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Casein kinase 2-interacting protein-1, an inflammatory signaling molecule interferes with TNF reverse signaling in human model cells

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ABSTRACT

When transmembrane form of tumor necrosis factor (mTNF) interacts with its cognate receptors or agonistic antibodies signaling pathways are activated in the ligand expressing cells. This "reverse signaling" appears a fine-tuning control mechanism in the immune response. Despite a clinical relevance key molecules of TNF reverse signaling and their functions remain elusive. We examined the role of CKIP-1, an interacting partner of the N terminal fragment of mTNF in inflammation and TNF reverse signaling. We found that CKIP-1 expression was elevated upon LPS challenge in THP-1 human monocyte model cells. Overexpression of CKIP-1 triggered classical activation of THP-1 cells and transactivated the human TNF promoter when co-expressed with c-Jun in the HEK293 model system. TNF reverse signaling induced a massive translocation of CKIP-1 from the plasma membrane to intracellular compartments in THP-1 cells. Expression of the N terminal fragment of mTNF in HEK293 cells resembled the effects of TNF reverse signaling with respect to relocalization of CKIP-1. In parallel with the translocation, CKIP-1-triggered activation of THP-1 cells was antagonized by TNF reverse signaling. Similarly, the presence of the N terminal fragment of mTNF inhibited CKIP-1 mediated TNF promoter activation in HEK293 cells. Both TNF reverse signaling in THP-1 cells and expression of the N terminal fragment of mTNF in HEK293 cells were found to induce apoptosis that could be prevented by overexpression of CKIP-1. Our findings demonstrate that CKIP-1 activates pro-inflammatory pathways and interferes with TNF reverse signaling induced apoptosis in human model cells.

1. Introduction

Members of the TNF and TNF receptor superfamilies are transmembrane proteins, but many of these have soluble forms liberated by TACE (TNF-alpha converting enzyme) [1]. When TNF superfamily ligands and their cognate receptors interact, signaling pathways can be activated in both ligand and receptor bearing cells, leading to activation, differentiation or apoptosis. This bidirectional communication has been reported for a number of TNF superfamily members [2–9] and considered as a fine-tuning mechanism during the immune response. We and others reported receptor-like properties of the transmembrane TNF (mTNF), the first described member of the superfamily [10–14]. Soluble ectodomains of receptor molecule (*e.g.* Etanercept) or anti-TNF antibodies (*e.g.* Infliximab, Adalimumab) can also elicit reverse signaling in mTNF expressing cells [15,16]. Therapeutic application of these agents in chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease [17] revealed effects of TNF reverse signaling on different immune cells mostly depending on the cell type. Infliximab treatment induced apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. In monocytes from patients with rheumatoid arthritis, signaling through mTNF attenuated pathologic interleukin-1 beta production and corrected deficient apoptosis [18]. Apoptosis induction through TNF reverse signaling has been reported *in vitro* upon treatment either with TNF antibodies [15] or with soluble TNFR [19]. Interestingly, TNF reverse signaling attenuated the proliferative potential of T helper cells,

Abbreviations: ACP, actin capping protein; ATM, ataxia telangiectasia mutated; CKIP-1, casein kinase 2-interacting protein-1; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; EGFP/ECFP/EYFP, enhanced green/cyan/yellow fluorescent protein; HBSS, Hank's buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hTIP, human TNF intracellular domain interacting protein; mTNF, transmembrane tumor necrosis factor; NLS, nuclear localization signal; TNFNterm, N terminal fragment of TNF.

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meanwhile increased the cytotoxic potential of CD8+ T lymphocytes [20]. Moreover, TNF reverse signaling has been implicated in undesirable side-effects of anti-TNF therapies, where secondary infections were observed [21].

Despite the apparent clinical relevance the molecular basis of TNF reverse signaling is largely unknown. The first reports revealed protein kinase C and MAPK/ERK to be involved in TNF reverse signaling triggered LPS resistance in myeloid cells [14]. We reported earlier that the cytoplasmic domain of TNF is serine phosphorylated in mTNF expressing cells [10]. Moreover, interaction of mTNF with its soluble receptor triggers rapid dephosphorylation of mTNF and concomitant Ca^{2+} signaling [10]. The cytoplasmic serine residues of mTNF and phosphorylation of c-Jun N-terminal kinase have been proved essential for Infliximab-induced interleukin-10 production, apoptosis, and G0/G1 cell cycle arrest, where Infliximab treatment was shown to upregulate Bax, Bak, and p21 expression [15]. The MAPK pathway and caspases have been implicated in downstream signaling events of TNF reverse signaling [14], whereas other possible participating molecules remained elusive.

Identifying signaling molecules recruited to the mTNF molecule and investigating their role may help to unravel molecular details of TNF reverse signaling. A novel protein that interacts with the intracellular domain of mTNF has been identified (hTIP, human TNF intracellular domain interacting protein [gi:9622148]) and proved to be identical to human casein kinase 2-interacting protein-1 (CKIP-1) [22]. CKIP-1 has been found to interact with a series of proteins involved in cellular functions like differentiation, cell motility and cell death [23,24]. Since CKIP-1 has been identified as an interacting partner of the pro-inflammatory mTNF, we aimed at elucidating the function of CKIP-1 in inflammation and in TNF reverse signaling. Here we show novel biological properties of CKIP-1 that was found to activate the inflammatory response and to interfere with reverse signaling elicited by anti-TNF in human THP-1 cells.

2. Materials and methods

2.1. Cell culture

HEK293 cells (ATCC, Manassas, VA) and their derivatives were grown in a (1:1) mixture of Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) and Ham's F-12 nutrient medium (Sigma) supplemented with 10% low endotoxin FCS (Sigma) and 2 mM L-glutamine (Sigma). THP-1 cells were maintained in Opti-MEM (Invitrogen) 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 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).

2.2. 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 mTNF (TNFNterm) was fused to 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. 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.

2.3. Transient transfections and reporter gene assays

Construct pGL3-luc-TNFprom (pGL3-luc from Promega) in which the luciferase gene was controlled by the TNF-alpha promoter (−801 to +1) was introduced into HEK293 cells by Lipofectamine-2000 (Invitrogen). HEK293 stable clones were isolated after G418 (Sigma) selection. Stable clones (2×10^5 cells) containing the luciferase reporter construct 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™ 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) or Lipofectamine-2000 (Invitrogen), respectively.

2.4. 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 HEPES (pH 7.3), 100 mM KCl, 0.2% Nonidet P-40, 1 mM PMSF). 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 on a 12.5% SDS-polyacrylamide gel. ^{35}S-labeled proteins were detected by autoradiography.

2.5. Cell lysis and immunoprecipitation

24 h post-transfection HEK293 cells were washed in ice-cold PBS, scraped in lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1%

NP-40, 5% glycerol, 1 mM EDTA, and protease inhibitor cocktail; Sigma) and incubated under constant agitation at 4 °C for 1 h. Total cell extracts were cleared by centrifugation at 4,000 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) antibodies, or left untreated. After washing the beads were added to 500 μl (1 mg/ml) cell lysate. Following overnight incubation at 4 °C on a rotator, the beads were thoroughly washed in lysis buffer and the bound proteins were eluted.

2.6. Immunoblotting

Protein samples were separated by SDS-PAGE (12.5%) and proteins were transferred to polyvinylidene difluoride (PVDF) 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 PVDF membrane, probed with anti-Flag M2 (Sigma) and HRP-conjugated anti-mouse-IgG secondary antibody (Sigma).

2.7. 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 C_t values. Primer sequences for hCKIP-1 (forward and reverse, respectively) were used as follows: CCACTCGAGACAGGGCAAAA and AGCCATTAGGTGTCCCCTTGT.

2.8. Flow cytometry

For apoptosis measurements, cells were transfected and treated or kept as control as indicated. 24 h after transfection 2.5×10^5 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 time course of apoptosis measurements, all cells were transfected and treated at the same time, then analyzed at different time points. Following FSC *vs.* SSC gating the EGFP (FITC) positive population was selected using an FSC *vs.* FITC dot plot.

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™) 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 (PI) (5 μg/ml, Sigma) exclusion.

2.9. Fluorescent microscopy and image analysis

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 100× objective (alpha plan, N.A. 1.45) [25]. ECFP and 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 mm × 0.9 mm) 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.

2.10. Ca^{2+} measurements

Adherent THP-1 cells were loaded with 5 μM Indo 1-AM (Invitrogen) for 20 min in the dark. After washing steps with Ca^{2+} containing HBSS (Invitrogen) live cells were monitored by fluorescence microscopy. Samples were excited using a mercury lamp (HBO100, Zeiss) at 333/30 nm and time-resolved fluorescence emissions at 405/20 nm and at 485/25 nm were detected simultaneously by Coolsnap HQ™ CCD cameras (Photometrics). 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. 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 a Matlab program.

2.11. 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 60× (N.A. 1.35) objective and standard filter settings. Live THP-1 cells transfected with pEGFPC1-CKIP-1 were visualized with a 40× (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 60× (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.

2.12. 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.

3. Results

3.1. 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) similar to inducible genes of inflammatory cytokines, where effects of LPS are known to be mediated by members of the NF-κB family of transcription factors in immune cells [26]. 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 cells were treated with LPS and harvested at different time points after stimulation. The expression of CKIP-1 was analyzed by qRT-PCR method. As shown in Fig. 1A, 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 exposure. Similar upregulation was found in protein levels followed by Western blot analysis (Fig. 1A, insert).

Next we tested whether CKIP-1 could influence differentiation steps in THP-1 cells. Anticipating low transfection efficiency in this cell type, CKIP-1-EGFP was transiently overexpressed in THP-1 cells, and only the CKIP-1 expressing population was analyzed in the experiments. Means of anti-CD86 or anti-DC-SIGN staining of positively transformed THP-1 cells are shown in Fig. 1B. CKIP-1 expression was found to facilitate the classical activation process as reflected in the significant increase in CD86 surface values. Meanwhile there was no influence on the alternative activation measured by DC-SIGN surface expression.

3.2. CKIP-1 transactivates the TNF promoter in HEK293 cells

As CKIP-1 was upregulated at inflammatory conditions in THP-1 cells and it has a Leu-Zipper domain at the C terminus we tested whether it is involved in the pro-inflammatory responses through interacting with transcription factors. CKIP-1 has also been reported as an interacting partner of c-Jun [24] that is known to transactivate the TNF promoter upon LPS challenge [27]. 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 clones. Although overexpression of neither c-Jun nor CKIP-1 transactivated the TNF promoter significantly, in cells transiently co-expressing both c-Jun and CKIP-1 a strong activation of the TNF promoter was observed (Fig. 1C). To elucidate if CKIP-1 activated c-Jun, wild type HEK293 cells were co-transfected with a plasmid coding for CKIP-1, with a pathway specific construct for c-Jun and with a reporter plasmid expressing luciferase. In these experiments the expression of luciferase showed c-Jun phosphorylation, where MEKK (Mitogen-activated protein kinase kinase kinase), a known upstream activator of c-Jun was used 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. 1D).

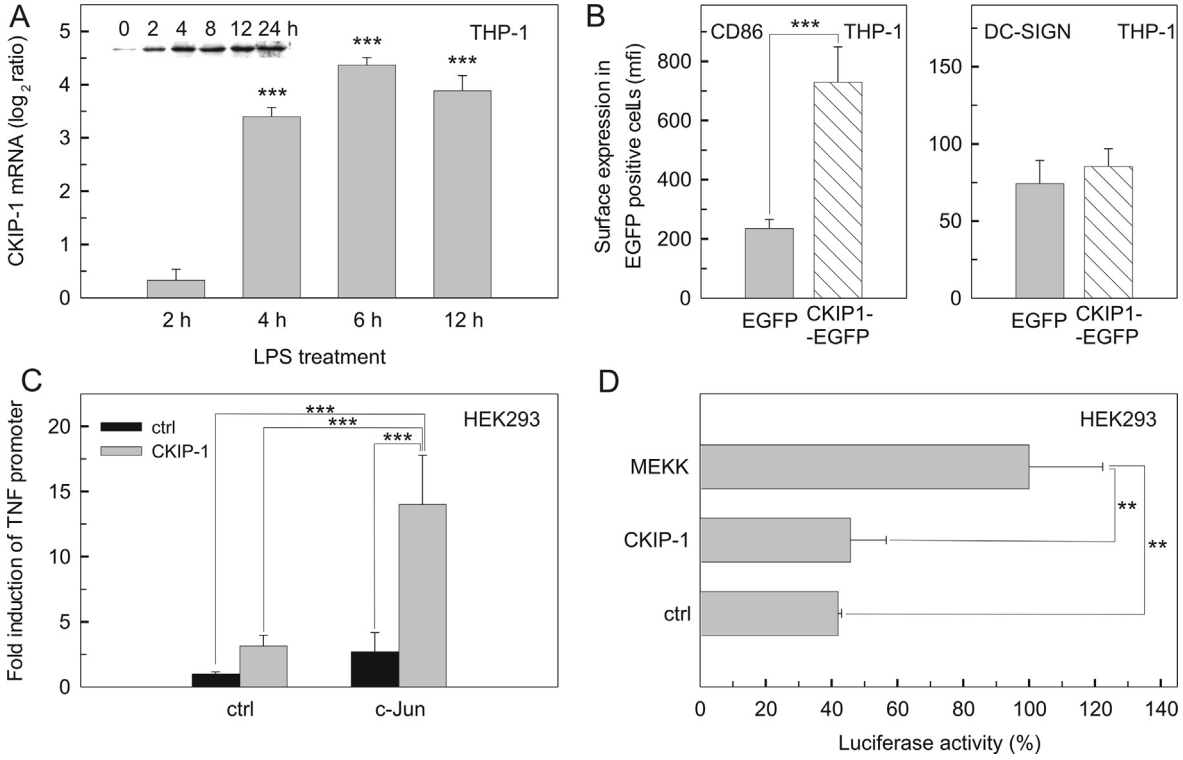

Fig. 1. CKIP-1 exerts a pro-inflammatory function. (A) LPS-induced CKIP-1 expression in THP-1 cells. THP-1 cells were exposed to LPS and harvested at indicated time points after stimulation. Changes in the transcript level of CKIP-1 in cells were measured by qRT-PCR method. Primers for 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase were used to normalize the values. Expression levels are shown as relative ratios ($n \geq 6$; mean ± SD). (insert) Protein level of CKIP-1 in control and LPS-treated cells were followed by Western blot analysis. (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. (C) 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. ($n \geq 3$; mean ± SD) (D) 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).

3.3. TNF reverse signaling induces Ca^{2+} oscillations and leads to relocalization of CKIP-1 in THP-1 cells

Anti-TNF treatments have been shown to induce reverse signaling in mTNF producing immune cells [16]. We therefore used Infliximab, a therapeutic TNF antibody to elicit reverse signaling in mTNF expressing THP-1 cells. PMA-induced adherent THP-1 cells were loaded with Indo-1-AM ratiometric dye, allowing Ca^{2+} signaling measurements. 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. 2A and B, treatment with the $F(ab)_2$ fragment triggered cells to specifically respond to a stimulation through the mTNF. This change was expressed in both the number of responsive cells and the amplitude of Ca^{2+} oscillations as compared to the effect of control solution (see also Supplementary videos 1 and 2 for control and $F(ab)_2$ treatment, respectively).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2013.04.001.

To explore potential changes in the subcellular distribution of CKIP-1 upon reverse signaling 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. 2C). To test whether an LPS challenge or TNF reverse signaling could influence the subcellular distribution of CKIP-1, transfected cells were treated with LPS, human immunoglobulin G (hIgG, as a control of Fc receptor mediated effects) or anti-TNF antibody (Infliximab). Because of the low transfection efficiency of this cell type, large scans of transfected THP-1 cells were imaged and analyzed. As shown in Fig. 2D, around 50% of the positively transfected cells already displayed both intracellular and plasma membrane localized CKIP-1 in untreated (ctrl) and LPS-treated samples. In contrast to hIgG control, Infliximab treatment resulted in a remarkable translocation of CKIP-1 from the plasma membrane to intracellular regions (see also Supplementary figure 1).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2013.04.001.

3.4. Expression of TNFNterm leads to relocalization of CKIP-1 in HEK293 cells

In mTNF overproducing cells we showed earlier intracellular and nuclear accumulation of TNFNterm as well as a regulatory effect of the peptide on cytokine gene transcription [13]. To explore the subcellular distribution of CKIP-1 and TNFNterm in HEK293 cells, fusion proteins CKIP-1-ECFP and TNFNterm-EYFP were transiently expressed and visualized by confocal microscopy. CKIP-1-ECFP was localized to the plasma membrane and intracellularly. TNFNterm-EYFP displayed intracellular localization, while the cells appeared to undergo morphological changes (Fig. 2E). Interestingly, when both fusion proteins were co-expressed no apparent morphological changes were observed. Nevertheless, a considerable relocalization of CKIP-1 from the plasma membrane to intracellular regions was measured upon TNFNterm co-expression (Fig. 2F and Supplementary figure 2).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2013.04.001.

3.5. TNFNterm interacts with CKIP-1

CKIP-1 has been identified as an interacting partner of the N terminal intracellular domain of the pro-inflammatory mTNF in a yeast two hybrid screen ([gi:9622148]). 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. 3 A 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. 3B).

Alternatively, co-immunoprecipitation analysis of TNFNterm-mCherry and CKIP-1-Flag was carried out in HEK293 cells. 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-mRFP 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. 3C). 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* pull down assays, as well (Fig. 3D).

3.6. 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 cellular localization of CKIP-1 we tested whether it influences the CKIP-1 mediated classical activation. 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. 4 A). 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. 4B, TNFNterm greatly reduced the co-operative effect of CKIP-1 and c-Jun on TNF promoter activation (see also Fig. 1C).

3.7. Expression of CKIP-1 prevents HEK293 cells from TNFNterm-induced apoptosis

Interestingly, in the imaging experiments (Fig. 2E) we observed morphological changes and decreased proliferation of HEK293 cells expressing TNFNterm. However, the morphological changes appeared to be counteracted in cells co-expressing both TNFNterm and CKIP-1. Since TNF reverse signaling has been reported to induce apoptosis in several types of immune cells [18,28–30] 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 (Fig. 5A). However, co-expression of CKIP-1

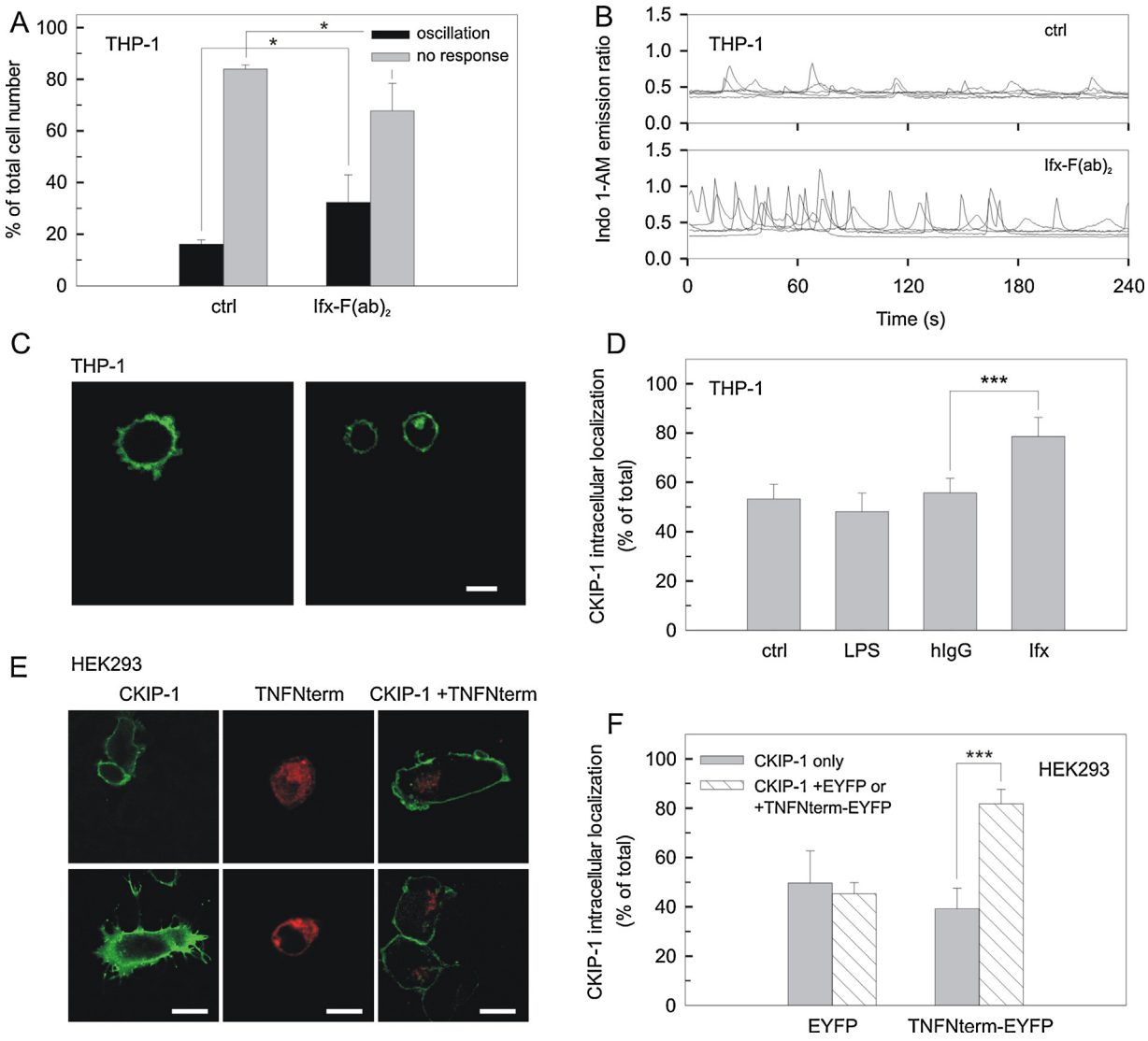

Fig. 2. TNF reverse signaling triggers CKIP-1 relocalization. (A) TNF reverse signaling induced Ca^{2+} oscillations in THP-1 cells. PMA-primed THP-1 cells were adhered on glass bottom dishes and loaded with Indo1-AM ratiometric dye. Fluorescence intensities were monitored in cells following treatment 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 videos 1A and B). ($n \geq 3$, 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. (C) 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). (D) 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 (see also Supplementary figure 2). ($n \geq 9$, where 14–75 cells were inspected for each sample; mean ± SD) (E) 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). (F) 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 (see also Supplementary figure 3). ($n \geq 3$, where 13–107 cells were inspected for each sample; mean ± SD).

almost completely prevented HEK293 cells from the apoptotic effect of TNFNterm (see also Supplementary figure 3).

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3.8. Expression of CKIP-1 interferes with TNF reverse signaling-induced apoptosis in THP-1 cells

Infliximab treatment has been shown to induce apoptosis in THP-1 cells [30]. As CKIP-1 could abolish the TNFNterm-induced apoptosis in HEK293 cells, we tested whether CKIP-1 could 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 different tumor cell types [24]. However, the presence of CKIP-1 did not just inhibit the apoptotic effect of Infliximab but reduced the apoptosis value below the control (hIgG) level (Fig. 5B and Supplementary figure 3). Note that CKIP-1

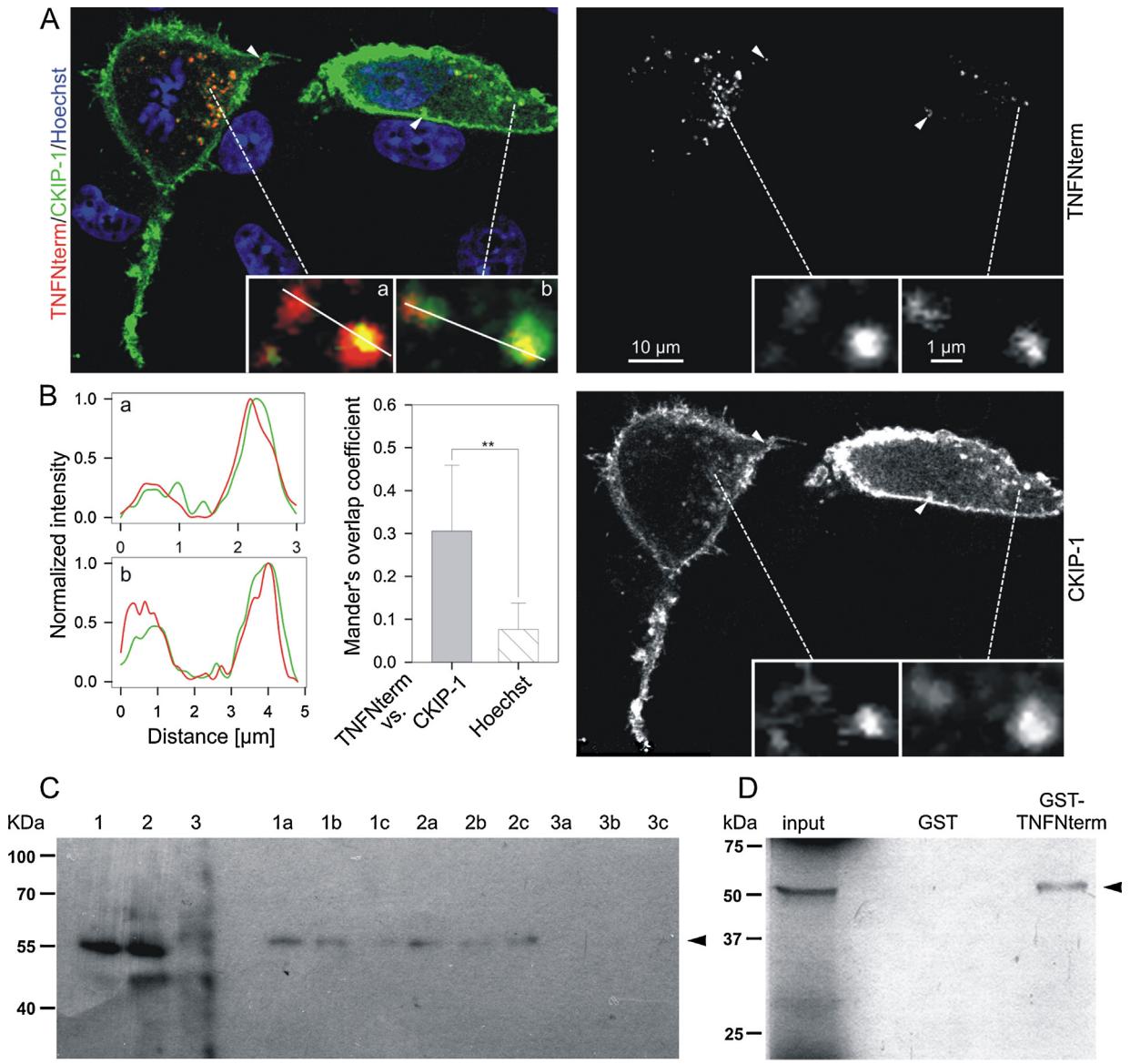

Fig. 3. TNFNterm interacts with CKIP-1. (A) Co-localization of TNFNterm and CKIP-1 in HEK293 cells. Fusion proteins TNFNterm-mCherry and CKIP-1-EGFP were transiently expressed in HEK293 cells. Cells were visualized by confocal microscopy. (B) Representative regions (inserts) were selected for line scan 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.

overexpression did not influence the surface expression of mTNF (data not shown). TNF reverse signaling is thought to induce caspase-3 activation and CKIP-1 has been shown to participate in caspase-3 mediated apoptosis [24]. To test whether CKIP-1 interferes with caspase-3 mediated apoptotic processes other than that induced by TNF reverse signaling, cells were treated with etoposide. Etoposide caused significant increase in the apoptosis values of control cells. Unlike Infliximab, etoposide further elevated the apoptosis value of THP-1 cells overexpressing CKIP-1 (Fig. 5C and Supplementary figure 3). Therefore CKIP-1 did not appear to interfere with etoposide-induced, caspase-3 mediated apoptosis.

3.9. 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. 5B). Since Infliximab treatment did not induce *ckip-1* expression in resting THP-1 cells (Supplementary figure 4), we tested whether TNF reverse signaling could influence the already induced CKIP-1 expression. THP-1 cells were challenged by LPS for 4 h, 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. 5D).

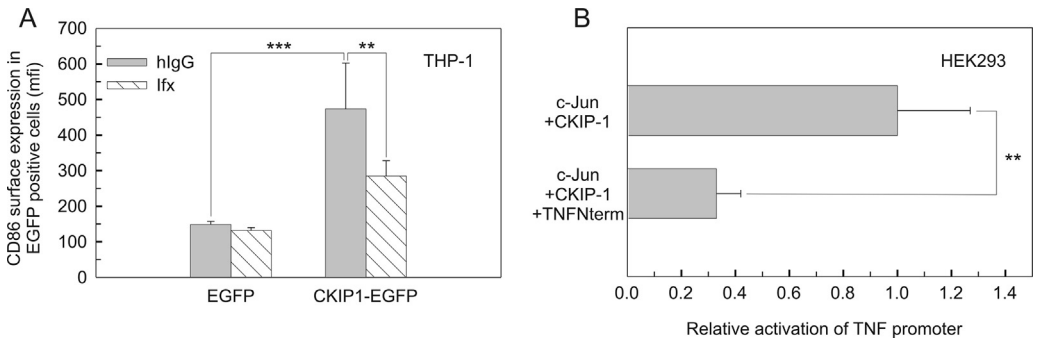

Fig. 4. TNF reverse signaling interferes with CKIP-1 mediated immune activation. (A) CD86 surface expression upon TNF reverse signaling. THP-1 cells transiently expressing EGFP or CKIP-1-EGFP were incubated with hIgG or Infliximab for 16 h. Cells stained with anti-CD86 and PI were analyzed by flow cytometry. Means of anti-CD86 staining of intact cells in EGFP positive subpopulations are shown. ($n = 4$; mean fluorescence intensity ± SD) Isotype values are 4.0 ± 0.4, 3.3 ± 0.4, 3.8 ± 0.5, 4.4 ± 1.2. (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. ($n \geq 5$; mean ± SD).

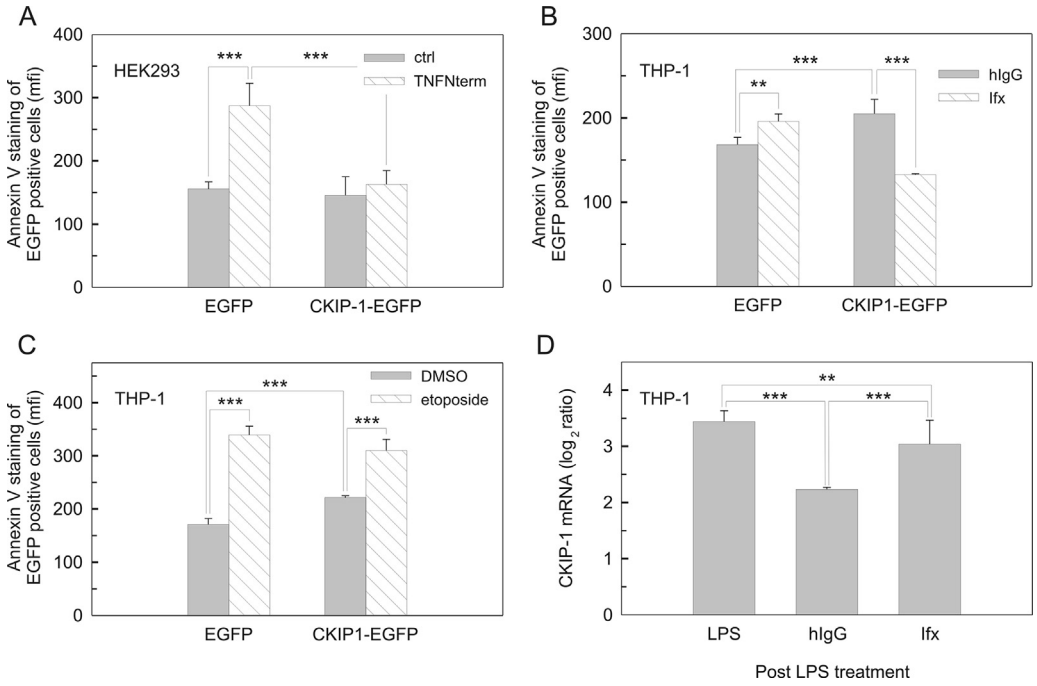

Fig. 5. CKIP-1 interferes with TNF reverse signaling-induced apoptosis. (A) Inhibition of TNFNterm-induced apoptosis by CKIP-1. HEK293 cells were transiently co-transfected with empty vector or TNFNterm plasmid and EGFP or CKIP-1-EGFP expressing vectors. 24 h after transfection cells were labeled with Annexin V-Alexa647 and PI and analyzed by flow cytomertry. Means of Annexin V-Alexa647 staining of cells are shown for EGFP positive subpopulations. ($n \geq 4$; mean fluorescence intensity ± SD) See also Supplementary figure 3 for time courses of dot plot display. (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) See also Supplementary figure 3 for time courses of dot plot display. (C) 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) See also Supplementary Fig. 3 for time courses of dot plot display. (D) 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).

Hence TNF reverse signaling appeared to interfere with relaxation of LPS-triggered CKIP-1 induction.

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4. Discussion

Receptor-like properties of the transmembrane form of the pro-inflammatory cytokine TNF have been reported by several laboratories [11–14]. 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 mTNF (TNFNterm) could accumulate in the nucleus and regulate the transcription of cytokines [13]. TNFNterm has been found to interact with a novel protein (hTIP, [gi:9622148]) in a yeast two hybrid assay when probed with an LPS-activated macrophage cDNA library. This protein has been proved identical to casein kinase-2 interacting protein-1 (CKIP-1). CKIP-1 has been shown to interact with c-Jun [24], actin capping protein subunits (ACP α and β) [23], ATM [31], Akt [32], IFP35 and Nmi [33] as well as Smurf-1 [34]. Since it has

been reported as a multifunctional protein interacting with the intracellular domain of mTNF, we assumed CKIP-1 to be involved in TNF reverse signaling.

CKIP-1 exhibited diverse functions in different cell types such as recruiting proteins to the plasma membrane [31,35] mediating regulation of the actin cytoskeleton [23], repressing cell survival signaling [24] and affecting cell differentiation [36]. Although CKIP-1 has been proposed to control cytokine signaling [33] its function in immunity is still unclear. Here we showed that the expression of CKIP-1 mRNA and the protein level were significantly elevated in THP-1 monocyte model cells exposed to LPS. Moreover, experiments with HEK293 model cells showed increased activity of the TNF promoter upon co-expression of CKIP-1 and c-Jun, where CKIP-1 did not facilitate activation through enhancing the phosphorylation of c-Jun. The pro-inflammatory role of 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, CKIP-1 exerted a positive regulatory role in the activation process of monocytes.

Therapeutically used soluble TNF receptor (Etanercept) or anti-TNF antibodies (Infliximab, Adalimumab) have been reported to elicit reverse signaling in mTNF producing immune cells [16]. Here we showed that Infliximab-induced TNF reverse signaling leads to Ca^{2+} oscillations in mTNF expressing THP-1 monocytes. Interestingly, we found that CKIP-1 re-localized from the plasma membrane to intracellular compartments upon TNF reverse signaling in THP-1 cells. A similar relocalization of CKIP-1 was shown for HEK293 cells when expressing TNFNterm. 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. CKIP-1 could indeed localize to the plasma membrane, in the cytosol as well as in the nucleus [23,24,35–37]. Moreover, a cell type and stimulus dependent relocalization of CKIP-1 from the plasma membrane to the nucleus has been reported [24,37]. Interestingly, in parallel with the relocalization of CKIP-1 in our model cells, 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 [12].

TNF reverse signaling has been shown to activate both pro-apoptotic and antiapoptotic signaling pathways, where fate of the cell depended on the degree of differentiation and crosstalk with other signaling pathways [18,38]. In NK cells or in different T cell subpopulations TNF reverse signaling has been shown to induce activation or differentiation [20,39–42]. In contrast, anti-TNF treatment has been reported to induce apoptosis in myeloid cells and in T lymphocytes [15,16,18,28–30]. In THP-1 monocytes reverse signaling has been shown to increase the release of TGF-beta1 from the cells, in turn to activate proapoptotic pathways [19]. Nevertheless, critical signaling molecules determining the fate of the cell upon TNF reverse signaling have not been revealed. In our experiments Infliximab treatment induced apoptosis in THP-1 cells. Moreover, overexpression of TNFNterm also induced apoptosis in HEK293, an apoptosis insensitive cell line. In addition to reverse signaling, CKIP-1 overexpression has been reported to promote apoptosis by forming a positive feedback loop between CKIP-1 and caspase-3 in different tumor cell lines [24]. In agreement, we observed a moderate apoptosis sensitizing effect of CKIP-1 in THP-1 cells. Interestingly, when TNF reverse signaling was activated LPS-triggered CKIP-1 induction in THP-1 monocytes was relaxed to a lesser extent, indicating the involvement of CKIP-1 in these signaling events. Importantly, overexpression of CKIP-1 interfered with TNF reverse signaling-induced apoptosis in THP-1 monocytes. Moreover, CKIP-1 overexpression almost completely counteracted the apoptotic effect of the co-expressed TNFNterm in HEK293 cells. Therefore, CKIP-1 appeared to be a critical factor determining the fate of monocytes interacting with anti-TNF agents.

Surprisingly, CKIP-1 could not only prevent THP-1 cells from TNF reverse signaling induced apoptosis but increased the survival of the CKIP-1 expressing cells upon Infliximab treatment. 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 [43]. Interestingly, CKIP-1 has also been shown to be cleaved by caspase-3 upon apoptotic signals [24]. Therefore we assumed a similar switch mechanism dependent on caspase-3 mediated cleavage of CKIP-1 upon reverse signaling elicited by anti-TNF treatment. However, in our experiments the antiapoptotic function of CKIP-1 was not activated upon treatment with etoposide, another apoptosis inducer acting through caspase-3. Hence, the switch in CKIP-1 function appeared to be more specific to anti-TNF treatment inducing reverse signaling. Interestingly enough, we showed earlier that the NLS containing TNFNterm could accumulate in the nucleus and regulate the transcription of cytokines [13]. A similar unusual phenomenon is known for Notch receptor, where upon ligand binding the cleaved intracellular domain of the transmembrane molecule translocates into the nucleus and acts as a transcriptional co-activator [44]. The apparent correlation between the relocalization of CKIP-1 and TNFNterm, as well as the specific interference of CKIP-1 with TNF reverse signaling indicate that CKIP-1 and TNFNterm may act through the same signaling complex. This idea is consistent with the findings of yeast two hybrid, *in vitro* pull down, co-immunoprecipitation and co-localization experiments, and further supported by the proposed scaffold nature of CKIP-1 [36] as well as the mutual influence on cellular activities of CKIP-1 and TNF reverse signaling shown in this work.

Taken together we showed novel functions of the signaling molecule CKIP-1 in THP-1 monocytes. CKIP-1 expression was induced upon LPS challenge and was shown to play an important stimulatory role in the pro-inflammatory process. CKIP-1 showed a counteracting effect on TNF reverse signaling-induced cell death, suggesting that it could act as an inhibitory molecule in the same immune cell. Since both signaling processes require CKIP-1, distinct activities of the scaffold protein CKIP-1 may be regulated by its localization, cleavage or interacting partners. Importantly, it has been suggested that secondary infections during anti-TNF therapies of chronic inflammatory diseases may originate from elimination of mTNF expressing immune cells. Our data suggest that expression level of CKIP-1 could regulate the response of immune cells upon TNF reverse signaling, therefore it 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.

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