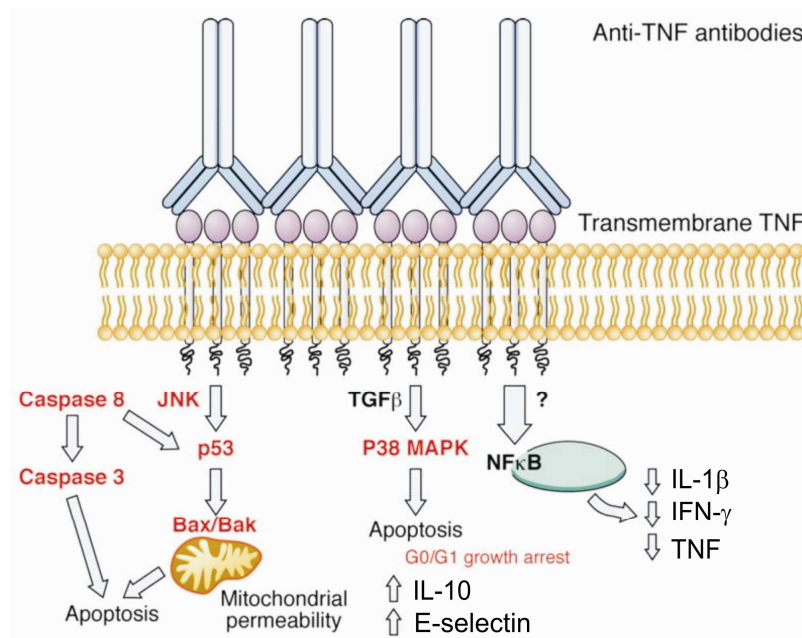
Fig. 3 Reverse signaling induced through tmTNF
Engagement of tmTNF by antibodies induces reverse (outside to inside) intracellular signaling cascade, which leads to cytokine production and E-selectin expression associated with caspase-dependent apoptosis. Caspase 8 acts to promote apoptosis through its downstream effects on caspase 3, as well as on proteins that influence mitochondrial permeability, Bax and Bak. TNF reverse signaling can inhibit the production of inflammatory cytokines through NF-kB activation, induces apoptosis or growth arrest through MAPK (adapted from Wong *et al.* 2008).

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

Identifying signaling molecules recruited to the tmTNF molecule and investigating their role may help to unravel molecular details of TNF reverse signaling. To find potential proteins which interact with the intracellular domain of the tmTNF molecule Kohchie and his colleagues screened a cDNA library of LPS-activated mouse macrophages by yeast two-hybrid method. LPS-stimulated mouse macrophage cells (RAW264.7) were used for the construction of the library and the intracellular domain of the mouse TNF was used as bait. Among several molecules (e.g. heat shock proteins) a novel protein was found, which activated the transcription of the reporter gene indicating an interaction with the intracellular domain of the tmTNF. The protein was termed as mouse TNF intracellular domain interacting protein (mTIP; AF168675.1). The human homologue (hTIP; AF168676.1) was cloned from a human lymph node plasmid library with a mouse probe. Subcloned cDNA coding for human and mouse TIP used in this study were kindly provided by Dr. Chie Kohchie (Hiroshima University, Japan). hTIP has been identified later as a human casein kinase 2-interacting protein-1 (hCKIP-1; AF217956.1) (Bosc *et al.*, 2000). Since then, CKIP-1 has been found to interact with a series of proteins involved in cellular functions like differentiation, cell motility and cell death.

CKIP-1 has a highly conserved DNA sequence in mammals, encoding a protein of 409 amino acid residues with a molecular mass of approximately 50 kDa. CKIP-1 is composed of a plecstrin homology (PH) domain at the N terminus, a leucine zipper (LZ) motif at the C terminus and five proline-rich motifs throughout the protein. Furthermore, the protein has many potential phosphorylation, glycosylation and N-myristoylation sites, indicating that extensive modification of the protein might alter its localization and activity.

PH domains are believed to target cellular membranes by binding specifically to phosphatidyl inositol derivatives (Bottomley *et al*., 1998). PH domains could also interact with other proteins and mediate protein-protein interactions (Lemmon *et*

al., 1995). Indeed, it has been shown, that phosphatidylinositide 3-kinase regulated the cellular localization of CKIP-1 as the PH domain binds to phosphatidylinositol 3-phosphate (Bosc *et al.*, 2000). It has been reported that CKIP-1 can bound to a broad spectrum of phospholipids *in vitro* through its PH domain and dimerize through the LZ motif (Olsten *et al*., 2004; Safi *et al*., 2004). However, the role of dimerization remains to be clarified. CKIP-1 has been shown to interact with c-Jun (Zhang *et al*., 2005), actin capping protein subunits (ACP α and β) (Canton *et al.*, 2005), ataxia telangiectasia mutated (ATM) kinase (Zhang *et al*., 2006), Akt (Tokuda *et al.*, 2007), interferon induced protein 35 (IFP35) and N-myc interacting protein (Nmi) (Zhang *et al*., 2007) as well as Smurf-1 (Lu *et al.*, 2008). CKIP-1 tested in different cell types exhibited diverse functionality. CKIP-1 has been reported to recruit proteins like CK2 and nuclear ATM to the plasma membrane in cancer cell lines (Olsten *et al*., 2004; Zhang *et al*., 2006). Moreover, it has been also shown that in various cancer cell lines, CKIP-1 forms a complex with Akt through its PH domain irrespective of Akt phosphorylation status and activity, while the LZ motif of CKIP-1 plays an important role in the suppression of Akt kinase activity suppressing tumor growth (Tokuda *et al.*, 2007). Similarly, CKIP-1 deficient mice undergo an age dependent increase in bone mass as a result of accelerated osteogenesis through decreased Smurf1 activity (Lu *et al.*, 2008). In osteosarcoma cells CKIP-1 affected the cell morphology through regulation of the actin cytoskeleton, interacted with actin capping protein and facilitated the phosphorylation of ACP by CK2 (Canton *et al.*, 2005). Recently, CKIP-1 has been shown to possess a consensus motif of CARMIL proteins, which inhibit actin capping protein, influencing actin-based lamellipodial dynamics (Takeda *et al.*, 2010). In several epitheloid cells, CKIP-1 was cleaved by caspase-3 and the cleaved CKIP-1 fragment repressed activator protein-1 (AP-1) activity and promoted apoptosis (Zhang *et al*., 2005). The C terminal domain of CKIP-1 possesses a LZ motif and is predicted to form coiled coils. Indeed, this C terminal fragment of the molecule has been shown to interact with c-Jun. However, CKIP-1 lacks an adjacent basic region to the LZ or any other DNA-binding motifs typical of basic LZ proteins, indicating that CKIP-1 is

not a transcription factor (Zhang *et al*., 2005). CKIP-1 exerts a positive function in skeletal muscle development. Its expression is up-regulated upon induction of C2C12 myoblast differentiation, and overexpression of CKIP-1 enhances myoblast differentiation (Safi *et al.*, 2004). It has been recently demonstrated that CKIP-1 is essential for muscle precursor elongation and fusion through regulation of cell morphology and lamellipodia formation (Baas *et al.*, 2012).

CKIP-1 is mainly localized to the plasma membrane. Interestingly, upon stimulation, a portion of CKIP-1 could translocate into the cytoplasm and to the nucleus (Zhang *et al*., 2005). Moreover, it has been shown that CKIP-1 is able to shuttle between the plasma membrane and the nucleus in myoblasts, where its PH domain and a C terminal autoinhibitory region co-ordinates the translocation (Xi *et al.*, 2010). Therefore, CKIP-1 appears to be a signaling molecule playing different regulatory roles in different cell types.

In peripheral blood mononuclear cells CKIP-1 is an interacting partner of IFP35 and Nmi, where the ratio of Nmi to CKIP-1 determines the stability of IFP35 thereby controlling cytokine signaling (Zhang *et al*., 2007). Data of expression studies suggest that CKIP-1 expression can be regulated in haemopoietic cell differentiation and enriched in certain subpopulations of blood cells. However, the role of CKIP- 1 in immunity is still unclear.

2 Aims of the study

The research interest of our group has been focusing on the biological role of the TNF molecule. Since a novel molecule CKIP-1 has been identified as an interacting partner of the pro-inflammatory tmTNF, we hypothesized that CKIP-1 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.

The thesis focuses on results with human model cells.

3 Materials and Methods

3.1 Cell culture

HEK293 cells (ATCC, Manassas, VA) and their derivatives were grown in a (1:1) mixture of Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) and Ham's F-12 nutrient medium (Sigma) supplemented with 10 % fetal calf serum (FCS) (Sigma) and 2 mM L-glutamine (Sigma). THP-1 cells were maintained in OptiMEM (Life Technologies, Carlsbad, CA) with 2 % FCS. For activation of THP-1 monocytes cells were challenged by a medium containing 10-1000 ng/ml LPS (Sigma), 50 µg/ml Infliximab (Remicade; Centocor B.V., The Netherlands), 150 µg/ml hIgG (Human gamma globulin; Human Bioplazma Kft., Hungary) or 5 µM etoposide (Sigma).

Adherent THP-1 cells were generated by priming the suspension cells with 25 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma) for 72 h. Adherent THP-1 cells were treated with $F(ab)_2$ fragment of Infliximab, generated by using $F(ab)_2$ Preparation Kit (Thermo Scientific, Rockford, IL).

3.2 Expression vectors

The human CKIP-1 coding sequence in pQBI-25 vector was kindly provided by Dr. Chie Kohchi (Hiroshima University, Japan). The coding sequence was cloned into pBluescript-SK+ (Stratagene, Santa Clara, CA) and into mammalian expression vectors pcDNA3 (Invitrogen), pEGFPC1, pECFPC1 (Clontech, Mountain View, CA) and p3xFlag-Myc-CMV-26 (Sigma). The 252 bp N terminal fragment of tmTNF (TNFNterm) was fused to glutathione S-transferase (GST) by cloning the cDNA into pGEX-4T1 (Amersham Biosciences, Germany) and was fused to EYFP or to mCherry by cloning into pEYFPN2 or pmCherryN2 (Clontech), respectively. In promoter activation experiments construct pGL3-TNFprom (pGL-3 from Promega) and pNF-κB-luc (Clontech) were used, in which the luciferase gene was controlled by the TNF promoter (-801 to +1) or a

promoter sequence containing 5x NF-κB binding sites, respectively. The Path Detect Trans Reporting System (pFA2-c-Jun, pFR-Luc, pFc-MEKK, pFC-dbd) was purchased from Stratagene. The mammalian expression plasmid for c-Jun (pcDNA3-c-Jun) was kindly provided by Dr. Imre Kacskovics (Eötvös L. University, Hungary). Enzymes used for *in vitro* recombinant DNA techniques were purchased from Fermentas and New England Biolabs.

3.3 Transient transfections and reporter gene assays

Plasmids pGL3-TNFprom and pNF-κB-luc were introduced into HEK293 cells by Lipofectamine-2000 (Life Technologies) and stable clones were isolated after G418 (Sigma) selection. Stable clones (2×10^5 cells) were transiently co-transfected in 24-well plates with 100 ng of expression vectors for CKIP-1 and c-Jun using JetPEI (Poly Transfections, France) according to the manufacturer's protocol. Total amount of DNA transfected was kept constant by adding empty vector. In luciferase assays cells were harvested 36 h after transfection and total cell extracts were prepared. Briefly, cells were harvested in 50 µl of lysis buffer (Promega, Madison, WI). Crude cell lysates were cleared by centrifugation, luciferase activity of 20 µl cell extracts was measured after injection of 20 µl Bright and Glow substrate (Promega) in a Luminoscan Ascent luminometer (Labsystems, Oy, Finland). To analyze the effect of CKIP-1 on the transcriptional activity of c-Jun the PathDetect^{TM} Trans-Reporting System was used (Stratagene). Transient transfections were performed using JetPEI. Briefly, 10^5 HEK293 cells were seeded in 24-well plates 16 h prior to transfection. Each point was co-transfected with 500 ng of pFR-Luc, 100 ng of pFA2-c-Jun and 100 ng of the positive control vector pFc-MEKK or 100 ng of pQBI-hCKIP-1. Parallel experiments were performed by co-transfecting the empty vector pQBI-25. Cells were grown in serum free medium for 6 h after transfection, then 10 % FCS containing culture medium was added and the cells were incubated for 24-36 h. Total cell extracts were prepared for luciferase assays as described above. For

flow cytometry and fluorescence microscopy experiments, THP-1 and HEK293 cells were transiently transfected by an Amaxa Nucleofector device (Amaxa, Germany) following the manufacturer's protocols and using Amaxa® Cell Line Nucleofector® Kit V (Lonza, Germany) or Lipofectamine-2000 (Invitrogen), respectively.

3.4 Protein expression, purification and GST-pull down assay

GST fusion proteins were purified from *E. coli* BL21 (DE3) lysates using 50 % slurry of glutathione-Sepharose beads (Amersham Pharmacia Biotech). pBS-hCKIP-1 (0.5 µg) was transcribed and translated *in vitro* using the TNT coupled reticulocyte system (Promega) in a total volume of 25 µl according to the manufacturer's protocol. In each of the pull-down experiments 10 µl of *in vitro* translated ^{35}S-labeled protein was diluted in 100 µl binding buffer (20 mM N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid (HEPES), 100 mM KCl, 0.2 % Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride; pH 7.3). About 2 µg of fusion protein or GST immobilized on glutathione-Sepharose beads were added, and the interactions were allowed to proceed by rotation at room temperature for 30 min. Beads were washed five times with binding buffer. Proteins bound were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5 %). ^{35}S-labeled proteins were detected by autoradiography.

3.5 Cell lysis and immunoprecipitation

24 h post-transfection HEK293 cells were washed in ice-cold phosphate buffered saline (PBS), scraped in lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 % NP-40, 5 % glycerol, 1 mM ethylenediaminetetraacetic acid, protease inhibitor cocktail; Sigma) and incubated under constant agitation at 4 °C for 1 h. Total cell extracts were cleared by centrifugation at 4000 g at 4 °C for 5 min. Total protein concentrations were measured using Bradford assay (Sigma). Immunoprecipitations were performed using the Pierce direct magnetic IP kit

(Thermo Scientific) according to the manufacturer's protocol. In brief, per IP 250 µg NHS-activated magnetic beads (Pierce) were covalently coupled with either 5 µg of monoclonal anti-Flag M2 (Sigma) or anti-RFP (Abcam, Cambridge, UK) antibodies, or left untreated. After washing beads were added to 500 µl (1 mg/ml) cell lysate. Following overnight incubation at 4 °C on a rotator, beads were thoroughly washed in lysis buffer and the bound proteins were eluted.

3.6 Immunoblotting

Protein samples were separated by SDS-PAGE (12.5 %) and proteins were transferred to polyvinylidene difluoride membranes. CKIP-1 protein levels in cell lysates were detected using anti-CKIP-1 antibody (Santa Cruz, Santa Cruz, CA) and the appropriate secondary antibody conjugated to horseradish peroxidase (Sigma), followed by detection with SuperSignal chemiluminescence kit (Thermo Scientific). Immunoprecipitation samples were separated by SDS-PAGE (10 %) and after blotting onto polyvinylidene difluoride ethylenediaminetetraacetic acid membrane, probed with anti-Flag M2 (Sigma) and HRP-conjugated anti-mouse-IgG secondary antibody (Sigma).

3.7 Expression Profile Verification

2 µg of total RNA from each sample was reverse transcribed and the cDNA was used for qRT-PCR. Reactions were carried out in ABsolute QPCR SYBR Green mix (ABGene, Epsom, UK) according to the manufacturer's instructions on a RotorGene 3000 instrument (Corbett Research, Sydney, Australia). Final relative gene expression ratios were calculated as delta-delta Ct values. Primer sequences for hCKIP-1 (forward and reverse, respectively) were used as follows: CCACTCGAGACAGGGCAAAA and AGCCATTAGGTGTCCCCTTGT.

3.8 Flow cytometry

For apoptosis measurements, 24 h after transfection 2.5x 10^5 cells were labeled with Annexin V-Alexa647 (Invitrogen) in staining buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) following the manufacturer´s instructions. For surface staining measurements, transfected cells were stained with Infliximab-A647 (labeled by Alexa Fluor 647 Microscale labeling kit, Invitrogen), anti-human B7/CD86-APC, anti-human DC-SIGN-APC or the appropriate isotype controls (RnD Systems, Minneapolis, MN), following the manufacturer´s protocols. Samples were analyzed by flow cytometry (BD FACSAria^{TM}) with an excitation at 488 nm and 633 nm using the bandpass filters 530/30 nm, 610/20 and 660/20 nm as appropriate. Cells with damaged membranes were gated by propidium-iodide (5 µg/ml, Sigma) exclusion. Following Forward Scatter (FSC) vs. Side Scatter (SSC) gating the EGFP (Enhanced Green Fluorescent Protein) positive population was selected using an FSC vs. Fluorescein isothiocyanate (FITC) dot plot.

3.9 Fluorescence microscopy

Cells analyzed by microscopy were grown either in suspension (THP-1) or on glass bottom dishes (HEK293) (35 mm in diameter Willco Wells BV, The Netherlands). Suspension THP-1 cells were dropped onto glass bottom dishes at 37 °C before visualizing with a custom designed instrument for large area fluorescence imaging (CytoScout®) based on an Axiovert 200 microscope (Zeiss, Germany) equipped with a 100x objective (alpha plan, N.A. 1.45) [25]. Enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) were excited at wavelengths of 405 nm and 514 nm and emissions were detected at 480/40 nm and 550/40 nm, respectively. Large areas of the samples (0.9 x 0.9 mm^2) were scanned by using the CytoScout® and Coolsnap HQ cameras (Photometrics, Tucson, AZ). Images were analyzed and overlayed for dual color experiments by V++. Localization of CKIP-1 was inspected in positively

transfected cells while applying manual threshold for subtracting autofluorescence.

3.10 Ca^{2+} measurements

Adherent THP-1 cells were loaded with 5 μM Indo 1-AM (Invitrogen) for 20 min at room temperature in the dark. Indo 1-AM allows monitoring changes in free cytoplasmic calcium levels (Grynkiewicz *et al*., 1985). After washing steps with Ca^{2+}-containing Hank's Balanced Salt Solution (Invitrogen) live cells were treated with 50 μg/ml F(ab)$_2$ fragment of TNF antibody (Infliximab) or control solution. F(ab)$_2$ fragment was produced by enzymatic reaction and subsequent separation using an F(ab)$_2$ fragment preparation kit (Thermo Scientific) following the manufacturer's instructions. *In situ* treated live cells were monitored by fluorescence microscopy. Samples were excited using a mercury lamp (HBO100, Zeiss, Germany) at 333/30 nm and time-resolved fluorescence emissions at 405/20 nm and at 485/25 nm were were collected via a 40x Neofluar objective (Zeiss, Germany) and detected simultaneously by Coolsnap HQ^{TM} CCD cameras (Photometrics, Tucson, AZ, USA). 240 frame videos were aquired at a frame rate of 0.5 frames per second, using an exposure time of 50 ms and 4x binning. Ratio of fluorescence emission at 405 nm (Ca^{2+}-bound dye) and at 485 nm (Ca^{2+}-free dye) was calculated for individual cells with the software Matlab® (MathWorks, Natick, MA, USA).

3.11 Confocal microscopy and image analysis

Constructs pECFPC1-CKIP-1 or pEYFP-N2-TNFNterm were transiently transfected into HEK293 cells as described above. ECFP and EYFP fluorescence in living cells was detected with an Olympus FV1000 confocal microscope (Olympus, Germany) using a 60x (N.A. 1.35) objective and standard filter settings. Live THP-1 cells transfected with pEGFPC1-CKIP-1 were visualized with a 40x (N.A. 1.30) objective.

For co-localization analysis, pEGFPC1-CKIP-1 or pmCherry-N2-TNFNterm were transiently transfected into HEK293 cells as described above. 48 h post transfection the cells were fixed in 4 % formaldehyde supplemented with 4 % sucrose at room temperature for 10 min. Fluorescence images were recorded with an Olympus FV10i confocal microscope (Olympus) using a 60x (N.A. 1.20) water immersion objective and standard filter settings. For quantification of the intracellular co-localization, the threshold was set manually to distinguish cytosolic CKIP-1 from CKIP-1 enriched intracellular regions. The Mander's overlap coefficients were calculated for TNFNterm-mCherry vs. CKIP-1-EGFP using the JACoP plugin for the ImageJ software (Wayne Rusband, NIH, Bethesda, USA). Hoechst 33342 (Invitrogen) labeled nuclei served as a control for Mander's co-localization analysis.

3.12 Statistical analysis

Experiments were carried out at least in duplicates. Data were analyzed by unpaired t-test and multiple comparisons were probed by ANOVA.

P values are shown by asterisks, where *, ** and *** correspond to p= 0.01 – 0.05, p= 0.001 – 0.01 and p< 0.001, respectively.

4 Results

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

Analysis of the promoter sequence of human *ckip-1* gene revealed several putative NF-κB binding sites (data not shown), similarly to inducible genes of inflammatory cytokines, where effects of extracellular agents e.g. LPS are known to be mediated by NF-κB transcription factors (Müller *et al*., 1993). To test whether CKIP-1 is involved in the inflammatory response, the effect of LPS on the transcriptional activity of *ckip-1* was followed in human monocytes. THP-1 as a human monocytic leukaemia cell line has been widely used as a surrogate for investigation human monocytes. Cells were treated with 100 ng/ ml LPS and harvested at different time points after stimulation. The expression of CKIP-1 was analyzed by qRT-PCR method. As shown in Fig. 4A, level of CKIP-1 mRNA was significantly elevated in cells exposed to LPS for 4 h, and a further increase was observed with a maximum at 6 h after LPS exposure. A similar upregulation was found in protein levels when followed by Western blot analysis (Fig. 4A, insert).

Next we tested whether elevated levels of CKIP-1 could influence differentiation steps in THP-1 monocytes. Anticipating low transfection efficiency in this cell type, CKIP-1-EGFP was transiently overexpressed in THP-1 cells, and only the successfully transfected subpopulation was analyzed in the experiments. 24 h after transfection live cells were stained for surface CD86 or Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) expression, for cellular surface markers of classical and alternative activation, respectively. Means of anti-CD86 or anti-DC-SIGN staining of positively transfected THP-1 cells are shown in Fig. 4B. CKIP-1 expression was found to facilitate the classical activation process as reflected in the significant increase in CD86 surface expression. Meanwhile, there was no influence on the alternative activation upon CKIP-1 overexpression measured by DC-SIGN surface expression.
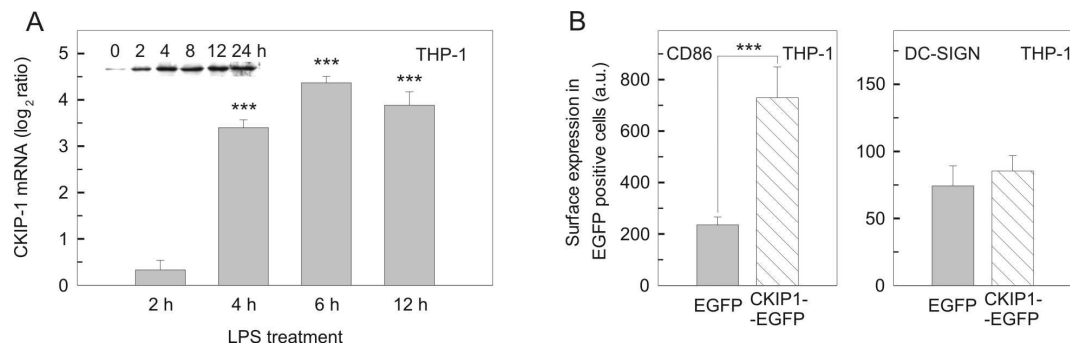
Fig. 4 CKIP-1 exerts pro-inflammatoy functions in THP-1 cells

(A) LPS-induced CKIP-1 expression in THP-1 cells. THP-1 cells were exposed to LPS and harvested at indicated time points after stimulation. Changes in the transcript level of CKIP-1 in cells were measured by qRT-PCR method. Primers for 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase were used to normalize the values. Expression levels are shown as relative ratios (n ≥ 6; mean ±SD). (insert) Protein level of CKIP-1 in control and LPS-treated cells were followed by Western blot analysis (a representative image shown). (B) CKIP-1 mediated classical activation of THP-1 cells. Surface expression of classical (CD86) and alternative (DC-SIGN) activation markers was measured by flow cytometry in THP-1 cells overexpressing CKIP-1 (n = 4 and n = 5 for CD86 and DC-SIGN staining, respectively; mean fluorescence intensity ±SD). Isotype values are 10.2 ±0.3, 13.6 ±1.2 and 27.9 ±2.4, 24.4 ±1.0 for CD86 and DC-SIGN staining, respectively.

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

Since we found CKIP-1 being upregulated at inflammatory conditions in THP-1 cells we tested whether CKIP-1 is involved in pro-inflammatory responses through activation of transcription factors. Members of the NF-κB family of transcription factors have been known to mediate the effects of LPS. Possible effect of CKIP-1 on activity of NF-κB transcription factors was tested using a luciferase reporter construct under the control of a promoter containing multiple NF-κB binding sites. Since transfection efficiency in THP-1 monocytes is known to be very low and the transfection procedure itself leads to enhanced cell death, an efficiently tranfectable human epitheloid cell line was used as a model cell system.

HEK293 cells were transfected with the reporter construct followed by isolation of stable clones. A number of clones were tested for inducibility by TNF treatment, a powerful activator of NF-κB. Transfection experiments were carried out with clones showing the highest response (data not shown).
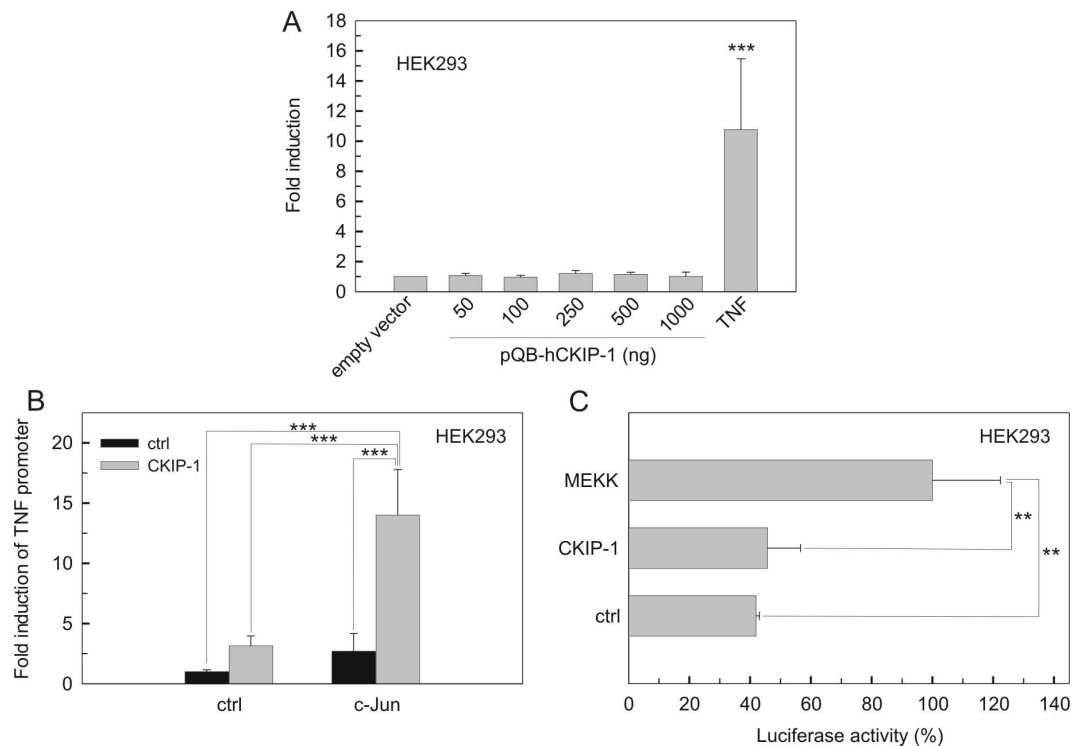
Fig. 5 Effect of CKIP-1 overexpression on transcription factor activation in HEK293 cells

(A) CKIP-1 overexpression does not induce NF-kappaB activation in HEK293 cells. Increasing amounts of expression vector for CKIP-1 were introduced into cells stably containing the NF-kappaB-luciferase construct. Luciferase activity in cell lysates was measured 36 h after transfection. Luciferase activity of cells treated with 12 U/ml TNF is shown as positive control. (B) Activation of TNF promoter by CKIP-1 and c-Jun in HEK293 cells. In stable clones carrying the luciferase gene under the control of the human TNF promoter c-Jun or CKIP-1 or c-Jun and CKIP-1 were transiently overexpressed. 24 h post-transfection luciferase activity was measured in cell lysates. (n ≥ 3; mean ±SD) (C) No influence of CKIP-1 on the activation of c-Jun in HEK293 cells. To evaluate c-Jun phosphorylation the Path-Detect Trans-Reporting System (Stratagene) was introduced into HEK293 cells. Effect of CKIP-1 expression measured as luciferase activity was compared to that induced by MEKK, used as a positive control. (n = 3; mean ±SD)

HEK293 cells containing the reporter plasmid were transfected with increasing amounts of CKIP-1 expression plasmid or empty vector and luciferase activity was measured in the cell lysates. Increased levels of CKIP-1 protein had practically no influence on the basal activity of the NF-κB transcription factors in HEK293 cells (Fig. 5A).

CKIP-1 has also been reported as an interacting partner of c-Jun (Zhang *et al.*, 2005) that is known to transactivate the TNF promoter upon LPS challenge (Rhoades *et al.*, 1992). To comprehend the effect of CKIP-1 on the transcriptional activity of the TNF gene, we generated stable clones of HEK293 cells carrying the

luciferase gene under the control of the human TNF promoter. Expression vectors coding for CKIP-1 and c-Jun were introduced into the cells. Protein expression levels for CKIP-1 and c-Jun were verified by Western blot analysis (data not shown). Although low amount of overexpressed c-Jun or CKIP-1 did not trans-activated the TNF promoter significantly, in cells transiently co-expressing both c-Jun and CKIP-1 a remarkable 13-fold activation of the TNF promoter was observed (Fig. 5B).

To elucidate whether CKIP-1 could participate in the activation of c-Jun we performed co-transfection experiments. Wild type HEK293 cells were transfected with the pathway specific construct pFA2-c-Jun encoding a fusion protein consisting of the DNA-binding domain of the yeast protein GAL4 and of the transactivator domain of c-Jun, with the reporter plasmid pFR-Luc and with the plasmid coding for CKIP-1. If the trans-activator domain of c-Jun becomes phosphorylated the fusion protein can bind to GAL4 binding sites in the promoter region of the luciferase reporter vector. Involvement of CKIP-1 in the activation of c-Jun was followed by measuring luciferase activity. Parallel experiments were carried out by co-transfection of plasmid encoding MEK kinase (MEKK), an upstream activator of c-Jun, as a positive control. Unlike in MEKK-transfected cells, overexpression of CKIP-1 did not facilitate the phosphorylation of c-Jun in HEK293 cells (Fig. 5C), pointing to other mechanisms leading to synergism between CKIP-1 and c-Jun on activation of TNF promoter in this cell line.

4.3 TNF reverse signaling induces Ca^{2+} oscillations in THP-1 cells

Anti-TNF treatments have been shown to induce reverse signaling in tmTNF producing immune cells (Mitoma *et al.*, 2008). 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. Since TNF reverse signaling has been shown to induce calcium signaling (Watts *et al.*, 1999), we tested whether Infliximab treatment leads to Ca^{2+} signaling in THP-1 cells. Since suspension THP-1 cells responded to mechanical stimuli with enhanced Ca^{2+} signaling, adherent THP-1 cells have been

used for the calcium measurements. PMA-induced adherent THP-1 cells were loaded with Indo-1-AM ratiometric dye and changes in the fluorescence intensities were followed by fluorescence live cell imaging. To avoid Fc-mediated effects, F(ab)$_2$ fragment of Infliximab was generated for these experiments and cells were treated either with F(ab)$_2$ fragment of Infliximab or with control solution. As shown in Fig. 6A and B, treatment with the F(ab)$_2$ fragment triggered THP-1 cells to specifically respond to a stimulation through tmTNF with Ca^{2+} signaling. The response was expressed in both the number of responsive cells and the amplitude of Ca^{2+} oscillations as compared to the effect of control solution.
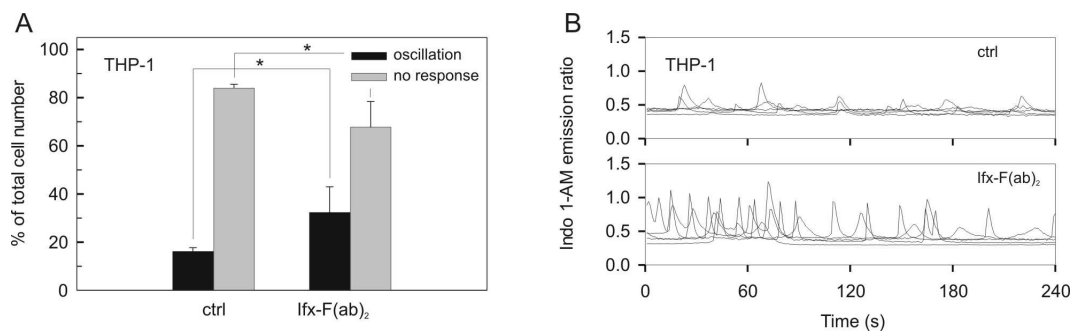

Fig. 6 TNF reverse signaling triggers Ca^{2+} signaling in THP-1 cells

(A). TNF reverse signaling induced Ca^{2+} oscillations in THP-1 cells PMA-primed THP-1 cells were adhered on glass bottom dishes and loaded with Indo1-AM ratiometric dye. Fluorescence intensities were monitored in cells following treatment with control solution or F(ab)$_2$ fragment of Infliximab (Ifx-F(ab)$_2$). Changes in the ratio of fluorescence emissions at 405 nm (Ca^{2+}-bound form) and 485 nm (Ca^{2+}-free form) in individual cells were analyzed by Matlab (see also Supplementary video 1A, B). (n ≥ 3, where 10-19 cells were inspected for each sample; mean ±SD) (B) Time courses of emission ratios are shown for representative cells treated with control solution or F(ab)$_2$ fragment of Infliximab.

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

CKIP-1 has been found to localize mainly to plasma membrane in different cell types. To explore the subcellular localization in THP-1 cells we fused the CKIP-1 sequence to a sequence of a fluorescent protein tag, ECFP. The fluorescent 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 (Fig. 7A).
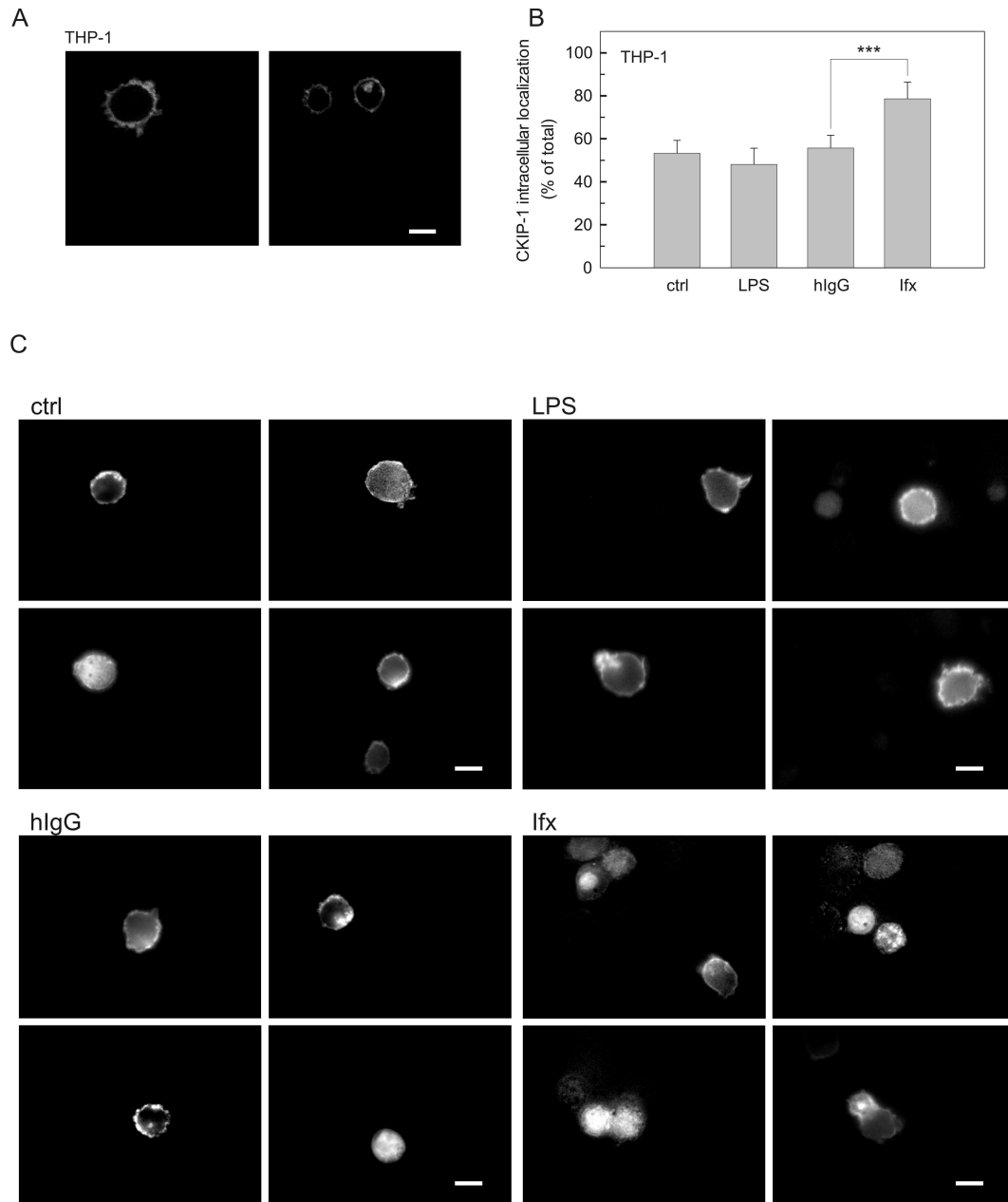
Fig. 7 TNF reverse signaling triggers CKIP-1 relocalization in THP-1 cells

(A) Representative subcellular localization of CKIP-1 in THP-1 cells. Plasma membrane and intracellular localization of CKIP-1-ECFP transiently overexpressed in THP-1 cells was visualized by confocal microscopy (scale bar= 10 µm). (B) Intracellular relocalization of CKIP-1 upon TNF reverse signaling in THP-1 cells. CKIP-1-ECFP was overexpressed in THP-1 cells. 6 h after transfection cells were treated with LPS, hIgG or Infliximab for 12-16 h. Localization of CKIP-1 was visualized by fluorescence microscopy taking large area scan images. Percentage of cells displaying intracellular CKIP-1 is shown for each treatment. (n ≥ 9, where 14-75 cells were inspected for each sample; mean ±SD) (C). Representative focus images from large images are shown for each condition (scale bar= 10 µm).

To test whether an LPS challenge or TNF reverse signaling could influence the subcellular distribution of CKIP-1, transfected THP-1 cells were treated with LPS, human immunoglobulin G (hIgG, as a control of Fc receptor mediated effects) or anti-TNF antibody (Infliximab), then visualized by fluorescence microscopy. Because of the low transfection efficiency of this cell type large (2 mm x 2 mm) scans of transfected THP-1 cells were imaged and analyzed.

As shown in Fig. 7B, around 50% of the positively transfected cells already displayed both intracellular and plasma membrane localized CKIP-1 in untreated (ctrl) cells. 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.

4.5 Expression of TNFNterm leads to a massive relocalization of CKIP-1 in HEK293 cells

In tmTNF overproducing cells we showed earlier intracellular and nuclear accumulation of the short N terminal peptide of tmTNF (TNFNterm). We showed a regulatory effect of the peptide on cytokine gene transcription and hypothesized the translocation of TNFNterm upon TNF reverse signaling (Domonkos *et al*., 2000). To explore the subcellular distribution of CKIP-1 and TNFNterm and their possible co-localization in HEK293 cells, fusion proteins CKIP-1-ECFP and TNFNterm-EYFP were transiently expressed and visualized by confocal microscopy. CKIP-1-ECFP was localized mainly to the plasma membrane and in the cytosol. TNFNterm displayed cytosolic and perinuclear localization (Fig. 8A). When fusion proteins were co-expressed in HEK293 cells CKIP-1 and TNFNterm seemed to partially co-localize. Moreover, a considerable relocalization of CKIP-1 from the plasma membrane to intracellular regions was observed upon TNFNterm co-expression (Fig. 8B).
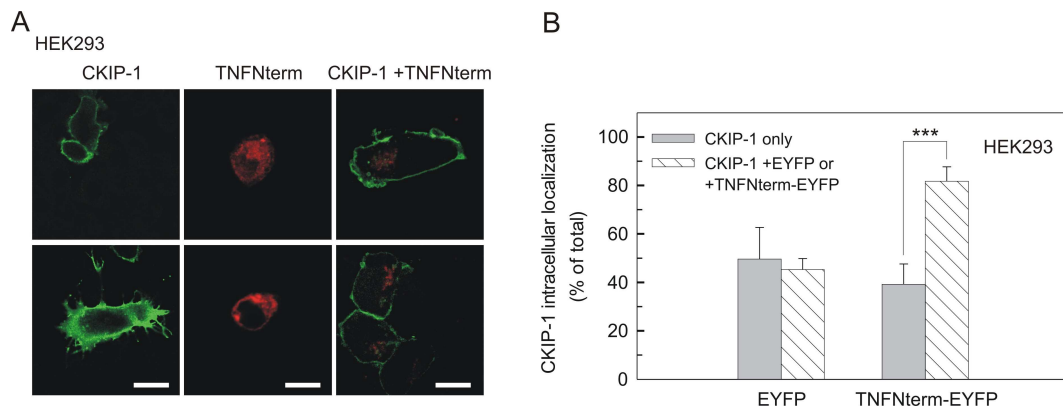
Fig. 8 Co-expression of CKIP-1 leads to relocalization of CKIP-1 in HEK293 cells

(A) Representative localization of CKIP-1 and TNFNterm in HEK293 cells. Fusion proteins CKIP-1-ECFP or TNFNterm-EYFP or both were transiently expressed in HEK293 cells. Cells were visualized by confocal microscopy (scale bar= 10 µm). (B) Intracellular relocalization of CKIP-1 upon TNFNterm expression. CKIP-1-ECFP and TNFNterm-EYFP or EYFP were transiently co-expressed in HEK293 cells and visualized by fluorescence microscopy. CKIP-1 localization was inspected in CKIP-1 positive cells of large area scans. Percentage of cells displaying intracellular CKIP-1 is shown for single CKIP-1-ECFP or double positive cells co-expressing CKIP-1-ECFP and EYFP or TNFNterm-EYFP. (scale bar= 10 µm) (n $\geq$ 3, where 13-107 cells were inspected for each sample; mean ±SD)

4.6 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. Given the relocalization of CKIP-1 upon co-expressing TNFNterm we further explored a possible physical interaction between TNFNterm-mCherry and CKIP-1-EGFP in HEK293 cells. As shown in Fig. 9A, TNFNterm and CKIP-1 frequently co-localize in the vicinity of the plasma membrane or in intracellular structures. In addition to representative images subjected to line scan analysis, the Mander's co-localization results revealed that a significant fraction of TNFNterm positive pixels overlap with those of positive for CKIP-1 (Fig. 9B).

Alternatively, co-immunoprecipitation analysis of TNFNterm-mCherry and CKIP-1-Flag was carried out in HEK293 cells.
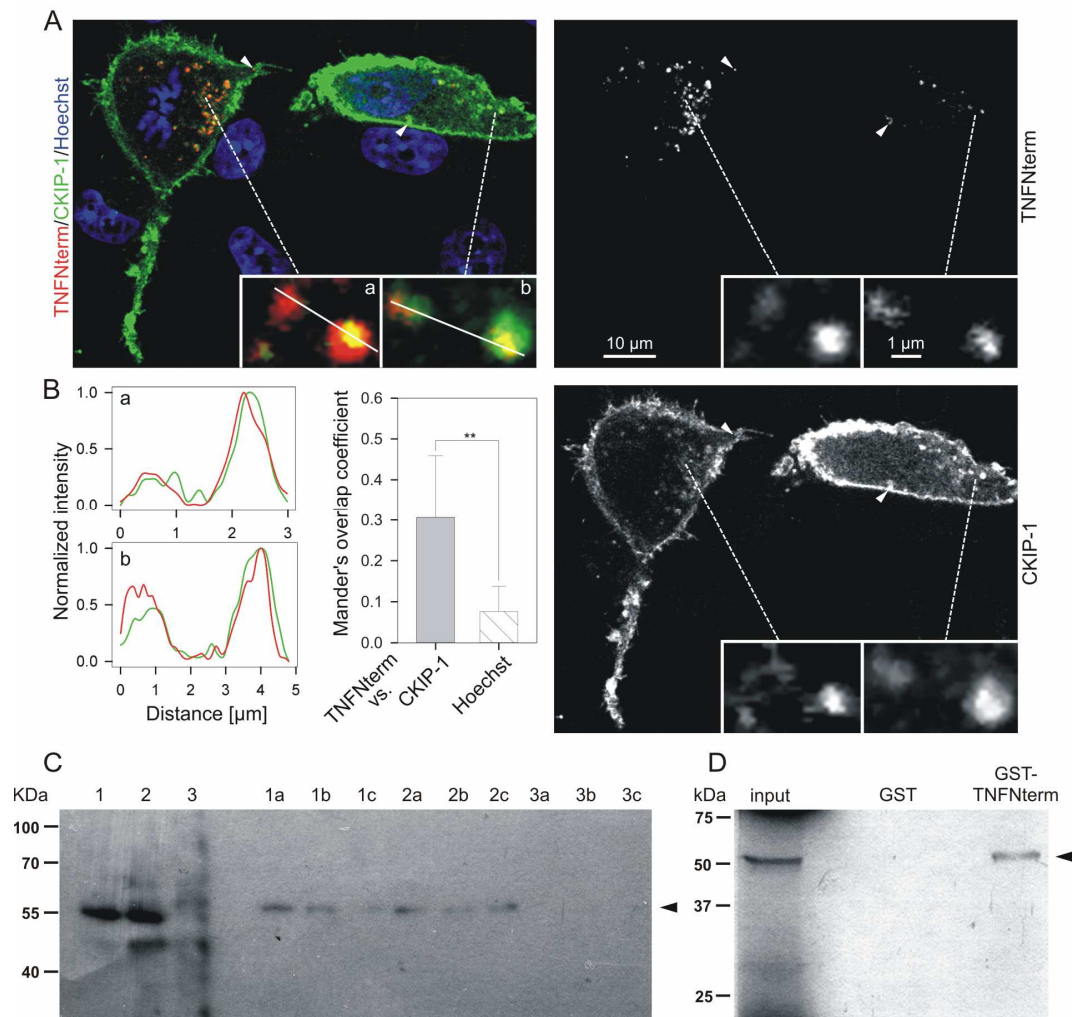
Fig. 9 Interaction of CKIP-1 with TNFNterm

(A) Co-localization of TNFNterm and CKIP-1 in HEK293 cells. Fusion proteins TNFNterm-mCherry and CKIP-1-EGFP were transiently expressed in HEK293 cells. Cells were visualized by confocal microscopy. (B) Representative regions (inserts) were selected for line scan analysis. TNFNterm and CKIP-1 frequently co-localize in intracellular structures (inserts) and at the plasma membrane (arrowheads). The Mander's overlap coefficients calculated for TNFNterm vs. CKIP-1 or Hoechst show a highly significant co-localization of TNFNterm and CKIP-1 (n= 5). (C) Immunoprecipitation Western blot analysis of TNFNterm and CKIP-1 in HEK293 cells. HEK293 cells were co-transfected with TNFNterm-mCherry and CKIP-1-Flag (1) or mCherry and CKIP-1-Flag (2) or TNFNterm-mCherry and Flag (3). Cell lysates were immunoprecipitated with beads coupled to anti-Flag (a) or anti-RFP (b) antibodies. Uncoupled beads (c) were used as a control. Total cell lysates (1-3) and immunoprecipitated lysates (1a-c, 2a-c, 3a-c) were probed for CKIP-1-Flag by Western blotting with anti-Flag antibody (a representative image shown, see arrowhead). Total cell lysates (1-3) represent 50 % of input used in immunoprecipitation. (D) Interaction of TNFNterm with CKIP-1 *in vitro*. The cDNA of TNFNterm was subcloned into a pGEX vector. Fusion protein product GST-TNFNterm or GST alone were bound to glutathione-Sepharose 4B beads and incubated with ^{35}S-labeled CKIP-1. After washing, bound proteins were separated by SDS-PAGE, and CKIP-1 was visualized by autoradiography (a representative image shown, see arrowhead). The lane „input" represents 40 % of the cell lysate used in the pull down experiments.

Cells were co-transfected with TNFNterm-mCherry and CKIP-1-Flag or with mCherry and CKIP-1-Flag as well as TNFNterm-mCherry and Flag, the latter two used as controls. The different cell lysates were incubated with anti-Flag, anti-RFP antibody or uncoated beads, followed by elution and Western blot analysis of the bound proteins. As expected, probing the samples for CKIP-1-Flag two of the three cell lysates (1, 2 but not 3) were found positive (Fig. 9C). As compared to uncoupled bead controls, an increased amount of CKIP-1-Flag was detected in anti-RFP immunoprecipitated lysates of HEK293 cells expressing TNFNterm-mCherry and CKIP-1-Flag (lane 1b vs. 1c). Meanwhile, this was not observed when the beads were incubated with lysates of HEK293 cells expressing CKIP-1-Flag and mCherry only (lane 2b vs. 2c). CKIP-1-Flag was captured with anti-Flag beads from lysates of HEK293 cells expressing CKIP-1-Flag (lanes 1a and 2a). There was no detectable CKIP-1-Flag from lysates of HEK293 cells expressing TNFNterm-mCherry and Flag only (lanes 3a-c). Finally, the interaction of TNFNterm and CKIP-1 was corroborated by *in vitro* GST pull down assays, as well. The fusion protein GST-TNFNterm or GST alone were expressed in *E. coli*, purified with glutation sepharose beads and incubated with *in vitro* translated ^{35}S-labeled CKIP-1. As shown in Fig. 9D, CKIP-1 interacted with GST-TNFNterm, but not with GST alone.

4.7 TNF reverse signaling reduces CKIP-1 mediated activation in THP-1 cells

CKIP-1 participated in the activation processes of inflammatory responses in both THP-1 cells and HEK293 model. Since reverse signaling affected the subcellular localization of CKIP-1 we tested whether it influences the CKIP-1 mediated activation of THP-1 cells. CKIP-1 overexpressing THP-1 cells were exposed to Infliximab or hIgG for 16 h followed by staining with anti-CD86 antibody. Remarkably, the CKIP-1 mediated increase in CD86 surface expression was significantly reduced upon Infliximab treatment (Fig. 10A). Since TNFNterm could also affect the localization of CKIP-1 in HEK293 cells, we tested its possible effect on the co-operative activation of the TNF promoter by CKIP-1 and

c-Jun. Stable clones containing the TNF promoter-driven luciferase reporter gene were co-transfected with expression plasmids for CKIP-1, c-Jun and TNFNterm. As shown in Fig. 10B, TNFNterm significantly reduced the co-operative effect of CKIP-1 and c-Jun on TNF promoter activation.
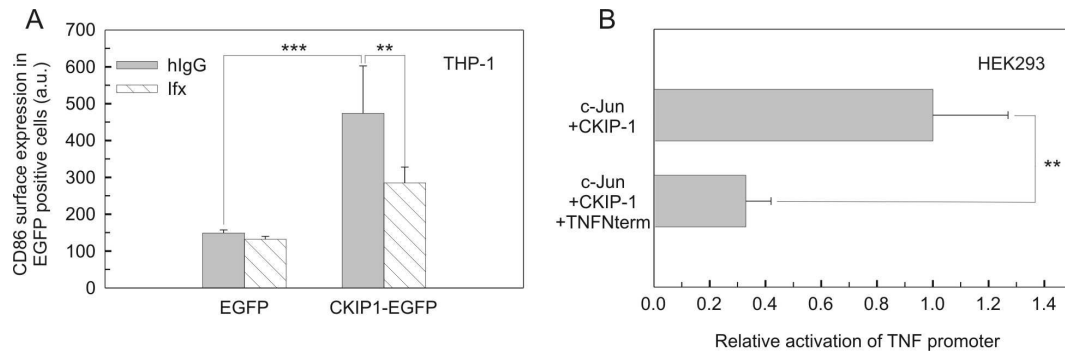

Fig. 10 TNF reverse signaling interferes with CKIP-1 mediated immune activation

(A) CD86 surface expression upon TNF reverse signaling. THP-1 cells transiently expressing EGFP or CKIP-1-EGFP were incubated with hIgG or Infliximab for 16 h. Cells stained with anti-CD86 and PI were analyzed by flow cytometry. Means of anti-CD86 staining of intact cells in EGFP positive subpopulations are shown. (n = 4; mean ±SD) (B) Inhibition of CKIP-1/c-Jun mediated activation of the TNF promoter by TNFNterm. CKIP-1 and c-Jun in the absence or presence of TNFNterm were transiently expressed in HEK293 clones carrying the luciferase gene under the control of the human TNF promoter. Luciferase activities are displayed in relation to the values measured for CKIP-1 and c-Jun co-operation. (n ≥ 5; mean fluorescence intensity ±SD) Isotype values are 4.0 ±0.4, 3.3 ±0.4, 3.8 ±0.5, 4.4 ±1.2.

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

Interestingly, in imaging experiments (Fig. 8A, 9A) we observed morphological changes and a decreased proliferation rate of HEK293 cells expressing TNFNterm. However, the morphological changes appeared to be counteracted in cells co-expressing both TNFNterm and CKIP-1. Since the morphological changes were similar to that of observed in apoptotic cells, we tested whether TNFNterm induces apoptosis in HEK293 cells and if CKIP-1 could influence this process. Expression vectors for CKIP-1-EGFP or EGFP and TNFNterm or empty vector were introduced into HEK293 cells. 24 h after transfection cells were analyzed by flow cytometry monitoring phosphatidyl-serine externalization by

Annexin V staining. Production of TNFNterm significantly increased the Annexin V staining of the transfected HEK293 cell population compared to control cells. Interestingly, co-expression of CKIP-1 almost completely prevented HEK293 cells from the apoptotic effect of TNFNterm (Fig. 11A).
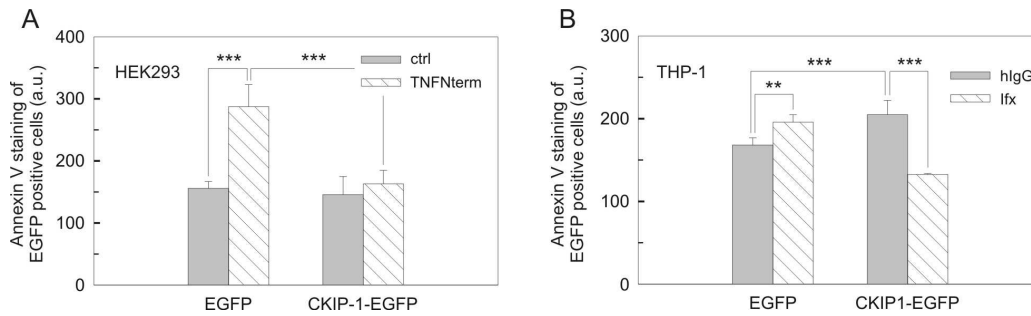

Fig. 11 CKIP-1 interferes with reverse signaling-induced apoptosis

(A) Inhibition of TNFNterm-induced apoptosis by CKIP-1. HEK293 cells were transiently co-transfected with empty vector or TNFNterm plasmid and EGFP or CKIP-1-EGFP expressing vectors. 24 h after transfection cells were labeled with Annexin V-Alexa647 and PI and analyzed by flow cytomertry. Means of Annexin V-Alexa647 staining of cells are shown for EGFP positive subpopulations. (n ≥ 4; mean fluorescence intensity ±SD) (B) Influence of CKIP-1 on Infliximab-induced apoptosis. THP-1 cells transiently expressing EGFP or CKIP-1-EGFP were incubated with hIgG or Infliximab for 16 h. Cells labeled with Annexin V-Alexa647 and PI were analyzed by flow cytometry. Means of Annexin V-Alexa647 staining of cells in EGFP positive subpopulations are shown. (n = 5; mean fluorescence intensity ±SD).
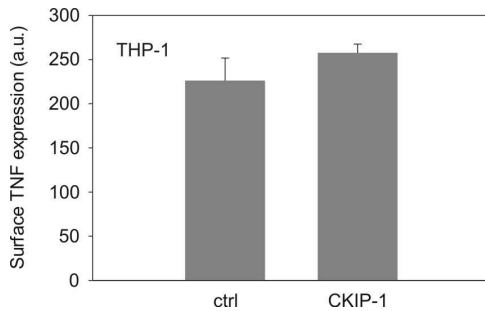

Fig. 12 Surface expression of tmTNF

THP-1 cells were transfected with CKIP-1-EGFP and EGFP expressing vectors. 24 h after transfection living cells were labeled with anti-TNF antibody and analysed by flow cytometry. Means of Ifx-Alexa647 staining of cells are shown for EGFP positive subpopulations. (n ≥ 4; mean fluorescence intensity ±SD)

Infliximab treatment has been shown to induce apoptosis in THP-1 cells (Shen *et al.*, 2005). As CKIP-1 could abolish the TNFNterm-induced apoptosis in HEK293 cells in the above experiments, we tested whether CKIP-1 could also affect the TNF reverse signaling-triggered apoptosis in THP-1 cells. Transfected cells were exposed to Infliximab or hIgG for 16 h followed by flow cytometry-based apotosis analysis. Infliximab treatment resulted in a significant increase in Annexin V staining of control cells. Noticeably, mere CKIP-1 overexpression sensitized THP-1 monocytes to apoptosis, a phenomenon that has been described for other cell types (Zhang *et al.*, 2005). However, the presence of CKIP-1 did not just inhibit the apoptotic effect of Infliximab but reduced the apoptosis below the control (hIgG) level (Fig. 11B), pointing to an activation of an additional survival signaling. Note that CKIP-1 overexpression did not influence the surface expression of tmTNF (Fig. 12).

4.9 CKIP-1 does not prevent cells from etoposide-induced apoptosis

TNF reverse signaling is thought to induce caspase-3 activation and CKIP-1 has been shown to participate in caspase-3 mediated apoptosis (Zhang *et al.*, 2005). To test whether CKIP-1 interferes also with other caspase-3 mediated apoptotic processes, cells were treated with etoposide. Etoposide treatment caused a significant increase in the apoptosis values of control cells. Unlike Infliximab, etoposide treatment further elevated the apoptosis value of THP-1 cells overexpressing CKIP-1 (Fig. 13).
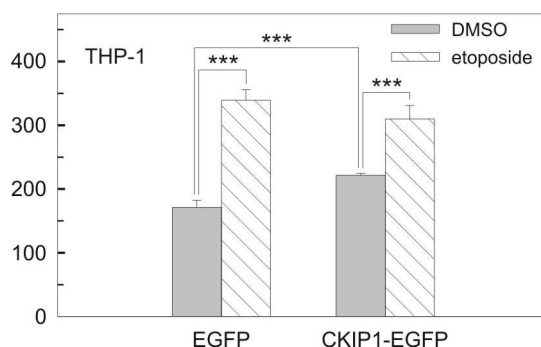

Fig. 13 Influence of CKIP-1 on etoposide-induced apoptosis.
THP-1 cells transiently expressing EGFP or CKIP-1-EGFP were incubated with etoposide or DMSO for 12 h. Cells labeled with Annexin V-Alexa647 and PI were analyzed by flow cytometry. Means of Annexin V-Alexa647 staining of cells in EGFP positive subpopulations are shown. (n = 5; mean fluorescence intensity ±SD)

4.10 Expression of CKIP-1 is controlled by TNF reverse signaling

Our data above showed that CKIP-1 expression can functionally interfere with TNF reverse signaling in THP-1 cells (Fig. 11B). We tested whether TNF reverse signaling could influence the already induced CKIP-1 expression. THP-1 cells were challenged by LPS for 4 h, and then subjected to LPS, hIgG or Infliximab for additional 4 h. Upon hIgG treatment the mRNA level of CKIP-1 was relaxed. Remarkably, upon Infliximab treatment the mRNA level of CKIP-1 was reduced to a lesser extent as compared to the hIgG control treatment (Fig. 14A). Hence TNF reverse signaling appeared to interfere with relaxation of LPS-triggered CKIP-1 induction. Note that Infliximab treatment did not induce *ckip-1* expression in resting THP-1 cells (Fig. 14B).
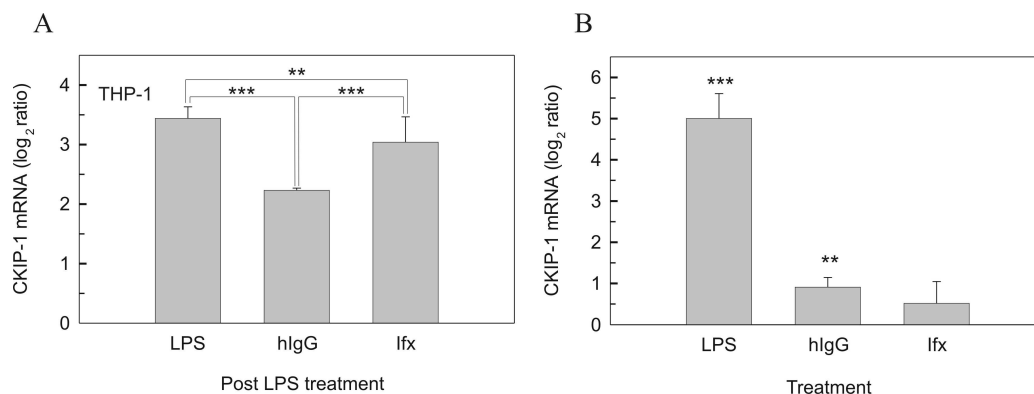
Fig. 14 Effect of TNF reverse signaling and LPS challenge on CKIP-1 expression

(A) CKIP-1 expression is maintained upon TNF reverse signaling. THP-1 cells were pretreated with LPS for 4 h. Following repeated washing steps cells were treated either with LPS or hIgG or Infliximab for 4 h. Samples were harvested and changes in the transcript level of CKIP-1 were measured by qRT-PCR. (n = 12; mean ±SD) (B) The effect of LPS challenge or TNF reverse signaling on CKIP-1 expression. THP-1 cells were treated with LPS, hIgG or Infliximab for 4 h. Samples were harvested and changes in the transcript level of CKIP-1 were measured by qRT-PCR. (n ≥ 3; mean ±SD)

5 Discussion

Activation of several intracellular signaling pathways triggered by the interaction of members of the TNF receptor superfamily with their cognate ligands leads either to cell death or to proliferation, differentiation and activation of the receptor bearing immune cells. These pathways are well known, but precious little is known about the molecular details of reverse signaling, the event elicited upon activation of the transmembrane forms of TNF superfamily ligands by their soluble or transmembrane receptors or agonistic antibodies in the ligand expressing cells. The phenomenon of reverse signaling was reported for a number of TNF superfamily members and appears to be a fine tuning control mechanism in the immune system (Eissner *et al.*, 2004). Receptor-like properties of the transmembrane form of the pro-inflammatory cytokine TNF (tmTNF) have been reported by several laboratories (Eissner *et al.*, 2000; Mitoma *et al.*, 2005; Watts *et al.*, 1999). Although TNF reverse signaling has been emerging as an important phenomenon in the immune response, its molecular basis remains elusive. We reported earlier that the NLS containing N terminal fragment of the tmTNF (TNFNterm) could accumulate in the nucleus and regulate transcription of cytokines (Domonkos *et al.*, 2000) . A shorter sequence of this TNFNterm has been found to interact with a novel protein, mouse TNF intracellular domain interacting protein (mTIP), and its human homologue (hTIP) has been cloned from a lymph node plasmid library.

This novel protein has been proved identical to casein kinase-2 interacting protein-1 (CKIP-1). CKIP-1 has been shown to interact with several intracellular proteins such as c-Jun (Zhang *et al.*, 2005), actin capping protein subunits (ACP α and β) (Canton *et al.*, 2005), ATM (Zhang *et al.*, 2006), Akt (Tokuda *et al.*, 2007), IFP35 and Nmi (Zhang *et al.*, 2007) as well as Smurf-1 (Lu *et al.*, 2008). Since CKIP-1 has been reported as a multifunctional protein and interacted with the intracellular domain of tmTNF, we assumed CKIP-1 to play a role in inflammation, as well.

CKIP-1 exhibited diverse functions in different cell types such as recruiting proteins to the plasma membrane (Olsten *et al.*, 2004; Zhang *et al.*, 2005), mediating regulation of the actin cytoskeleton (Canton *et al.*, 2005), repressing cell survival signaling (Zhang *et al*., 2005) or affecting cell differentiation (Safi *et al.*, 2004). Although CKIP-1 has been reported to influence interferon-gamma (IFN-γ) induced signaling through controlling the stability of IFP35 (Zhang *et al*., 2007) its function in immunity remained unclear.

In this study we showed that expression of human CKIP-1 was significantly elevated in THP-1 monocytes exposed to LPS, both at mRNA and protein levels. Moreover, model experiments with HEK293 cells showed an increased activity of the TNF promoter upon co-expression of CKIP-1 and c-Jun. Both c-Jun and CKIP-1 was able to significantly transactivate the TNF promoter in a concentration dependent manner (data not shown), however, the highest activities were found upon co-expression at lower expression levels, pointing to a strong synergism between the two proteins. Noteworthy, this co-operative effect could not be attributed to the direct phosphorylation of the transcription factor c-Jun, as measured by *cis*-activating pathway specific transfection experiments. Therefore, CKIP-1 appears to act indirectly in HEK293 cells, leading to an enhanced activity of the TNF promoter upon c-Jun overexpression. Interestingly, CKIP-1 overexpression clearly facilitated an increase in c-Jun activity in a concentration-dependent manner in other cell lines, where the N-terminal half of the protein failed to activate c-Jun in those cells (data not shown). Since intracellular signaling might vary among different experimental systems and cell types, these data further support cell type dependent functions of CKIP-1. The pro-inflammatory role of human CKIP-1 was further supported by the fact of CKIP-1-mediated increase in surface expression of CD86, a cell surface marker of classical monocyte activation. Therefore, we conclude that CKIP-1 exerts a positive regulatory role in the activation process of human monocytes.

Overexpression of mouse CKIP-1 in mouse macrophages had no detectable effect on the basic transcriptional level of a set of inflammatory cytokines. Interestingly, upon LPS challenge IL-1, IL-6 and TNF were induced more dramatically in the

CKIP-1 overexpressing cells (data not shown). Another interesting observation was that CKIP-1 overproducing mouse macrophages did not show *in vitro* LPS tolerance, the phenomenon of decreased or absent reaction to bacterial endotoxin after an initial treatment with the same agent (data not shown). These findings indicate that both human and mouse CKIP-1 is involved in the regulation of inflammatory cytokine expression.

The human and the mouse CKIP-1 transcripts encode proteins of 409 and 408 amino acid residues, respectively. The proteins with a calculated molecular mass of approx. 50 kDa exhibit a highly conserved amino acid sequence, with 90.2% identity. However, in contrast to human cells, mouse macrophage cells showed a clear decrease in mRNA levels of CKIP-1 upon LPS challenge (data not shown). In addition, sequence analysis revealed several NF-κB bindig sites in the human and several Sp1 sites in the mouse CKIP-1 promoter, suggesting an inducible and a constitutive expression, respectively. Since regulation of human and mouse CKIP-1 seemed to be different, we aimed at elucidating the role of human CKIP-1 in immune cells in particular in TNF reverse signaling.

Several monoclonal TNF antibodies and a soluble TNF receptor are licensed for clinical use in immune mediated inflammatory diseases, where they bind to soluble and tmTNF and can neutralize the pathological effects of TNF. Most of these drugs have been reported to elicit reverse signaling in tmTNF producing immune cells (Mitoma *et al.*, 2008).

Infliximab, used in this study, is a chimeric (25 % mouse and 75 % human) monoclonal antibody constructed by linking the variable regions of a mouse anti-human TNF monoclonal antibody to a human Fc part. Infliximab binds with a high affinity and specificity to human soluble TNF and tmTNF. It is routinely administered intravenously in several diseases. Here we showed that Infliximab treatment induced Ca^{2+} oscillations in tmTNF expressing THP-1 monocytes. In accordance, TNF reverse signaling induced Ca-signaling has been reported in mouse macrophages (Watts *et al*., 1999). Since frequency of Ca^{2+} oscillations is known to optimize gene expression (Dolmetsch *et al.*, 1997), TNF reverse signaling might lead to transcriptional changes in human monocytes, as well.

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. The subcellular localization of CKIP-1 is highly dependent on the cell type and the stimulus. CKIP-1 could be localized to the plasma membrane, in the cytoplasm and in the nucleus, depending on the cell type examined. Interestingly, the localization of CKIP-1 could be regulated by extracellular signals, such as insulin treatment (Safi *et al.*, 2004). In response to TNF, CKIP-1 has been reported to translocate from the plasma membrane to the cytoplasm and then to the nucleus accompanied by caspase-3 cleavage (Zhang *et al.*, 2005). These observations suggest that CKIP-1 shuttles between various intracellular compartments and imply that CKIP-1 may bind to different partners in different compartments.

Intriguingly, we found that CKIP-1 relocalized from the plasma membrane to intracellular compartments upon TNF reverse signaling in THP-1 cells.

Remarkably, a similar relocalization of CKIP-1 was shown for HEK293 cells when expressing TNFNterm, a short N terminal fragment of tmTNF. We found this short peptide of 10kDa, a residual fragment of tmTNF after cleavage by TACE, to translocate into the nucleus and regulate cytokine expression. We hypothezised that upon TNF reverse signaling tmTNF can be dephosphorylated by an unknown phosphatase and after cleavage the remaining N terminal fragment translocates to the nucleus (Domonkos *et al.*, 2000). A similar unusual phenomenon is known for Notch, where upon ligand binding the cleaved intracellular domain of the transmembrane molecule translocates into the nucleus and acts as a transcriptional co-activator (Oswald *et al.*, 2001). Moreover, intramembrane proteolysis by signal peptide peptidase-like proteases and subsequent nuclear translocation have been reported for the tmTNF (Friedmann *et al.*, 2006) and for the FasL (Kirkin *et al.*, 2007).

Since we observed a mutual influence on the subcellular distribution of TNFNterm and CKIP-1, we hypothesized that TNF reverse signaling and CKIP-1 overexpression could interfere with each other in cellular functions. Indeed, in parallel with the relocalization of CKIP-1, Infliximab-induced TNF reverse

signaling or expression of TNFNterm antagonized the pro-inflammatory effects of CKIP-1. In fact, upon TNF reverse signaling CKIP-1 mediated increase in CD86 surface expression was diminished in THP-1 cells. Moreover, expressing TNFNterm in HEK293 cells drastically inhibited the co-operative activation of the TNF promoter by CKIP-1 and c-Jun. 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 (Eissner *et al*., 2000). This negative regulatory role of TNF reverse signaling might operate partly through sequestration of CKIP-1 from the activating protein complexes.

TNF reverse signaling has been shown to activate both proapoptotic and antiapoptotic signaling pathways, where fate of the cell depended on the degree of differentiation and crosstalk with other signaling pathways (Meusch *et al*., 2009; Xin *et al*., 2006). In THP-1 monocytes reverse signaling has been shown to increase the release of TGF-beta 1 from the cells, in turn to activate proapoptotic pathways (Waetzig *et al*., 2005). In our experiments Infliximab treatment induced apoptosis in THP-1 cells. Moreover, overexpression of TNFNterm could induce apoptosis in HEK293, which is known as an apoptosis insensitive cell line. Nevertheless, critical signaling molecules determining the fate of the cell upon TNF reverse signaling have not been revealed.

CKIP-1 overexpression has been reported to promote apoptosis by forming a positive feedback loop between CKIP-1 and caspase-3 (Zhang *et al*., 2005). Increased numbers of cleaved poly(ADP-ribose) polymerase and cleaved caspase-3 fragments have been reported by others after etoposide treatment in CKIP-1 stable transfectants. It has also been reported, that CKIP-1 expression enhances the sensitivity to anticancer drugs by suppressing Akt activity by targeting the Akt PH domain (Tokuda *et al.*, 2007). In agreement, we observed a moderate apoptosis sensitizing effect of CKIP-1 in THP-1 cells.

Importantly, overexpression of CKIP-1 interfered with TNF reverse signaling induced apoptosis in THP-1 monocytes. In accordance, CKIP-1 overexpression almost completely counteracted the apoptotic effect of the co-expressed

TNFNterm in HEK293 cells. Therefore we hypothesize, that CKIP-1 prevents cells form TNF reverse signaling induced apoptosis by interfering with TNFNterm functions.

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. These data indicate that besides sensitizing to apoptosis CKIP-1 might play an antiapoptotic role as well. A similar but opposing switch between pro- and antiapoptotic functions has been shown for Livin, where the antiapoptotic function of the protein turned to proapoptotic activity through a caspase-3 mediated cleavage (Abd-Elrahman *et al.*, 2009). Interestingly, CKIP-1 has been shown to be cleaved by caspase-3 upon apoptotic signals in different epitheloid cells (Zhang *et al.*, 2005). However, in our experiments the antiapoptotic function of CKIP-1 was not activated upon treatment with etoposide, another apoptosis inducer acting through caspase-3. Although we did not test the antiapoptotic switch in the presence of a caspase-3 inhibitor, we can assume a similar switch mechanism dependent on caspase-3 mediated cleavage of CKIP-1 upon TNF reverse signaling. Hence, the switch in CKIP-1 function appears to be rather specific to anti-TNF treatment.

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 physically interact. This idea is consistent with the findings of yeast two-hybrid and *in vitro* GST pull down assays, co-immunoprecipitation and co-localization experiments Thus, we speculate that in immune cells they likely interact in a protein complex that is supported by the proposed scaffold nature of CKIP-1 (Safi *et al.*, 2004) as well as the mutual influence on cellular activities of CKIP-1 and TNF reverse signaling shown in this study.

The molecular mechanism of the proapoptotic activity of TNF reverse signaling remains elusive. However, activation of apoptotic signaling through a secondary autocrine loop of TGF-beta signaling has been reported for THP-1 monocytes, where TNF reverse signaling increased the constitutive release of TGF-beta from

the cells. TNF reverse signaling has been proposed to sensitize THP-1 cells to TGF-beta induced apoptosis, as TGF-beta alone is not cytotoxic for cells at the concentration measured in those experiments (Waetzig *et al*., 2005).

Based on experimental data we hypothesize that activation tmTNF on the surface of THP-1 cells by transmembrane or soluble receptors or agonistic antibodies leads to dephosphorylation of the intracellular domain of tmTNF and subsequent recruitment of interacting signaling proteins which govern the subcellular localization of the complex. The earlier identified, functional NLS of TNF might play an important role in further nuclear translocation of later complexes interacting with transcription factors localized in nucleus. The high number of interacting partners and the stoichiometric interaction between CKIP-1 and c-Jun suggest that CKIP-1 may be a scaffold protein that coordinates signal transduction events, providing a platform for other interacting molecules. These proteins appear to impose an architecture that physically separates signaling molecules of similar pathways, thereby providing the means for specific subpathway selection. Excess CKIP-1 might inhibit TNF revese signaling induced apoptosis by interfering either with the autocrine TGF-beta signaling or the sensitizing effect of TNF reverse signaling through influencing the localization of TNFNterm. Since CKIP-1 has been shown to interact with Smurf-1 (Lu *et al.*, 2008) that is involved in TGF-beta signaling, there is a possibility that CKIP-1 interferes with the TGF-beta induced apoptotic pathway, as well. CKIP-1 overexpression might shift the balance between p38 and ERK1/2 pathways, thereby promoting survival of the cells (Fig. 15).
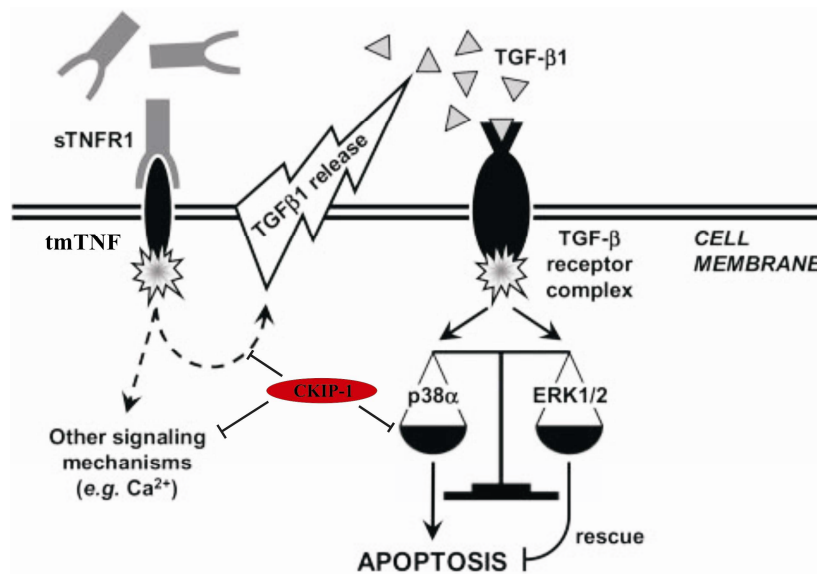
Fig. 15 A proposed schematic model for the role of CKIP-1 in TNF reverse signaling in THP-1 monocytes
TNF reverse signaling leads to enhanced TGF-beta1 release. Autocrine TGF-beta1 activates the proapoptotic effector p38 leading to cell death. Overexpression of CKIP-1 might inhibit signaling events through the N terminal fragment of tmTNF and/or shifting the balance to ERK1/2 signaling leading to enhanced survival (adapted from Waetzig *et al.*, 2005).

Notably, LPS-triggered CKIP-1 induction was relaxed to a lesser extent when TNF reverse signaling was activated, further indicating the involvement of CKIP-1 in this signaling pathway. Thus, CKIP-1 appeared to be a critical factor determining the fate of THP-1 monocytes interacting with anti-TNF antibody.

Importantly, it has been suggested that secondary infections during anti-TNF therapies in chronic inflammatory diseases may partly originate from elimination of immune cells expressing tmTNF. 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.

6 Summary

When transmembrane form of tumor necrosis factor (tmTNF) interacts with its cognate receptors or agonistic antibodies signaling pathways are activated in the ligand expressing cells. The phenomenon called reverse signaling has been considered as a fine-tuning control mechanism in the immune response and has been reported in anti-TNF therapies, as well. Despite a clinical relevance the key participating molecules of TNF reverse signaling and their functions remain elusive. Here we examined the role of CKIP-1, an interacting partner of the N terminal fragment of tmTNF (TNFNterm) in inflammation and TNF reverse signaling. We showed that CKIP-1 expression was elevated upon LPS challenge in THP-1 monocytes. Overexpression of CKIP-1 triggered classical activation of THP-1 cells and trans-activated the human TNF promoter when co-expressed with c-Jun in the HEK293 model system. Upon TNF reverse signaling a massive translocation of CKIP-1 from the plasma membrane to intracellular compartments was observed in THP-1 cells. A similar relocalization of CKIP-1 was shown in HEK293 cells upon expression of TNFNterm. In parallel with the translocation, CKIP-1-triggered activation of THP-1 cells was diminished upon TNF reverse signaling. Similarly, the presence of TNFNterm inhibited CKIP-1 mediated TNF promoter activation in HEK293 cells. Both TNF reverse signaling in THP-1 monocytes and expression of the TNFNterm in HEK293 cells were found to induce apoptosis. Intriguingly, overexpression of CKIP-1 greatly interfered with apoptotic triggers elicited through tmTNF in both THP-1 monocytes and HEK293 cells. We showed novel functions of the signaling molecule CKIP-1 in monocytes. First, CKIP-1 was shown to play an important stimulatory role in the pro-inflammatory process. Second, CKIP-1 showed a counteracting effect on TNF reverse signaling-induced cell death, suggesting that it could act as well as an inhibitory molecule in immune cells. Since both signaling processes require CKIP-1, distinct activities of the scaffold protein CKIP-1 may be regulated by its localization and interacting partners.

7 Highlights of the study

Involvement of CKIP-1 in monocyte activation and TNF reverse signaling was examined.

CKIP-1 expression is elevated upon LPS challenge in human monocyte model cells

Excess CKIP-1 results in activation of monocyte model cells.

TNF reverse signaling triggers translocation of CKIP-1, while CKIP-1-mediated activation is decreased.

CKIP-1 expression counteracts TNF reverse signaling induced apoptosis.

CKIP-1 is proposed as a candidate for regulating the fate of tmTNF expressing cells upon TNF reverse signaling.

8 Összefoglalás

A tumor nekrózis faktor (TNF), a TNF szupercsalád névadója, egy tipikus gyulladásos citokin, amely receptorához kötődve génexpressziót, differenciációt és sejthalált is képes kiváltani. A membrán kötött és szolublis formában is megtalálható TNF-et főként immunsejtek, de más sejttípusok is termelik. Mivel nagyon hatásos citokin, termelésének hibás szabályozása több - gyakran súlyos következményekkel járó - patológiás elváltozás okozója lehet. Ilyen például az LPS indukálta szeptikus sokk, vagy több autoimmun betegség mint a reumatoid artritisz vagy a Crohn betegség. Ezekben a betegségekben évek óta sikeresen alkalmazzák az anti-TNF terápiát, ahol a kóros mennyiségű TNF-et ellenanyaggal semlegesítik.

Az utóbbi évtized áttörő felismerése volt, hogy a TNF szupercsalád membránkötött ligandjai képesek kétirányú jelátviteli folyamatokat kiváltani. A receptorával vagy agonista ellenanyaggal is aktiválható, ligandon induló jelátviteli folyamat vagy *reverz jelátvitel* az egészséges szervezetben az immunválasz finomhangolását teszi lehetővé. Ilyen receptor-szerű viselkedést a transzmembrán TNF (tmTNF) esetében is megfigyeltek, ahol a reverz jelátvitel monocitákban anergiás állapotot idéz elő, míg egyes T sejt típusokban génexpressziót fokoz. A tmTNF-en induló reverz jelátvitelt anti-TNF terapiák alkalmazása során is megfigyelték, ahol gyakran a tmTNF-et expresszáló különféle immunsejtek aktiválódását vagy apoptózisát okozza. Klinikai relevanciájának ellenére a tmTNF-en induló reverz jelátvitel molekuláris mechanizmusa napjainkban sem egészen ismert.

A jelenség mechanizmusának feltérképezésében komoly segítséget jelenthet a tmTNF N terminális, intracelluláris doménjével kölcsönható fehérjék azonosítása. Az egyik ilyen kölcsönható molekulát élesztő két hibrid technikával azonosították LPS-sel kezelt egér makrofágok cDNS könyvtárát vizsgálva. A fehérje humán megfelelője azonosnak bizonyult a szintén újabban felfedezett CKIP-1 (kazein kináz-2-vel kölcsönható protein) fehérjével. Azóta a CKIP-1 több funkcióját is leírták különböző sejttípusokban. A CKIP-1 fehérje aktívan részt vehet

differenciációban, sejtmozgásban vagy sejthalálban is. Ennek megfelelően, kölcsönhatását a kazein kináz-2 mellett, több intracelluláris fehérjével is kimutatták. Kölcsönhat például a c-Jun transzkripciós faktorral, aktin kötő fehérje alegységekkel vagy a tumorsejtek túlélésében részt vevő Akt fehérjével is. Mivel a gyulladásos válaszban részt vevő tmTNF kölcsönható partnereként is azonosították, célul tűztük ki a CKIP-1 immunrendszerben betöltött szerepének tanulmányozását. Vizsgáltuk a CKIP-1 fehérje esteleges szerepét monociták gyulladásos válaszában és a tmTNF-en induló reverz jelátvitelben.

Kimutattuk, hogy a CKIP-1 expressziója LPS kezelés hatására THP-1 humán monocita sejtekben megemelkedik. Túltermelése a sejtek klasszikus aktivációját okozza, amelyet a CD86 sejtfelszíni expresszió növekedése is jelez. Modell rendszerként alkalmazott humán embrionális vesesejtekben (HEK293) a CKIP-1 túltermelése a TNF promóter aktiválódásához vezet, amelyben erős szinergizmust mutat a c-Jun transzkripciós faktorral. Így elmondható, hogy a CKIP-1 egy, a gyulladásos immunválaszban pozitív aktivációs szereppel rendelkező fehérje.

Kísérleteinkben a TNF reverz jelátvitel kiváltására az infliximab nevű rekombináns monoklonális anti-TNF-et használtuk. Ezt a humanizált ellenanyagot évek óta sikeresen alkalmazzák a kóros mennyiségű TNF neutralizálására különböző gyulladásos kórképek klinikai terápiája során. Irodalmi adatokkal egybecsengően, az infliximab kezelés az általunk vizsgált THP-1 monocitákban is képes volt kalcium-oszcillációt indítani. Mitöbb, THP-1 sejtekben az eredetileg főként plazma membrán közelében elhelyezkedő CKIP-1 jókora hányada TNF reverz jelátvitel hatására citoplazmatikus elhelyezkedest mutatott. Hasonló transzlokációt figyeltünk meg HEK293 modell sejtekben a tmTNF N terminális doménjének túltermelésekor.

A CKIP-1 meglehetősen összetett doménszerkezetű és több lehetséges poszttranszlációs módosítóhellyel is rendelkezik. A módosítások és más fehérjékkel való kölcsönhatásai befolyásolhatják a CKIP-1 lokalizációját és aktivitását. ennek megfelelően, a CKIP-1 molekula a sejten belül stimulus és sejttípus függően elhelyezkedhet a plazma membránban, a citoplazmában vagy

akár a sejtmagban is. Érdekes, hogy a modell sejtjeinkben megfigyelt transzlokációval párhuzamosan az infliximab által indukált TNF reverz jelátvitel vagy a TNF N terminalis peptidjének túltermelése a CKIP-1 különböző aktivitásainak csökkenéséhez vezetett. Így feltételezhető, hogy a tmTNF-en induló reverz jelátvitel gyulladásos válaszban korábban leírt negatív szabályozó szerepe részben a CKIP-1 molekulán keresztül valósulhat meg.

Anti-TNF kezelés hatására bekövetkező apoptózist monocita sejtek esetében és T limfocitákban is megfigyeltek már korábban. Ezzel egybecsengően, kísérleteinkben az infliximabbal kezelt THP-1 sejtek egy része apoptózist szenvedett, ami morfológiai változásokon túl a plazma membrán külső felszínén megjelenő foszfatidil-szerin mennyiségének növekedésében is megnyilvánult. Hasonló apoptótikus jegyeket figyeltünk meg a tmTNF N terminális doménjét termelő, egyébként apoptózisra nem érzékeny, HEK293 modell sejtekben is.

Érdekes, hogy a CKIP-1 molekula túltermelése mindkét vizsgált sejttípusban gátolta az apoptótikus hatást. Ráadásul a CKIP-1 expressziója nemcsak megvédte a sejteket a TNF reverz jelátvitel által kiváltott apoptózistól, hanem a túlélő sejtek számát is megnövelte. A jelenséget egy másik, szintén kaszpáz-3 molekulán keresztül érvényesülő apoptózis-indukáló szer, az etopozid estében nem figyeltük meg. Kísérleti erdményeink alapján valószínű, hogy a CKIP-1 túltermelése a tmTNF-en induló reverz jelátvitel által indított apoptózist specifikusan képes ellensúlyozni. Így a CKIP-1 sejten belüli mennyisége meghatározhatja az anti-TNF-fel kölcsönható immunsejtek sorsát. Fontos megjegyezni, hogy a CKIP-1 túltermelés nem befolyásolta a sejtfelszíni tmTNF mennyiségét, igy a megnövekedett túlélő sejtek száma valószínűleg egy funkcióváltás eredménye.

A tmTNF-en induló reverz jelátvitel pontos molekuláris mechizmusa egyelőre nem teljesen ismert. Transzmembrán TNF-et termelő sejtekben a tmTNF citoplazmatikus doménje szerin-foszforilált, amit a kazein kinaz 1 végez. TNF reverz jelátvitel során egy eddig ismeretlen szerin/treonin foszfatáz által defoszforiláció következik be, amelyet intracelluláris kalciumion koncentráció-növekedés kísér. A folyamatban feltételezhetően aktiválódik a molekula N

terminális peptidrészlete, amely más jelátviteli fehérjékkel kölcsönhatva a sejt típusától és állapotától függően citokin gének expresszióváltozását idézi elő vagy apoptózist indukál. Kísérleti eredményeink alapján feltételezzük, hogy TNF reverz jelátvitel során a tmTNF N terminális peptidje es a CKIP-1 egy közös molekulakomplex részét képezik, amelyet a két molekula lokalizációban es aktivitásban tapasztalt kölcsönös egymásra gyakorolt hatása és a CKIP-1 molekula vázfehérje jellege is alátamaszt.

Élő, humán sejtes modell rendszereken végzett kísérleteinkben a CKIP-1 fehérje új funkcióit mutattuk meg. A humán CKIP-1 fehérje a gyulladásos immunválaszban mint aktivaciót serkentő molekula vesz részt. A CKIP-1 sejten belüli mennyisége gyulladásos körülmények között megnő és a monociták éresét, klasszikus aktivációját serkenti. Megemelkedett mennyiségének ugyanakkor specifikus gatló hatása van a TNF reverz jelátvitel által indukált sejthalálra. Kísérleti eredményeink alapján a CKIP-1 fehérje különböző aktivitásai a molekula lokalizációjának és más fehérjékkel való kölcsönhatásának közvetlen eredménye lehet.

A tmTNF-en induló reverz jelátvitel mind proapoptótikus és antiapoptótikus jelátviteli útvonalakat is képes indítani, ahol a sejtek sorsa a differenciáció fokától és más jelátviteli utakkal való kölcsönhatástól függ. Anti-TNF terápiák során az általunk vizsgált CKIP-1, az ellenanyaggal kölcsönható sejtek sorsát befolyásoló kulcsmolekulák egyike lehet. Ezért különböző immunsejtekben betöltött szerepének további kutatását fontosnak tartjuk az immunterápiák fejlesztése szempontjából is.

Aknowledgement

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