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Toso: A novel regulator of signal transduction for survival and apoptosis in lymphocytes

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To my Great Grandpa,
Bravery, Strength and Will

To my Parents,
with love

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A. List of abbreviations

%	Percent
°C	Degree Celsius
AICD	Activation induced cell death
AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis protease-activating factor
APC	Allophycocyanin
BCR	B-cell receptor
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CD	Cluster of differentiation
cDNA	Complementary DNA
c-FLIP	Cellular FLICE-inhibitory protein
CLL	Chronic lymphocytic leukemia
cm	Centimeter
CMV	Cytomegalovirus
CNS	Central nervous system
Cyt *c*	Cytochrome C
DCC	Deleted in colorectal cancer
DcR3	Decoy receptor 3
DD	Death domain
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR(s)	Death receptors
dsDNA	Double-stranded DNA
EDTA	Ethylene diamine tetracetic acid
EndoG	Endonuclease G
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell scanning

FADD	FAS-associated death domain
Faim3	Fas apoptosis inhibitory molecule 3
FasL	Fas ligand
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward-scatter
GFP	Green fluorescent protein
HSV	Herpes simplex virus
IAP(s)	Inhibitors of apoptosis protein
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
M	Molar
MACS	Magnetic-associated cell sorting
MAPK	Mitogen-activated protein kinase
mFasL	Membrane-bound FasL
mg	Milligram
miRNA(s)	Micro RNA(s)
ml	Milliliter
mm	Millimeter
mM	Millimolar
MOMP	Mitochondrial outer membrane permeabilization
MS	Multiple sclerosis
NF-κB	Nuclear factor kappa B
ng	Nanogram
nm	Nanometer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
pIC	Polyinosinic-polycytidylic acid
PLCγ	Phospholipase Cγ
p-value	Probability value
qPCR	Quantitative, real time PCR

RIP1	Receptor-interacting protein 1
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEB	Staphylococcus enterotoxin B
sFasL	Soluble FasL
SLE	Systemic lupus erythematosus
Smac	Second mitochondria-derived activator of caspases
SPOTS	Signaling protein oligomerization structures
SSC	Sideward-scatter
ssDNA	Single-stranded DNA
TAE	Tris/acetate acid/EDTA
tBid	Truncated form of Bid
TNF	Tumor necrosis factor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
Tris	Trishydroxymethylaminomethane
TRADD	TNF-receptor-associated death domain
WT	Wild-type
x *g*	Gravity force
µg	Microgram
µl	Microliter

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1. Introduction

1.1 Apoptotic cell death

As the two sides of the same coin, proliferation and cell death go together and require one another. During the evolution of multi-cellular organisms, the issue of maintaining both body and organ size conducive with health and viability became a challenge. The evolutionarily derived answer to the problem came in the form of programmed cell death or apoptosis (Yin and Dong, 2009). The term “apoptosis” literally means ”leaves falling from a tree” in Greek and was coined to describe the morphological features observed during this coordinated deconstruction and packaging of the cell for elimination by phagocytes and neighboring cells (Lockshin and Zakeri, 2001). Apoptosis is a strictly regulated process, which plays a key role in development, morphogenesis, tissue remodeling and immune response, mediating the ordered removal of superfluous, aged or damaged cells (Debatin and Fulda, 2006). In contrast to necrotic cells, which can elicit an inflammatory reaction, apoptotic cells are removed in an inconspicuous fashion, mainly by phagocytosis by neighboring cells or by specialized macrophage-like cells (Krammer *et al.*, 2007). A key feature of apoptosis is a cleavage of cytoskeletal proteins by aspartate-specific proteases, which thereby collapses subcellular components. Other characteristic features are chromatin condensation, nuclear fragmentation, and the formation of plasma-membrane blebs (Hotchkiss *et al.*, 2009).

There are at least two broad pathways that lead to apoptosis depending on the mechanism of initiation: the intrinsic and extrinsic pathways. The initiators of the intrinsic pathway include increased intracellular reactive oxygen species, DNA damage, the unfolded protein response, and the deprivation of growth factors. These initiators ultimately lead to increase mitochondrial permeability, thereby promoting the release of a number of factors, leading to the formation of the apoptosome, which is comprised of the adapter protein Apaf-1, cytochrome c, and caspase-9. Following activation, caspase-9 in turn can activate caspase-3 leading to apoptosis (Hotchkiss *et al.*, 2009). The extrinsic pathway begins outside the cell and is initiated by extracellular stimuli, when conditions in the extracellular environment determine that a cell must die. Extrinsic pathway is activated when members of the tumor necrosis factor (TNF) superfamily bind to the cell-surface “death receptors”, members of the TNF-receptor family (Green, 2005). Death receptors (DRs) reported so far are TNF-R1

(CD120a), CD95 (APO-1, Fas), DR3 (APO-3, LARD, TRAMP, WSL1), TRAIL-R1 (APO-2, DR4), TRAIL-R2 (DR5, KILLER, TRICK2), and DR6 (Guicciardi and Gores, 2009). The receptor cross-linking either with agonistic antibodies or with death ligands initiates the formation of a multimolecular complex of proteins called death-inducing signaling complex (DISC) (Kischkel *et al.*, 1995; Marsden and Strasser, 2003; Newton *et al.*, 1998). Aggregation of this complex causes conformation change in its components that trigger the catalytic activity of caspase-8, a central mediator of apoptosis (Hotchkiss *et al.*, 2009). The Fas death receptor, which conveys apoptotic signals through binding to its cognate ligand, FasL, is the best characterized member of the death receptor family.

1.2 Fas

Fas (CD95/Apo-1/TNFRSF6) is a prototypic death receptor belonging to the tumor necrosis factor (TNF) receptor superfamily (Ashkenazi and Dixit, 1998; Li-Weber and Krammer, 2003; Tibbetts *et al.*, 2003). The Fas molecule was originally identified in 1989 by two independent groups who described a monoclonal antibody that recognized a 52 kDa cell surface protein and strongly induced apoptosis in several cell lines (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). To date, Fas is the best characterized member of death receptors in the immune system that activate the extrinsic apoptosis pathway (Barnhart *et al.*, 2003; Wajant, 2006).

Fas is a type I glycoprotein expressed on the cell surface as preassociated homotrimers (Siegel *et al.*, 2000). This 45–54 kDa glycosylated cell-surface protein is ubiquitously expressed in various tissues, but particularly abundant in the liver, heart, kidney, pancreas, brain, thymus, and activated mature lymphocytes, as well as virus-transformed lymphocytes (Salido and Rosado, 2009; Yin and Dong, 2009). The broad expression pattern of Fas suggests that many tissues are ready to activate the apoptotic program upon request from outside, i.e., via the extrinsic apoptotic pathway (Wajant, 2006).

Fas localizes on the plasma membrane as well as in the cytosol, in particular, in the Golgi complex and the trans-Golgi network (Bennett *et al.*, 1998; Sodeman *et al.*, 2000). Translocation of Fas-containing vesicles to the cell surface has been observed upon stimulation, providing an effective mechanism to regulate the plasma membrane density of the death receptor and avoid its spontaneous activation (Feng and Kaplowitz, 2000; Sodeman

et al., 2000). Fas-mediated apoptosis can also be modulated by posttranslational modifications such as glycosylation (Peter *et al.*, 1995) or palmitoylation of the receptor (Feig *et al.*, 2007), as well as at the transcriptional level, by directly regulating Fas expression (Chan *et al.*, 1999; Muller *et al.*, 1998).

1.2.1 The physiological function of Fas

Apoptosis is tightly controlled in the immune system to maintain normal immune cell homeostasis. The adaptive immune response is characterized by the dramatic expansion of antigen specific lymphocytes against a pathogen which is an essential aspect for proper immune competence. However, the elimination of activated lymphocytes after an immune response by apoptosis is also critical for maintaining lymphocyte homeostasis and controlling immunological tolerance. The discovery of genetic mutants of Fas has led to a remarkable improvement in our understanding of lymphocyte apoptosis and immune disorders.

Natural occurring mutations of Fas or its ligand have been found in autoimmune strains of mice (i.e. *lpr* mice and *gld* mice, respectively) and in patients with an autoimmune lymphoproliferative syndrome (ALPS) (Bettinardi *et al.*, 1997). The deficiency of Fas or Fas ligand primarily affects the immune system and reveals the essential role of Fas in regulating the immune response. These genetic abnormalities in Fas signal transduction result in massive lymphadenopathy and disruption of lymphocyte homeostasis due to a lack of activation induced apoptosis in lymphocytes. Mice lacking Fas or FasL develop progressive lymphadenopathy and splenomegaly which predominantly involves the unusual $CD4^-CD8^-$ T cells, which are thought to derive from conventional T cells that have been repeatedly activated *in vivo* via their TCR complex (Landolfi *et al.*, 1993; Takahashi *et al.*, 1994; Watanabe-Fukunaga *et al.*, 1992). These mutant mice do, however, also accumulate several-fold increases in conventional $CD4^+$ as well as $CD8^+$ T lymphocytes and B lymphocytes (Takahashi *et al.*, 1994; Watanabe-Fukunaga *et al.*, 1992). The same but even more pronounced phenotype has been reported for mice in which Fas has been inactivated by gene targeting (Adachi *et al.*, 1995). This led to the conclusion that FasL-Fas signaling plays a critical role in the homeostasis of the lymphoid system, most likely by killing unwanted cells at one or several developmental checkpoints.

Fas-mediated apoptosis also plays an important role in the control of B cell development and B cell-mediated immune responses (Krammer, 2000; Nagata, 1997). FasL and Fas play critical roles in the control of B cell survival during the germinal center reactions (Strasser *et al.*, 2009). Activated B cells bearing low-affinity BCR are killed when Fas on their membrane surface is ligated by FasL expressed on intrafollicular $CD4^+$ T cells, and they fail to receive a pro-survival signal through their BCR and CD40 (Rathmell *et al.*, 1995). During development, immature B cells that encounter and bind self-antigen in the bone marrow are deleted from the immune repertoire in a process analogous to the negative selection of T cells (Tiegs *et al.*, 1993). It was found that both Fas signaling as well as the Bcl-2-regulated apoptotic pathway contribute to B cell deletion (Strasser *et al.*, 1995). In accordance, the loss of Bim (Hughes *et al.*, 2008) or overexpression of Bcl-2 (Hughes *et al.*, 2008; Hutcheson *et al.*, 2008; Weant *et al.*, 2008) lead to extreme lymphadenopathy and autoimmunity in Fas- or FasL-deficient mice. On the basis of recent studies, complementary roles for both Fas and Bim have been proposed in the regulation of T and B cell apoptosis during the course of immune responses (Hughes *et al.*, 2008; Hutcheson *et al.*, 2008; Weant *et al.*, 2008).

A human counterpart to the *lpr/gld* phenotype in mice is known as an autoimmune lymphoproliferative syndrome (ALPS) (Rieux-Laucat *et al.*, 2003). The ALPS patients, most of whom are children, carry mutations in either the Fas gene (APLS la) or the FasL gene (APLS lb), which generates defective proteins that either lack their normal function or work as dominant-negative when expressed with normal Fas or FasL (Wajant, 2006; Yin and Dong, 2009). As a consequence, these patients are unable to effectively downregulate the immune reaction and develop lymphadenopathy, splenomegaly, and hypergammaglobulinemia. Furthermore, ALPS patients and *lpr* and *gld* mice are phenotypically associated with systemic autoimmunity and produce a repertoire of antoantibodies similar to that found in human systemic lupus erythematosus (SLE) (Cohen and Eisenberg, 1991). These findings suggest the critical role of Fas to control immunological tolerance to self antigens and autoimmunity. Therefore, excessive or defective susceptibilities to Fas-induced apoptosis have been proposed as a major pathogenetic mechanism in a variety of autoimmune diseases.

The physiological roles of the FasL-Fas system also include tumor surveillance. Dysregulation or mutations of Fas or Fas ligand gene have been described to be associated with cancer (Bettinardi *et al.*, 1997; Davidson *et al.*, 1998; Drappa *et al.*, 1996; O' Reilly *et*

al., 2009) which suggests that Fas function is involved in the pathogenesis of malignancies. However, accumulating recent data also suggest that depending on the cellular context, Fas can activate not only apoptotic signaling, but also activating or survival signaling. This Fas mediated non-apoptotic signaling is also involved in promoting tumor progression (Hao *et al.*, 2004; Ma *et al.*, 2004; Matsumoto *et al.*, 2007; O' Reilly *et al.*, 2009). Therefore, it remains still unclear whether the immunologic abnormalities and malignancies observed in Fas deficiency *in vivo* result from either the non-apoptotic and/or apoptotic signaling defects mediated by Fas.

1.2.2 Fas mediated apoptotic signaling

The early events of death signaling downstream of Fas receptors have been well characterized. Upon FasL binding, Fas undergoes a conformational change to reveal its cytoplasmic death domain (DD) to favor homotypic interactions with other DD-containing proteins (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995; Itoh and Nagata, 1993; Papoff *et al.*, 1999). The FasL-induced Fas conformational change leads to the recruitment of the adaptor molecule FADD (Fas-associated protein with death domain). FADD is a ubiquitously expressed, 28 kDa cytosolic protein with a C-terminal death domain (DD), and a death effector domain (DED) at the N-terminus. FADD associates with Fas receptor through its DD, while DED is required for self-association and binding procaspase 8 (Figure 1.1) (Chinnaiyan *et al.*, 1996; Hill *et al.*, 2004; Kischkel *et al.*, 1995; Scott *et al.*, 2009). Recently, the x-ray crystal structure of the Fas-FADD death domain complex revealed a tetrameric arrangement of four primary Fas/FADD complexes (Scott *et al.*, 2009).

The structure studies showed that only when sufficient numbers of Fas molecules are in close proximity, as it is expected upon Fas ligand binding, the open forms of Fas receptor can be stabilized. This open form of Fas enables to bind to FADD, thereby further fostering the death inducing signaling complex formation (Scott *et al.*, 2009). The death-inducing signaling complex, termed DISC, is a multi-protein complex formed by members of the death receptor family of apoptosis-inducing cellular receptors. Fas receptor forms the DISC upon trimerization as a result of its ligand (FasL) binding and the DISC is composed of the death receptor, FADD, and caspase-8 (Kischkel *et al.*, 1995; Medema *et al.*, 1997). It transduces a downstream signal cascade resulting in apoptosis.

Once caspase-8 associates with FADD, the high local concentration of caspase-8 leads to its autoproteolytic cleavage and activation. Aside from its two DED, caspase-8 contains a protease domain consisting of two subunits. Following the autoproteolytic cleavage of the enzyme, caspase-8 is released from the DISC as an active heterotetramer containing two p18 and two p10 subunits leading to the activation of caspase-3 and further downstream death effectors. Thus, efficient DISC assembly provides a molecular scaffold concentrating cysteine proteases to induce the activation of caspase-8 and, in turn, subsequent progression of the apoptotic program (Donepudi *et al.*, 2003; Medema *et al.*, 1997; Muzio *et al.*, 1996).

Fas mediated apoptosis has also been described to be transduced in two general modes (Figure 1.1) (Algeciras-Schimnich and Peter, 2003; Algeciras-Schimnich *et al.*, 2003; Scaffidi *et al.*, 1998; Scaffidi *et al.*, 1999). Type I cells, including BJAB (an EBV transformed human Burkitt's lymphoma cell line) and primary T cells, demonstrate rapid Fas internalization and efficient DISC assembly. Caspase-8 is mainly activated at the DISC and is responsible for direct cleavage and activation of the effector caspase-3. In these cells, Fas is associated with membrane rafts, which favors its internalization (Hueber *et al.*, 2002). Colocalization of Fas with a lipid raft is an essential step, as disruption of lipid raft structure by cholesterol depletion inhibits Fas-mediated apoptosis in Type I cells (Scaffidi *et al.*, 1998). Type I cells undergo Fas-mediated apoptosis without significant mitochondrial contribution. Although active caspase-8 generated at the DISC also activates mitochondria through cleavage of Bid, overexpression of the anti-apoptotic proteins Bcl-2 or Bcl-XL does not prevent activation of caspase-8 or caspase-3 in these cells, nor does it inhibit apoptosis (Barnhart *et al.*, 2003).

In contrast to Type I cells, DISC formation in Type II cells is delayed and strongly reduced and in these cells the Fas-mediated apoptotic signal relies on an apoptotic amplification loop triggered by the mitochondria (Figure 1.1) (Eramo *et al.*, 2004). In Type II cells, the low level of DISC-generated active caspase-8 is sufficient to cleave a small amount of Bid to generate tBid, but insufficient to directly cleave and activate caspase-3 (Li *et al.*, 1998; Luo *et al.*, 1998). tBid, in turn, translocates to the mitochondria and induces the release of apoptogenic factors, leading to the formation of the apoptosome, which is comprised of the adapter protein Apaf-1, cytochrome *c*, and caspase-9 (Acehan *et al.*, 2002; Bratton *et al.*, 2001; Gross *et al.*, 1999). Through an energy-requiring reaction, procaspase-9 is processed to

the mature enzyme and, in turn, activates caspase-3, starting a caspase cascade downstream of the mitochondria (Li *et al.*, 1997; Zou *et al.*, 1997).

Recently, Muppidi and Siegel refined this model by showing that the enrichment of Fas into or outside lipid rafts was responsible for the Type I and Type II phenotypes, respectively. After ligation, Fas does not fully colocalize with lipid rafts and is not efficiently internalized in Type II cells (Muppidi and Siegel, 2004). In Type II cells, the activation of caspases occurs mainly downstream of mitochondria, and caspase activation and apoptosis can be blocked by overexpression of Bcl-2 or Bcl-XL (Barnhart *et al.*, 2003), suggesting an essential role of mitochondria for the execution of the apoptotic program.

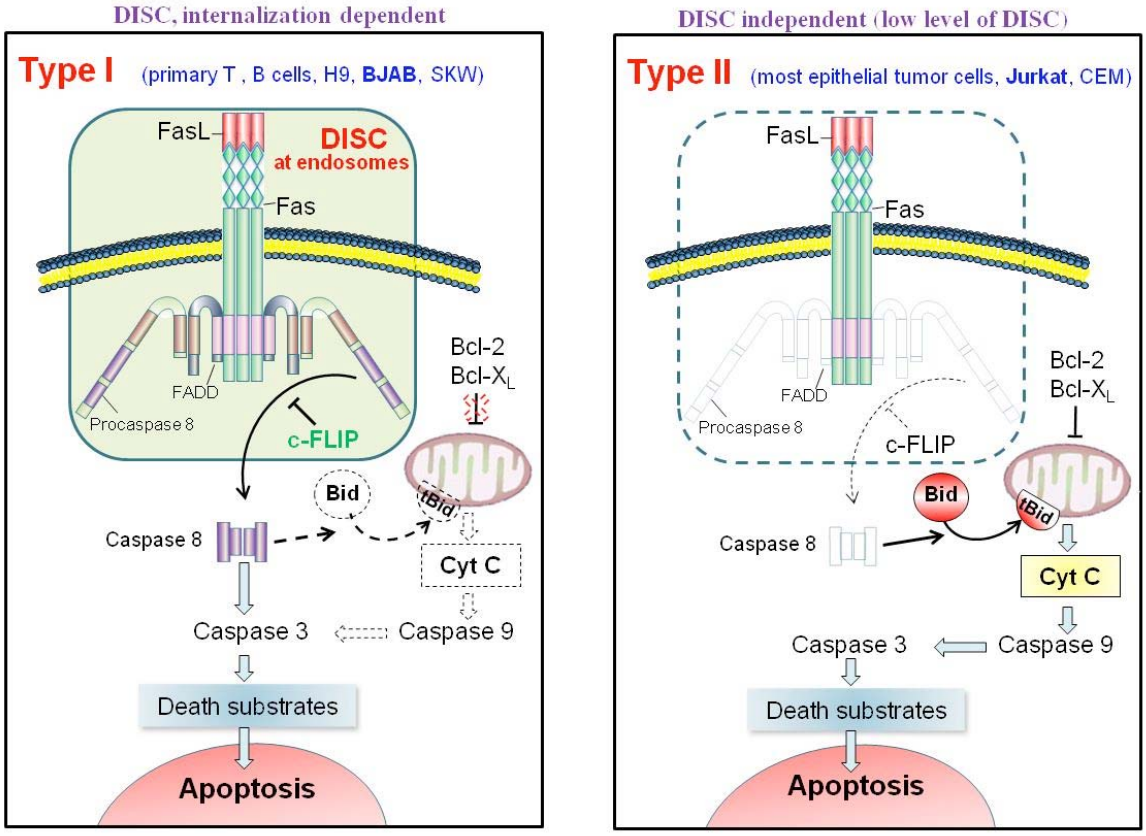


Figure 1.1: The two pathway model of Fas signaling.

(A) The Type I signaling pathway. In Type I cells, Fas engagement is resulted in an efficient formation of the death inducing signaling complex (DISC), which contains FADD, procaspase-8 and c-FLIP, and ultimately in large amounts of caspase-8 activation. This large amount of caspase-8 is able to directly cleave several substrates, including caspase-3 and also activate the apoptotic function of mitochondria. Blockage of mitochondrial apoptotic function by Bcl-2 or Bcl- XL is not able to inhibit apoptosis in Type I cells due to the ability of the cells to directly activate caspase-3, leading to apoptosis. (B) The Type II signaling pathway. In Type II cells, Fas ligation leads to the formation of a poor or undetectable DISC and the production of very little caspase-8. These quantities are sufficient to cleave a small amount of Bid but insufficient to activate caspase-3.

Therefore, these cells require a mitochondrial amplification loop, which is mediated through Bid cleavage. Mitochondria-released cytochrome c (Cyt c) then activates caspase-9. Active caspase-9, in turn activates caspase-3, starting a caspase cascade downstream of the mitochondria, leading to apoptosis. The reliance of these cells on the mitochondrial apoptotic signal amplification function results in a blockage of that signal upon expression of Bcl-2 or Bcl-XL.(Adapted from Scaffidi et al., 1998)

Recent studies have also demonstrated that within seconds of ligand binding, the preassociated receptors form SDS- and β-mercaptoethanol-stable Fas microaggregates associated with palmitoylation of the membrane proximal cysteine 199 in the receptor (Chakrabandhu *et al.*, 2007; Feig *et al.*, 2007). Palmitoylation is the targeting signal for Fas localization into lipid rafts, and facilitates the subsequent internalization of the receptor and caspase-8 activation (Algeciras-Schimnich *et al.*, 2002; Henkler *et al.*, 2005; Siegel *et al.*, 2004) in Type I cells. Upon ligand triggering, the receptor complexes organize into higher-order aggregates through interactions with actin filaments forming signaling protein oligomerization transduction structures (SPOTS) which are visible by immunofluorescence microscopy (Henkler *et al.*, 2005; Siegel *et al.*, 2004). The SPOTS formation drives further clustering of Fas receptors to form large lipid raft platforms (a process referred to as “receptor capping”) that are internalized into endosomal vesicles through clathrin-mediated endocytosis (Eramo *et al.*, 2004; Lee *et al.*, 2006).

1.2.3 Fas mediated non-apoptotic signaling

It is firmly established that the dual outcome, including ligand-induced activation and apoptosis, appears to be a common property of signaling through several members of the TNFR family, including death receptors, TNF-R1 and Fas. Although Fas signaling has been mainly considered with respect to its death-inducing function, accumulating data suggest that depending on the cellular context, Fas can also induce activating signals, such as the activation of NF-κB and MAPK pathways (Lee *et al.*, 2006). Such non-apoptotic signaling activities of Fas are thought to mediate proinflammatory responses in immune cells (e.g. chemokine and cytokine production), neuronal tissue remodeling and promote tumor progression (Hao *et al.*, 2004; Ma *et al.*, 2004; Matsumoto *et al.*, 2007).

Already in 1993, there was data showed that Fas triggering enhances proliferation of TCR-stimulated T cells and thymocytes (Alderson *et al.*, 1993). Several other studies demonstrate that T cell proliferation induced by suboptimal anti-CD3 stimulation is enhanced when Fas is co-triggered (Kennedy *et al.*, 1999; Sun *et al.*, 2006; Suzuki and Fink, 1998; Suzuki *et al.*, 2000). Furthermore, deletion of Fas in T lymphocytes causes lymphopenia in mice (Hao *et al.*, 2004), suggesting that Fas expression by T cells is required for their survival, proliferation, and/or activation. It has also been reported that Fas-FasL interactions enhance activation through the Toll-like receptor pathway and promote chronic inflammation (Ma *et al.*, 2004). Fas was reported to promote proliferation of fibroblasts (Aggarwal *et al.*, 1995) and maturation of dendritic cells in culture (Rescigno *et al.*, 2000).

Fas is highly expressed in liver cells (hepatocytes). Liver cells also die through Fas-mediated apoptosis during viral hepatitis, liver cirrhosis, and Wilson's disease. However, Fas is involved in liver regeneration subsequent to partial hepatectomy (Desbarats and Newell, 2000). Indeed, injection of the normally hepatotoxic the anti-mouse Fas antibody into partially hepatectomized mice actually accelerates liver regeneration. Liver damage is associated with the activation of anti-apoptotic signaling pathways (Akt, STAT3, and NF-κB) (Diehl, 2000) that protect against Fas-mediated cell death and thus may help to switch Fas-mediated signals from primarily apoptotic to non-apoptotic.

Fas mediated non-apoptotic activities have been also described in other tissues such as central nervous system (CNS), colon, pancreas, and heart (Apostolou *et al.*, 2003; Badorff *et al.*, 2002; Tamm *et al.*, 2004; Zuliani *et al.*, 2006). Such non-apoptotic signaling activities of Fas are thought to mediate proinflammatory responses in immune cells (e.g. chemokine and cytokine production), neuronal tissue remodeling and promote tumor progression (Hao *et al.*, 2004; Ma *et al.*, 2004; Matsumoto *et al.*, 2007; O' Reilly *et al.*, 2009). Treatment of FasL-resistant MCF7 cells with anti-APO-1 antibody or soluble FasL (which does not induce Fas internalization) promotes tumor-cell motility and invasiveness (Barnhart *et al.*, 2004; O' Reilly *et al.*, 2009). These data provided the molecular basis for the assumption that FasL-induced non-apoptotic processes, including the MAPK and NF-κB signaling, play a role in the tumorigenesis of Fas-resistant tumors (Barnhart *et al.*, 2004; Lee *et al.*, 2006).

The physiological importance of Fas-induced activation signals is further underscored by recent studies on gene-targeted mice that express either only soluble FasL (sFasL) or only

membrane bound FasL (mFasL) (O' Reilly *et al.*, 2009). Mice lacking sFasL appeared normal and T cells from these mice showed normal apoptotic activity. In contrast, mice lacking mFasL showed defective apoptosis, and had phenotypic manifestations of lymphadenopathy, splenomegaly, skin lesions, and immune-mediated kidney injury analogous to lupus nephritis. These mice produced anti-DNA antibodies, showed increased nuclear factor NF-κB activation, had elevated levels of pro-inflammatory cytokines, and developed hepatic histiocytic sarcoma. The inflammatory manifestations in mice lacking mFasL were more severe than those in FasL deficient mice (*gld/gld*), which expressed a mutant form of FasL that could not bind Fas. These studies thus suggest that mFasL, but not sFasL, is critical for the activation-induced death of T cells, while sFasL contributes to inflammatory processes through non-apoptotic signaling pathways that are critical to autoimmunity and tumorigenesis (O' Reilly *et al.*, 2009). The execution and regulation of Fas-mediated non-apoptotic signaling, in particular with respect to its contribution to autoimmunity or tumorigenesis is, however, still largely unclear.

Recent reports have suggested that membrane dynamics, sub-cellular localization, and internalization process play critical roles for the balancing apoptotic and non-apoptotic pathways mediated by Fas. Fas ligand engagement induces spatial and conformational alterations in Fas receptor (Algeciras-Schimnich *et al.*, 2002). An additional requirement for the oligomerized Fas receptor to induce apoptosis is the internalization of Fas through a clathrin-mediated pathway and delivery of Fas containing vesicles to early endosomal compartments (Lee *et al.*, 2006). Recent studies have demonstrated that the endosomal compartments are major sites for the initiation of Fas mediated death signaling complex formation and apoptosis. The assembly of the death-inducing signaling complex (DISC) and caspase-activation predominantly occur at endosomal compartments after Fas receptor internalization (Lee *et al.*, 2006) (Figure 1.2). Consequently, blockade of Fas-receptor endocytosis impairs DISC formation and apoptosis. Furthermore, in cells with disabled Fas endocytosis, the receptor aggregates induce activation of pro-survival signals, such as Erk and NF-κB activation (Figure 1.2), demonstrating that compartmentalization of the Fas signal is critical to determine the cellular outcome after Fas stimulation (Lee *et al.*, 2006).

Hence, the subcellular localization and endocytic pathways of Fas play important roles in controlling the activation of distinct signaling cascades to determine either cellular survival or cell death. These findings are not only important for the understanding death receptor

biology, but also provide a mechanistic basis for the capacity of a given receptor to induce distinct cellular fates within an identical cellular context. These data also suggest a different paradigm for transmembrane signaling and emphasizes the role of the endocytic pathway for Fas receptor degradation as well as signal propagation.

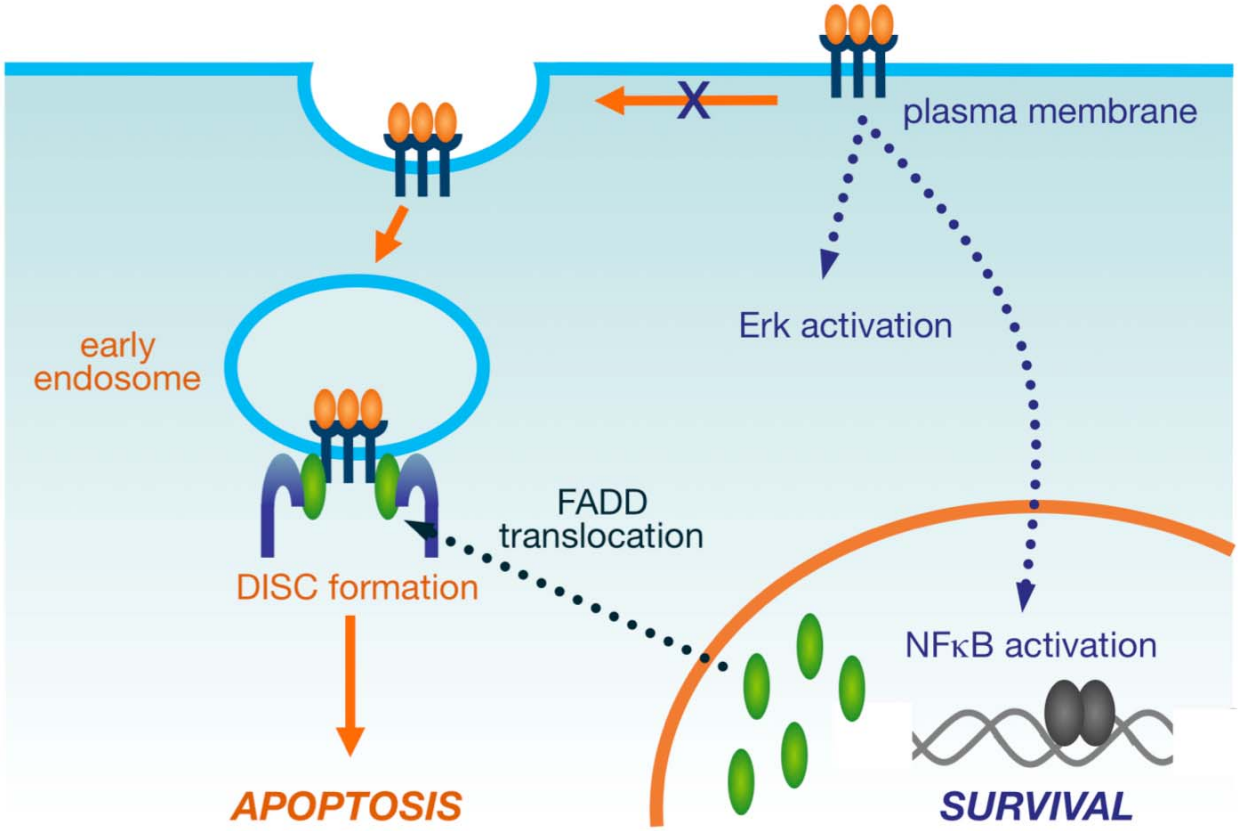


Figure 1.2: Model for Fas signaling.

FasL triggering induces the clustering of Fas at the plasma membrane. Subsequently, activated Fas receptors get internalized and delivered to endosomal compartments. The death adaptor FADD translocates from the nucleus to the cytoplasm, where it is recruited to Fas at early endosomes and promotes efficient formation of the death inducing signaling complex (DISC). The resulting activation of caspase-8 induces further activation of downstream death effectors, ultimately leading to cellular apoptosis. In contrast, FasL stimulation of cells unable to internalize Fas results in activation of anti-apoptotic signaling pathways, including the activation of Erk and NF-κB. Hence, Fas signaling can activate divergent biochemical pathways to promote distinct cellular fates (survival vs. apoptosis). (Adapted from Lee *et al.*, EMBO J. 2006)

1.3 Toso

Toso, also known as Faim3, is a transmembrane protein belonging to the immunoglobulin gene superfamily. Using retroviral cDNA library-based functional cloning, Toso was originally identified as a surface molecule with negative regulatory function on death receptor mediated apoptosis in immune cells (Hitoshi *et al.*, 1998). Sequence analysis predicted that the 390-amino acid type I integral membrane protein has a signal peptide and an extracellular region homologous to immunoglobulin (Ig) variable domains but with 2 additional cysteines. The cytoplasmic region of Toso contains a basic region, an acidic region, and a proline-rich region which serves as a potential docking site for SH3 domain-containing signaling proteins (Figure 1.3) (Hitoshi *et al.*, 1998; Pallasch *et al.*, 2008).

Microarray analysis (Abbas *et al.*, 2005) and RT-PCR (Hitoshi *et al.*, 1998) has demonstrated that Toso is exclusively expressed in cells of hematopoietic origin. The expression of Toso is restricted to lymphoid organs, where it is particularly highly expressed in T, B and NK cells (Hitoshi *et al.*, 1998).

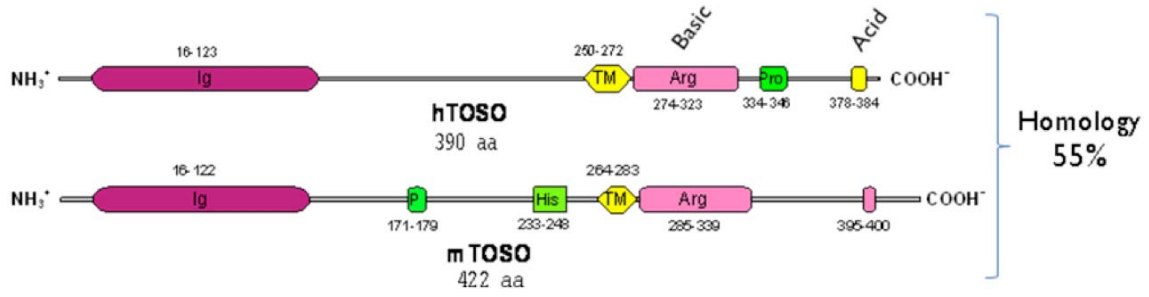


Figure 1.3: Computer analysis of mouse and human Toso.

Mouse and human protein sequences of Toso share 55% homology. The computer analysis indicates that Toso has a large extracellular domain with homology to immunoglobulin variable domains and a smaller intracellular domain which has a basic amino acid-rich region.

It has also been observed that human Toso gene expression is tightly regulated during T cell activation. Toso is highly expressed in naïve and memory T cells, but it is strongly down-regulated in activated T cells, as revealed by microarray analysis (Abbas *et al.*, 2005). Studies employing RT-PCR in an allogenic stimulation system have reported a transient increase in Toso gene expression during early stages of T cell activation, while Toso

expression was no longer detectable in later stages of T cell activation (Hitoshi *et al.*, 1998). It has been suggested that this transient up-regulation of Toso expression in newly activated T cells might be responsible for the temporary resistance of T cells to the death receptor mediated apoptosis during the early state of an immune response. Toso expression is also up-regulated by B-cell receptor (BCR) activation while CD40L triggering significantly reduces Toso expression (Pallasch *et al.*, 2008).

Functional analysis has shown that the overexpression of Toso in Jurkat leukemic T cells inhibits CD95 and TNFR induced apoptotic signaling (Hitoshi *et al.*, 1998). Consistently, overexpression of the mouse Toso in primary T cells results in resistance to Fas/FasL induced apoptosis (Song and Jacob, 2005). Mutational analysis indicates that the immunoglobulin domain and the transmembrane region of Toso, but not the cytoplasmic domain, are required to inhibit Fas-induced apoptosis and capase-8 activation (Hitoshi *et al.*, 1998). All previous reports suggest that Toso functions as a negative regulator of death receptor mediated apoptotic signaling, most likely acting at very proximal level in the apoptotic signaling cascade. The biological function of Toso is, however, elusive. No ligand for Toso has yet been identified and the exact molecular mechanism of how Toso blocks death receptor mediated apoptosis, is still unresolved. Controversial findings have been reported: one group has suggested that Toso inhibits capase-8 processing through the induction of the caspase inhibitor FLIP (Hitoshi *et al.*, 1998), while another research group reported that the cytoplasmic domain of Toso can directly bind to FADD, thereby blocking caspase-8 activation in response to Fas (Song and Jacob, 2005). Thus further in depth analysis is required to better define the mechanisms of Toso protein function. Moreover, an alternative concept should also be considered in which Toso may affect death receptor dynamics at the plasma membrane, such as receptor aggregation and subsequent internalization.

The human Toso gene has been mapped to 1q31 – q32 (Hitoshi *et al.*, 1998), a region which has frequent changes in hematopoietic malignancies and solid tumors. Studies in nude mice have demonstrated that duplication of the chromosome segment of this region is associated with proliferation and metastasis of human chronic lymphocytic leukemic B-cells (Ghose *et al.*, 1990). A serial analysis of gene expression (SAGE) and microarray analysis revealed that Toso was identified as over-expressed candidate gene in chronic lymphocytic leukemia (CLL) cells (Pallasch *et al.*, 2008; Proto-Siqueira *et al.*, 2008). In CLL cells, high levels of

TOSO expression have been correlated with aggressive disease, being associated with high leukocyte count, advanced Binet stage and also, the CD38(+) CLL subset with proliferative activity showed enhanced TOSO expression. B-cell receptor-stimulation was identified as a positive regulator of TOSO expression and the specific induction of TOSO via the BCR suggest autoreactive BCR signaling involving TOSO as a mediator of resistance to apoptosis in CLL (Pallasch *et al.*, 2008).

In T cells, the overexpression of Toso is also associated with autoimmune disease, as illustrated by gene chip analysis. Toso is down-regulated in fully activated T cells under normal stimulation conditions, while, in marked contrast, Toso is constitutively expressed in autoreactive T cells isolated from the inflamed joint area of systemic lupus erythematosus (SLE) patients. Moreover, Toso has been identified as a significantly up-regulated gene in $CD4^+$ T cells of SLE patients, associated with the disease severe active state (Deng *et al.*, 2006). In addition, overexpression of Toso has also been observed in peripheral blood mononuclear cells from acute multiple sclerosis (MS) patients (Achiron *et al.*, 2007). This remarkable up-regulation of Toso in autoreactive T cells under inflammatory conditions might contribute to the survival of activated T cells by rendering them resistant to apoptosis.
Whether Toso may activate non-apoptotic signal pathways through death receptors in autoreactive T cells or CLL cancer cells under particular pathological conditions is an interesting possibility that should also be investigated. Further analysis of the Toso pathway coupled with gene disruption analysis in mice will likely provide important clues about the overall role that Toso plays in regulating cellular apoptosis and survival in the immune system.

2. The goal of the study

Apoptosis plays fundamental roles in the development and the homeostasis of immune cells. The goal of the present study was to investigate how the surface molecule Toso participates in the regulation of apoptotic and survival signaling pathways in lymphocytes. The study should particularly focus on the mechanisms of Toso function in the regulation of Fas (CD95) mediated apoptotic and non-apoptotic signal transduction. To address these important biological questions, the study has followed a combined approach by applying biochemical, cell biological and genetic methods to dissect the molecular and regulatory mechanisms of Toso function in Fas mediated apoptosis. Employing gene knock-down or overexpression studies, the study should examine the anti-apoptotic function of Toso in response to Fas stimulation. The study should also identify and characterize down-stream effector molecules and regulatory mechanisms that are involved in the function of Toso. Furthermore, the potential capacity of Toso to activate survival signaling pathways in response to death receptor stimulation should also be analyzed.

Together, this study aimed at advancing our knowledge of the complex regulatory mechanisms of death receptor signaling that determines cellular apoptotic and survival fates in immune cells.

3. Materials and Methods

3.1 Materials

3.1.1 Laboratory equipments

PRODUCT	COMPANY
Automacs	Miltenyi Biotec, Germany
Balance (max. 5400 g)	Kern, Germany
Bench	Thermo scientific, Germany
Centricon Plus-20	Millipore, Ireland
Centrifuge 5415R	Eppendorf, Germany
Centrifuge 5810R	Eppendorf, Germany
Centrifuge Rotina 16 R	Hettich Zentrifugen, Germany
FACS Calibur flow cytometer	BD Biosciences, Germany
Fluorescence microscope (Diaphot 300)	Nikon, Japan
Gel Doc XR	Bio Rad, Germany
Glassware	Duran Group, Germany
Incubator	Memmert, Germany
Incubator Heracell 150	Thermo scientific, Germany
Light cycler capillaries	Roche, Germany
Light cycler centrifuge adapters	Roche, USA
Macs multi stand	Miltenyi Biotec, Germany
MACS separation columns	Miltenyi Biotec, Germany
Magnetic stirrer	IKA, Germany
Microplate shaker	Laborbedarf Hassa, Germany
Microscope	Olympus Optical, Japan
Microwave oven (1026L)	Privileg, Korea
Nucleofector	Amaxa, Germany
pH Meter	Baack Laborbedarf, Germany
Photometer (automatic)	Eppendorf, Germany
Pipettes	Eppendorf, Germany
Power supply	Consort, Belgium

Refrigerator	Liebherr profiline, Germany
Rotator driver STR4	Stuart scientific, U.K.
Shaker for bacterial cultures Inova 4230	Brunswick scientific, USA
Sorvall RC 5C Plus centrifuge	Thermo scientific, Germany
Thermocycler	Eppendorf, Germany
Thermomixer	Eppendorf, Germany
Vortex	Heidolph, Germany
Waterbath	Memmert, Germany
Water-purification system Milli-Q	Millipore, Eschwege
XCell II™ Blot Module	Invitrogen, U.K.
Xcell SureLock™ Mini-Cell	Invitrogen, U.K.

3.1.2 Chemicals and reagents

Reagents listed below were used either in purity grades pro analysis or in HPLC-grade.

PRODUCT	Company
Acetic Acid	Roth, Germany
Acrylamide/bis-acrylamide, 40% solution	Biorad, Germany
Agarose	Invitrogen, U.K.
Amersham Hyperfilm ECL	GE Healthcare, U.K.
Aminoacids	PAA Laboratories, Austria
Ammonium chlorid	Roth, Germany
Ammonium persulfate	Biorad, Germany
Ampicillin	Roth, Germany
AnnexinV binding buffer	BD Pharmingen, Germany
Aqua B. Braun H2O	Melsungen, Germany
Bacto Agar	BD Pharmingen, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Germany
Bromophenol blue	Sigma-Aldrich, Germany
Concanavalin A	GE Healthcare, Sweden
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Germany
Disodium hydrogen phosphate (Na2HPO4)	Merck, Germany
DMEM	PAA Laboratories, Austria

DNA molecular weight marker	Quiagen, Germany
dNTPs (10 mM)	Invitrogen, U.K.
EDTA 1 % (w/v) in PBS	Biochrom, Germany
Ethanol (C_2H_5OH)	Roth, Germany
Ethidium bromide	Biorad, Germany
Ethylene diamine tetraacetic acid (EDTA)	Roth, Germany
Expand high fidelity enzyme blend	Roche, Germany
FasL	Alexis Biochemical, Germany
Fetal calf serum (FCS)	Biochrom, Germany
Ficoll-plaque	GE Healthcare, Sweden
Gel blotting paper	Roth, Germany
Glycerol	Sigma-Aldrich, Germany
Glycine	Sigma-Aldrich, Germany
Hydrochloric acid (HCl)	Merck, Germany
Isopropanol (C_3H_8O)	Roth, Germany
Kanamycin	Roth, Germany
L-glutamine with penicillin/streptomycin	PAA Laboratories, Austria
L-glutamine, 200 mM	Biochrom, Germany
Loading dye (6-fold)	Biolabs, Germany
Lysine	Merck, Germany
Methanol	Roth, Germany
$MgCl_2$	Roth, Germany
NP-40	Thermo scientific, Germany
Nupage MOPS SDS running buffer	Invitrogen, U.K.
NUPAGE Transfer buffer	Invitrogen, U.K.
Paraformaldehyde	Sigma-Aldrich, Germany
PBS for cell culture (without Magnesium, without Calcium)	PAA Laboratories, Austria
Penicillin/streptomycin (10.000 U/10.000 µg/mL)	Biochrom, Germany
Potassium chloride (KCl)	Merck, Germany
Potassium phosphate (KH_2PO_4)	Merck, Germany
Protein A/G PLUS-Agarose	Santa Cruz Biotechnology, Germany
Restriction enzymes	Biolabs, UK
RPMI-1640	Gibco, Germany
Sigmafast protease inhibitor	Sigma-Aldrich, Germany

Sodium azide (NaN_3)	Sigma-Aldrich, Germany
Sodium carbonate (Na_2CO_3)	Roth, Germany
Sodium chloride (NaCl)	Roth, Germany
Sodium dodecyl sulfate (SDS)	Fluka, Germany
Sodium fluoride	Sigma-Aldrich, Germany
Sodium hydroxide (NaOH)	Roth, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Germany
Sodium pyruvat ($C_3H_3NaO_3$)	PAA Laboratories, Austria
Superblock T20 blocking buffer	Thermo scientific, Germany
T4 DNA ligase	Biolabs, UK
Taq DNA polymerase	Invitrogen, U.K.
Tris	Serva GmbH, Germany
Triton X-100	Sigma-Aldrich, Germany
Trypan blue (0.5% w/v in physiological saline)	Gibco, U.S.A.
Trypsin/EDTA, 0,05 %/0,02 % (w/v) in PBS	Gibco, U.S.A.
Trypton	Sigma-Aldrich, Germany
Tween 20	Sigma-Aldrich, Germany
TNFα	BASF Bioresearch, U.S.A.
Western blot detection reagent	Thermo scientific, Germany
Western blotting molecular weight markers	GE Healthcare, U.K.
Yeast extract	Roth, Germany
β-mercaptoethanol	Sigma-Aldrich, Germany

3.1.3 Solutions and media for bacterial culture

Specification	Composition	
LB medium (pH 7,4)	1 % (w/v) 1 % (w/v) 0,5 % (w/v)	NaCl Yeast extract Trypton in distilled H_2O
LB agar (pH 7,4)	1 % (w/v) 1 % (w/v) 0,5 % (w/v)	NaCl Yeast extract Trypton

		in distilled H_2O
Ampicillin stock solution	10 mg/mL	Ampicillin in distilled H_2O
Kanamycin stock solution	10 mg/mL	Kanamycin in distilled H_2O
Spectinomycin stock solution	10 mg/mL	Spectinomycin in distilled H_2O

3.1.4 Molecular and cell biology kits

Kit	Description	Company
Amaxa Nucleofector kit	Programs and cell-type-specific solutions lead to excellent transfection performance.	Amaxa, Germany
BLOCK-iT Lentiviral Pol II miR RNAi Expression System	A Gateway-adapted, lentiviral destination vector for expression of microRNA or cDNA in mammalian cells	Invitrogen, Germany
BLOCK-iT Pol II miR RNAi expression vector kit	Gateway-adapted expression vector for the RNAi analysis of a target gene in mammalian cells	Invitrogen, Germany
Dead cell removal kit	To remove dead cell	Miltenyi Biotec, Germany
FastLane cell cDNA kit	For high-speed preparation of cDNA without RNA purification for use in real-time RT-PCR	Qiagen, Germany
LightCycler TaqMan Master kit	Ready-to-use hot start reaction mix for PCR on the LightCycler Carousel-Based System using TaqMan Probes	Roche, Germany
pENTR/D-TOPO Cloning Kit	Cloning of blunt-end PCR products into an entry vector for the Gateway System	Invitrogen, Germany
QIAprep miniprep/maxiprep	Plasmid DNA extraction from bacteria	Qiagen, Germany
QIAquick gel extraction	Extraction of nucleotide fragments from agarose gels	Qiagen, Germany
QIAquick PCR purification	To cleanup DNA fragments from enzymatic reactions	Qiagen, Germany

QuickChange site-directed mutagenesis kit	To make point mutations, switch amino acids, delete or insert single or multiple amino acids in a vector construct sequence	Stratagene, Netherlands
RosetteSep human T cell enrichment cocktail	To enrich human T cells from whole blood.	Stemcell Technologies, Austria

3.1.5 Plasmid constructs

Plasmid name	Vector	Insert
miToso1-pcDNA6.2	pcDNA6.2-GW/EGFP-miR	mi-hToso1
miToso2-pcDNA6.2	pcDNA6.2-GW/EGFP-miR	mi-hToso2
miToso3-pcDNA6.2	pcDNA6.2-GW/EGFP-miR	mi-hToso3
miNegative-pcDNA6.2	pcDNA6.2-GW/ miR-neg	miRNA negavite
miToso1-pLenti6	pLenti6/V5-DEST	mi-hToso1
miToso2-pLenti6	pLenti6/V5-DEST	mi-hToso2
miToso3-pLenti6	pLenti6/V5-DEST	mi-hToso3
miNegative-pLenti6	pLenti6/V5-DEST	miRNA negavite
Toso-pLenti6	pLenti6/V5-DEST	human Toso
SM-Toso-pLenti6	pLenti6/V5-DEST	silent mutated-human Toso
Toso-pIRES2	pIRES2-EGFP	human Toso
Rip1-pIRES2	pIRES2-EGFP	human Rip1
Rip1-K377R-pIRES2	pIRES2-EGFP	human Rip1-K377R

3.1.6 MicroRNA (miRNA) sequences

Name	Targets	Sequences (5’ → 3’)
mi-hToso1	Human Toso	*Top strand:* TGCTGTTGTATTCTGCCTTGATGAAGGTTTTGGCCACTGACTGACCTTC AT CAGC AGAATACAA *Bottom strand:* CCTGTTGTATTCTGCTGATGAAGGTCAGTCAGTGGCCAAAACCTTCATC AAGGCAGAATACAAC

mi-hToso2	Human Toso	*Top strand:* TGCTGTATGCAGGCATCTGGAACAAAGTTTTGGCCACTGACTGACTTT GTTCCATGCCTGCATA *Bottom strand:* CCTGTATGCAGGCATGGAACAAAGTCAGTCAGTGGCCAAAACTTTGTT CCAGATGCCTGCATAC
mi-hToso2	Human Toso	*Top strand:* TGCTGTGAAGATGCTCTGGACACTCGGTTTTGGCCACTGACTGACCGA GTGTCGAGCATCTTCA *Bottom strand:* CCTGTGAAGATGCTCGACACTCGGTCAGTCAGTGGCCAAAACCGAGTG TCCAGAGCATCTTCAC

3.1.7 PCR primers

Primers for PCR and sequencing were purchased from Metabion (Martinsried, Germany) at stock concentration of 100 μM. Following primer pairs were used:

Name	Specificity	Sequence (5' → 3')
CMV-F	CMV promoter	CGCAAATGGGCGGTAGGCGTG
hTOSO-ExGW-F	Toso	CACCATGGACTTCTGGCTTTGGCCACTTTACTTCCTGCC
hTOSO-ExGW-R	Toso	CTTGTCATCGTCGTCCTTGTAGTCGCCTCCGGCAGGAACA TTGATGTAGTCATCTGAATC
miRNA-LoopF	miRNA loop region	GTTTTGGCCACTGACTGAC
hTOSO-IRES-F	Toso	CACCTAGCTAGCGCCGCCACCATGGACTTCTGGCTTTGGC CACTTTACTTCC
hTOSO- IRES -R	Toso	CTCGAGGAATTCTCACTTGTCATCGTCGTCCTTGTAGTCG CCTCCGGCAGGAACATTGATGTAGTCATCTGAATC
Rip1-PIRES-F	Rip1	CACCTAGCTAGCGCCGCCACCATGGACTACAAGGACGAC

		GATGACAAGCAACCAGACATGTCCTTG
Rip1-PIRES-R	Rip1	CTCGAGGAATTCTTAGTTCTGGCTGACGTAAATCAAGCTGCT
SM-hTOSO1-F	Toso	CGTGGTATCCACCACCAACTTTATTAAAGCTGAGTATAAGGGCCGAGTTACTCTGAAGC
SM-hTOSO1-R	Toso	GCTTCAGAGTAACTCGGCCCTTATACTCAGCTTTAATAAAGTTGGTGGTGGATACCACG

3.1.8 Cell lines

Cell line	Description	Source
BJAB	Burkitt's lymphoma cell line	Immunobiology lab, FZB*
Jurkat	T cell leukemia established from the peripheral blood	ATCC
HEK 293WT	Human embryonic kidney cell line	ATCC
HEK 293FT	HEK 293F- derived cell line, stably expresses the SV40 large T antigen which is controlled by CMV promoter.	Invitrogen

*FZB: Research Center Borstel, Leibniz Center for Medicine and Biosciences, Germany

3.1.9 Antibodies

Primary antibody

Specification	species/isotype	source/reference
anti-CD4-Alexa Fluor 488	mouse monoclonal IgG1	ImmunoTools
anti-CD8-Alexa Fluor 488	mouse monoclonal IgG1	BD Pharmingen
anti-CD3-FITC	mouse monoclonal IgG1	ImmunoTools
anti-CD95-FITC	hamster monoclonal IgG2	BD Pharmingen

anti-CD3-APC	mouse monoclonal IgG1	ImmunoTools
anti-CD95-APC (DX2)	mouse monoclonal IgG1	eBioscience
anti-CD8-Alexa Fluor 488	mouse monoclonal IgG1	BD Pharmingen
anti-CD95 (C-20)	rabbit polyclonal IgG	Santa Cruz
anti-CD3	mouse monoclonal IgG1	BD Pharmingen
anti-CD28	mouse monoclonal IgG1	BD Pharmingen
anti-RIP1 (H207)	rabbit polyclonal IgG	Santa Cruz
anti-FADD (H-181)	rabbit polyclonal IgG	Santa Cruz
anti-FLAG (M2)	mouse monoclonal IgG1	Sigma-Aldrich
anti-Toso (6B10)	rat monoclonal IgG1	Pallasch et al., 2009
anti-Toso	polyclonal rat serum	Molecular immunology, FZB*
anti-Toso (A36)	rat monoclonal IgG1	Molecular immunology, FZB*
anti-Toso (A36)	rat monoclonal IgG1	Molecular immunology, FZB*
anti-CD69-FITC	mouse monoclonal IgG1	DAKO
anti-β actin (AC-15)	mouse monoclonal IgG1	Sigma-Aldrich
anti-RIP1 (D94C12)	rabbit monoclonal IgG	Cell Signaling
anti-CD95	mouse monoclonal IgG1	Santa Cruz
anti-pErk	rabbit polyclonal IgG	Cell Signaling

*FZB: Research Center Borstel, Leibniz Center for Medicine and Biosciences, Germany

Secondary reagents

Reagents	Company
Goat anti-mouse IgG, Cy5-conjugated	Caltag
Donkey anti-rat IgG, Cy5-conjugated	Jackson Immuno Research
Goat anti-mouse IgG, horseradish peroxidase-conjugated	Jackson Immuno Research
Goat anti-rat IgG (light chain), horseradish peroxidase-conjugated	Jackson Immuno Research
Streptavidin, Cy5-conjugated	Caltac
Streptavidin, FITC-conjugated	BD Pharmingen
Streptavidin, horseradish peroxidase-conjugated	Jackson Immuno Research
Donkey anti-rabbit IgG, horseradish peroxidase-conjugated	GR Healthcare

3.1.10 Laboratory supplies

PRODUCT	COMPANY
Anopore membrane (0.02 µm, 10 µm)	Nunc, Dennmark
Anopore membrane (0.2 µm, 10 µm)	Nunc, Dennmark
Gel casting tray	Bio-Rad Laboratories, Germany
Microcentrifuge tubes (1.5 ml, 2 ml)	Sarstedt, Germany
Microscope cover slips (24 x 40 mm)	Gerhard Menzel Glasbearbeitungswerk, Germany
Microscope slides (76 x 26 mm)	Waldemar Knittel, Germany
Multidish with 24 wells	Nunc, Dennmark
Multidish with 48 wells	Nunc, Dennmark
Multidish with 96 wells	Nunc, Dennmark
Needles (23 G, 27 G)	Becton Dickinson S.A., Spain
Neubauer counting chamber	Paul Marienfeld, Germany
Petri dish (100 x 15 mm, sterile)	Becton Dickinson Labware, U.S.A.
Plastic pipette tips	Sarstedt, Germany
Plastic pipettes (5 ml, 10 ml, 25 ml)	Greiner bio-one, Germany
Plastic tubes (15 ml, 50 ml)	Sarstedt, Germany
Syringe (1 ml, 5 ml, 10 ml)	Becton Dickinson S.A., Spain
Tissue culture flask	Greiner bio-one, Germany
Tubes for FACS (5 ml, 75 x 12 mm)	Sarstedt, Germany

3.1.11 Software

Name	Company
Cell Quest	Becton Dickinson, Immunocytometry Systems, U.S.A.
Microsoft Office 2007	Microsoft Cooperation, U.S.A.
FlowJo	Tree Star, Inc., U.S.A.
LightCycler Software version 3	Roche, Germany

3.2 Methods

3.2.1 Cell culture techniques

3.2.1.1 Cultivation of mammalian cells

All cell lines were kept in an incubator at 37°C, 95% humidity, and an atmosphere of 5% CO_2.

BJAB and Jurkat cells were cultured in a RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 50 mM β-Mercaptoethanol, penicillin/streptomycin (50 µg/ml of each), and 2 mM glutamine. Cells were seeded at a density of 0.2 x 10^6 cells/ml and passaged every 2-3 days.

HEK293FT cells were cultured in DMEM medium supplemented with 10% FCS, 1% P/S, 500 µg/ml Geneticin, 0.1 mM MEM Non-Essential Amino Acids, 1 mM Sodium Pyruvate, and 6 mM L-glutamine. Every 2-3 days, cells were sub-cultured. To detach the cells, all medium from the flask were removed and the cells were washed once with 10 ml of PBS. Then, 2 ml of trypsin/versene (EDTA) solution was added to the monolayer and incubated for 2 minutes at room temperature (RT) until cells detach. The detachment of cells was confirmed by a microscope. After most of the cells have detached, 8 ml of complete medium containing Geneticin were added and the cell suspension was transferred into a 15 ml sterile, conical tube to determine viable, total cell counts (3.2.1.2). Finally, cells were seeded at a density of of 1x10^5 cells/ml.

Right after isolation, primary human T cells were cultured in the RPMI 1640 complete medium at the concentration of 2.5 x 10^6 cells/ml in 6-well plate that was pre-coated with 1 µg/ml of anti-CD3 antibody and 5 µg/ml of anti-CD28 antibody. In 7-day cultures, 2 ng/ml of recombinant human IL-2 was added to the cultures after 48 hours of priming, and restimulated every week with 1 µg/ml of anti-CD3 antibody and 5 µg/ml of anti-CD28 antibody.

3.2.1.2 Cell counting

Counting of cells was performed with exclusion of dead cells by trypan blue. Dead cells appear blue due to the permeation of trypan blue into the membrane of only dead cells.

Working solution of trypan blue was prepared by diluting trypan blue 1:10 in 0.9% NaCl. The cell suspension was diluted 1:10 in the working solution of trypan blue and cells were counted on a hemacytometer counting chamber under an optical microscope.

3.2.1.3 Thawing and freezing of cell lines

For preservation of cell lines in liquid nitrogen, 5 x10^6 cells were washed once with PBS and collected by centrifugation at 1000 *g* for 5 minutes at room temperature (RT). Thereafter, the cell pellet was resuspended in 1 ml of freezing medium (10% DMSO in FCS), transferred directly into a cryovial, and the following freezing steps were performed: 30 minutes on ice, 16 hours in a freezing device, which had been filled with isopropanol, 2 days in -80°C freezer, and finally in a liquid nitrogen tank for long term storage.

For thawing cells, cryovial was removed from the liquid nitrogen tank and incubated immediately in a 37°C water bath by slow agitation for 30 seconds. The cells were then resuspended in 30 ml of the appropriate cold growth medium and centrifuged at 1000 *g* for 5 minutes at RT. Finally, the cell pellet was seeded on a fresh tissue culture flask containing pre-warmed growth medium.

3.2.1.4 Human peripheral blood T cells isolation

Human peripheral blood was collected by venipuncture from healthy adult volunteers. Buffy coat was prepared by spin a whole blood sample at 1000 *g* for 10 minutes at RT with the brake off, and then the concentrated leukocyte band (buffy coat) was removed and adjusted volume to 5 x 10^7 cells/ml. The T cells were isolated by using RosetteSep® kit (StemCell Technologies, Germany). Briefly, 50 µl of RosetteSep cocktail was added into every 1 ml of buffy coat suspension, gently mixed, and incubated for 20 minutes at RT. Thereafter, the sample was diluted with 2 times the volume of PBS + 2% FCS and mixed gently. 3 ml of diluted sample was layered on top of 1.5 ml Ficoll solution (specific density 1.077 g/ml, Biochrom) and separated by centrifugation at 1000 *g* for 30 minutes at RT, with the break off. The interphase containing enriched cells was carefully transferred into a fresh tube and washed twice with 50 ml of PBS + 2% FCS. The cells were then resuspended in the RPMI 1640 complete medium at the concentration of 2.5 x 10^6 cells/ml and cultured in pre-coated

anti-CD3/anti-CD28 6-well plate (3.2.1.1). The purity of the T-cell fraction (usually > 90%) was determined by FACS analysis with anti-CD3 antibody (3.2.4.4).

3.2.1.5 Stimulation of mammalian cells

Cells were washed once with growth medium and stimulated by appropriate grow medium supplemented with corresponding stimuli. The duration of stimulation as well as final concentration of stimuli are indicated in the Results section. The various stimuli used in this study are listed in the Materials section.

3.2.1.6 Transfection of mammalian cells

Amaxa nucleofection

For nucleofection of the cells, Amaxa Nucleofector Device (Amaxa GmbH) and Nucleofector Solutions (Amaxa GmbH) were used. The transfection was performed according to the manufacturer's protocol. Briefly, 4 x 10^6 cells were pelleted by centrifugation at 1000 *g* for 5 minutes at RT, and the supernatant was completely discarded. Cell pellet was re-suspended in 100 µl of nucleofection solution containing 10 µg of corresponding DNA plasmid(s) (used DNA plasmid(s) are indicated in Results section). After the pulse using suitable nucleofection program (table 3.1), 500 µl of the cell culture medium was immediately added to neutralize the nucleofection solution. Cells were seeded in 3 ml of the pre-warmed culture medium on a 6-well plate, following by incubated in a humidified 37°C/5% CO_2 incubator.

Table 3.1: The nucleofection programs and solutions.

Cell lines	Cell amount	Program	Solution	DNA plasmid
BJAB	4 x 10^6	C-009	T	10 µg
Jurkat	4 x 10^6	X-001	V	10 µg
Human primary T cells	4 x 10^6	T-023	Human T cell	10 µg

Transfection by lipofectamine

DNA-Lipofectamine™ 2000 complexes were prepared as follows for the transfection of pIRES2-Toso construct into HEK cells: 9 µg pIRES2-Toso DNA plasmids were diluted and gently mixed in 0.6 ml of Opti-MEM® I medium without serum in a sterile 1 ml eppendorf tube. In parallel, 18 µl of Lipofectamine™ 2000 was diluted in a separate 1 ml tube in 0.6 ml of Opti-MEM® I medium without serum, gently mixed, and incubated for 5 minutes at RT. Thereafter, the diluted DNA and the diluted Lipofectamine™ 2000 were combined, mixed gently, and incubated for 20 minutes at RT. Meanwhile, HEK WT cells were trypsinized, pelleted, and resuspended at a density of 1.2 x 10^6 cells/ml in Opti-MEM® I medium containing serum, yet without antibiotics. The DNA-Lipofectamine™ 2000 complexes were added into a 6-well plate. Next, 1.8 ml of the HEK WT cell suspension was added into the plate, mixed gently, and incubated overnight in the humidified 37°C/5% CO_2 incubator. The next day, the transfection medium was replaced by the normal HEK WT growth medium.

3.2.1.7 Death cell removal

To remove death cells, Death Cell Removal Kit (Miltenyi-Biotec) was used. Briefly, up to 10^7 cells were pelleted by centrifugation at 1000 *g* for 5 minutes at RT, and the supernatant was completely discarded. Cell pellet was resuspended in 100 µl of Dead Cell Removal MicroBeads and incubated for 30 minutes at RT. Magnetic separation was carried out with a mini-MACS® Separator (Miltenyi-Biotec) using a positive selection MS column (Miltenyi-Biotec). At the end of the separation, viable cells were resuspended in cell culture medium.

3.2.2 Molecular biology

3.2.2.1 RNA isolation and cDNA synthesis

RNA isolation

RNA was isolated from cells using PureLink™ RNA Mini Kit (Invitrogen). Briefly, 1×10^6 cells were pelleted by centrifugation at 1000 *g* for 5 minutes at RT, re-suspended in 300 µl of Lysis Buffer prepared with 2-mercaptoethanol, and vortexed until the cell pellet is completely

dispersed. The lysate was then passed 10 times through an 18–21-gauge needle attached to a RNase-free syringe. 300 µl of 70% ethanol was added to cell homogenate, mixed thoroughly, transferred into the Spin Cartridge, and centrifuged at 10000 *g* for 15 seconds at RT. The flow-through was discarded, and the Spin Cartridge was washed several times by using Wash Buffer I, Wash Buffer II. At the last step, RNA was eluted by 30 µl of RNase–Free Water. RNA quality and quantity were determined by UV absorbance at 260 nm (3.2.2.2).

cDNA synthesis

Complementary DNA (cDNA) was synthesized from purified RNA by using Superscript™ III first-strand synthesis kit (Invitrogen). Briefly, 50 ng random primers, 1 µl annealing buffer, and 5 µg RNA were added to RNase/DNase-free water to get the total volume of 8 µl. The reaction was incubated for 5 minutes at 65°C and then immediately placed on ice for 3 minutes. Contents were collected by briefly centrifugation and cDNA synthesis was performed by addition of 10 µl of 2X first-strand reaction mix and 2 µl of a Superscript™ III/RNaseOUT™ enzyme mix. The reaction was incubated for 10 minutes at 25°C, followed by 50 minutes at 50°C, and terminated at 85°C for 5 minutes, and then immediately chills on ice to proceed directly to PCR or real-time PCR, or store at -20°C.

cDNA synthesis by using FastLane cDNA kit

For quantitative real-time PCR analysis, cDNA was prepared from 0.5 x 10^6 cultured cells by using the FastLane Cell cDNA kit (QIAGEN) following the manufacturer's instructions.

3.2.2.2 Determination of nucleic acid concentration

A dilution of 1/79 in Braun H_2O was performed on all nucleic acid samples. Diluted samples were placed in a silica cuvette (Hellma). Absorbance was then read against a Braun H_2O blank with a BioPhotometer (Eppendorf, Germany) at 260 nm and 280 nm.

The following equations were applied to calculate RNA and DNA concentration in the samples:

$$[\text{RNA in } \mu g/mL] = \lambda_{260nm} \text{ x dilution factor x } 40$$

$$[\text{DNA in } \mu g/mL] = \lambda_{260nm} \text{ x dilution factor x } 50$$

A ratio of $\lambda_{260nm}/\lambda_{260nm}$ between 1.8 and 2.0 indicates a satisfactory purity of the extracted DNA or RNA.

3.2.2.3 Polymerase chain reaction (PCR)

Standard PCR

The sequences of the primers used for PCR are listed in 3.1.7. PCR was performed by using 1 U of *Taq* DNA polymerase in a PCR-reaction mixture of 50 µl. Samples were amplified in a DNA Thermocycler for 30 cycles. Each cycle consisted of denaturation at 95°C for 30 seconds, annealing for 30 seconds, and elongation at 72°C, proceeded by initial denaturation at 95°C for 5 minutes and followed by a final extension step at 72°C for 5 minutes. Annealing temperature for each primer was experimentally determined by running the same reaction at different annealing temperatures using a gradient thermocycler. Annealing time was based on the size of the amplified fragments. For cloning, sequences were amplified up to 30 cycles using *P*wo polymerase that has a proofreading activity. A mock PCR (without template) was included to exclude contamination in all experiments.

Colony PCR

A sterile toothpick was dabbed onto a bacterial colony then directly soaked into PCR-reaction mix of 25 µl. The initial denaturing step of PCR program was at 95^{o}C for 10 minutes, followed by 30 cycles of denaturation at 95° C for 30 seconds, annealing for 30 seconds, and elongation at 72° C for 1 minute, and the final extension step was performed at 72°C for 5 minutes. Annealing temperature for each primer pair was experimentally determined by running the same reaction at different annealing temperatures using a gradient thermocycler.

Quantitative real-time PCR

For quantitative PCR, optimal sets of primers were designed for each studied gene by using the "ProbeFinder" (Roche Applied Bioscience) website interface. Reaction was made with Real-time PCR Master mix (Applied Biosystems), 0.1 µM of a labeled probe, 1 pmole of each primer, and template (1 µl) in 10 µl reaction volume. The reference gene used for all experiments was HPRT. Quantitative, real time PCR (qPCR) analysis was performed on an LightCycler 1.5 (Roche, Germany) by using the standard cycling conditions as follows: one

95°C for 10 minutes, and 45 cycles of 95°C for 10 seconds denaturation, 55°C for 30 seconds annealing, and 72°C for 5 seconds extension. All reactions were performed triplicate. The threshold cycle number for product detection (ΔC_T value) was used to calculate the relative expression levels using the $\Delta\Delta C_T$ method.

3.2.2.4 Agarose gel electrophoresis of DNA

Agarose gel (1% w/v) was prepared by heat-dissolving agarose in Tris/acetate acid/EDTA (TAE) using a microwave oven. Melted agarose was allowed to cool to approximately 55°C before a volume of 40 ml was poured into a beaker. To this volume, 1 µl of 10 mg/ml ethidium bromide was added, gently swirled, and poured into a gel casting tray fitted with a 12-well comb. The polymerized gel was placed in an electrophoresis tank filled with the running buffer. Samples were loaded at volumes of 24 µl which contained 20 µl PCR product plus 4 µl of 6-fold loading dye along with 0.1 µg of molecular weight ladder. Gel electrophoresis was carried out in TAE buffer at 100 V for 50 minutes. The gel was finally visualized by using UV light and photographed (Gel Doc XR device and software, Bio-Rad).

3.2.2.5 Restriction digest of DNA

In the course of a cloning procedure using conventional ligation step, DNA plasmid and PCR-amplified DNA fragments were digested with appropriate enzymes (NEB Biolabs). Three units of each restriction enzyme were added into 50 µl of digestion reaction containing 5 µg of DNA plasmid or 1 µg of PCR-amplified DNA fragments. The restriction digest was performed in a suitable restriction enzyme buffer for overnight at 37°C and the digested fragments were visualized by agarose gel electrophoresis (3.2.2.4) and purified from a gel (3.2.2.6).

Prior to sequencing, the identity of DNA was confirmed by an analytical restriction digest. Usually, 1-3 units of each restriction endonuclease were used per 10 µl batch volume and ≈500 ng of DNA. The restriction digest was performed in a suitable restriction enzyme buffer for overnight at 37°C. Following this, the presence and size of DNA fragments were analyzed by agarose gel electrophoresis (3.2.2.4).

3.2.2.6 Purification of DNA

Isolation of DNA from agarose gel

After electrophoresis (3.2.2.4), DNA fragments of the right size were excised from the gel by using a clean, sharp, razor blade and transferred into a sterile Eppendorf tube. Subsequently, the excised DNA fragments were extracted using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. At the last step, DNA was eluted from the column with 30 µl of dH_2O, and directly used or stored at -20°C.

Isolation of DNA from solution

After PCR or restriction enzyme digestion, DNA fragments were purified using a QIAquick PCR purification Kit (Qiagen) following the manufacturer's instructions. At the last step, DNA was eluted from column with 30 µl dH_2O and directly used or stored at -20°C.

3.2.2.7 Cloning

3.2.2.7.1 Cloning using ligation reaction

In the course of a cloning procedure, the vector and insert, which had been digested to obtain compatible ends, were ligated by using T4-DNA ligase (NEB Biolabs). Generally, 50 ng of vector DNA with 1:3 molar ratio of insert was ligated. Both insert and vector were mixed and the resulting volume was filled up with water to 10 µl. Afterwards, 2 µl of 10x ligase buffer, 7 µl of H_2O, and 1 µl of T4 DNA ligase were added to obtain a total ligation volume of 20 µl. Ligation reaction was performed for 1 hour at RT and 2 µl of the ligation mixture was subsequently transformed into appropriate competent bacterial cells (3.2.2.10).

3.2.2.7.2 Gateway cloning

BP Cloning Reaction

Gel purified amplification product was mixed with 150 ng of pDONR™201 vector (Invitrogen), BP reaction buffer and 2 µl of BP Clonase™ enzyme mix (Invitrogen) in a final volume of 10 µl. The reaction was incubated for 1 hour at 25°C. 2 µl of proteinase K solution was then added, and the mixture was incubated at 37°C for 10 minutes.

LR Cloning Reaction

Entry clone plasmid preparation (200 ng) was mixed with 300 ng of destination vector, LR reaction buffer and 4 µl of LR Clonase™ enzyme mix (Invitrogen) in a final volume of 20 µl. The reaction was then incubated 1 hour at 25°C. Finally, 2 µl of proteinase K solution were added and the mixture was incubated at 37°C for 10 minutes.

3.2.2.7.3 Cloning of the miRNA expression vectors

An expression vector for the miRNA was cloned according to the "Block-iT^{TM} Pol II miRNAi Expression Vector Kits". Initially, the single-stranded DNA (ssDNA) oligos (top strand and bottom strand) were reconstituted with TE buffer to a final concentration of 200 µM. Thereafter, the bottom and top oligo were annealed for each miRNA targeting position by the following annealing reaction (table 3.2) at RT:

Table 3.2: The components required to anneal the ssDNA oligos.

Reagent	Amount
Top strand DNA oligo (200 µM)	5 µl
Bottom strand DNA oligo (200 µM)	5 µl
10X Oligo Annealing Buffer	2 µl
DNase/RNase-Free Water	8 µl
Total volume	20 µl

This mixture was then incubated at 95°C for 4 minutes. The single-stranded oligos were allowed to anneal by cooling down the reaction mixture to RT for 5 - 10 minutes. This 50 µM stock solution of double-stranded DNA (dsDNA) oligos was then diluted via serial dilutions to 10 nM solution with water and 10X oligo annealing buffer. Thereafter, the dsDNA oligos

were ligated into the pCDNA6.2-GW/EmGFP vector by following ligation reaction (table 3.3) at RT.

Table 3.3: The components required for ligation of the dsDNA into vector.

Reagent	Amount
5X Ligation Buffer	4 µl
pcDNA™6.2-GW/EmGFP-miR, linearized (5 ng/µl)	2 µl
miR-ds oligo (10 nM)	4 µl
DNase/RNase-Free Water	9 µl
T4 DNA Ligase (1 U/µL)	1 µl
Total volume	20 µl

This mixture was mixed well by pipetting up and down, incubated for 5 minutes at RT and transformed into OneShot TOP10 competent *E. coli* (3.2.2.9). After incubated at 37^{o}C for 1 hour, the bacteria were plated on spectinomycin (50 µg/ml) selective agar plates. The next day, single bacterial colonies were screened by PCR with mi-LoopF (targeting to the miRNA loop sequence) and M13-R primers (primer sequences are listed in 3.1.7). Thereafter, positive colonies were transferred into LB medium (containing 50 µg/ml of spectinomycin), grown overnight, and subjected to plasmid-preparation (3.2.2.8). Plasmids were analyzed via sequencing (3.2.2.9) using EmGFP primer (primer sequence is listed in 3.1.7).

3.2.2.8 Preparation of plasmid DNA

Small-scale plasmid isolation

A single colony was used to inoculate 5 ml of LB broth supplemented with appropriate antibiotic for plasmid selection. Bacterial culture was grown overnight at 37^{o}C with constant shaking at 200 rpm. The plasmid DNA was isolated from the overnight culture using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

Large-scale plasmid isolation

A single colony was used to inoculate 5 ml of LB broth supplemented with appropriate antibiotic for plasmid selection, at 37°C for 8 hours. This pre-culture was subsequently used to inoculate 250 ml of LB broth containing the respective antibiotic. Bacterial culture was grown overnight at 37°C with constant shaking at 200 rpm, and then cells were pelleted by centrifugation at 6000 *g* for 5 minutes at RT. Plasmid purification was subsequently carried out by using the EndoFree Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in dH_2O and adjusted to the concentration of 1-3 µg/ml.

3.2.2.9 Transformation of competent bacteria with plasmid DNA

For the transformation of plasmid DNA into bacteria, 2 µl of ligation reaction or 10 ng of purified plasmid DNA was added onto appropriate competent *E. coli* previously thawed on ice. After gentle mix, the bacterial suspension was incubated for 30 minutes on ice. Then, the bacteria were submitted to a heat shock at 42°C for 45 seconds and subsequently chilled on ice for 2 minutes. Thereafter, 200 µl of pre-warmed SOC medium was added and the bacteria were incubated at 37°C for 60 minutes with rotation. 100 µl of transformed bacteria was streaked on suitable LB-antibiotic agar plate and incubated overnight at 37°C.

3.2.2.10 Site directed mutagenesis

The high fidelity, PCR based QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene) was used to introduce the mutations into expression vector Toso-pLenti5/V5-DEST. The mutagenic primers (listed in 3.1.7) were designed to substitute C (201) to T, C (204) to T, G (207) to A, A (210) to T, A (213) to G, and C (216) to T. The composition of PCR mutagenesis reaction was: 5 µl of 10X *Pfu* Turbo polymerase buffer (Stratagene); 125 nM of dNTPs; 125 ng of each primer; 30 ng of pLenti6-Toso plasmid; H_2O to 49 µl; and 1 µl (2.5 U) of *PfuUltra* high-fidelity polymerase. Sample was amplified in a DNA Thermocycler for 18 cycles. Each cycle consisted of denaturation at 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 10 minutes, proceeded by initial denaturation at 95°C for 1 minute. After the PCR cycle, the reaction mixture was subjected to DpnI digestion by incubation with 2 µl of DpnI enzyme for 30 minutes at RT to selectively digest the methylated parental

supercoiled plasmids. Thereafter, 2 µl of digested reaction was transformed into XL1-Blue competent *E. coli* cells (3.2.2.9) with a selection for resistance to ampicillin (100 µg/ml), and successful mutagenesis was confirmed by sequencing using CMV-F primer (listed in 3.1.7).

3.2.3 RNAi design and lentiviral methods

3.2.3.1 Design of miRNAs targeting human Toso

For RNAi-mediated knock-down of Toso in BJAB and Jurkat cells, the online program, "BLOCK-iT™ RNAi Designer, from Invitrogen website was used to design three different miRNAs targeting three different regions of the Toso mRNA (accession number: AF057557). The sequences of the miRNAs are listed in 3.1.6.

3.2.3.2 Production of lentivirus in HEK293FT cells

To knock-down or overexpress Toso using a Lentiviral transfection system, four different lentiviruses were produced according to the "Block-iT™ Lentiviral Pol II miRNAi Expression System" (Invitrogen). For the miRNA expression system, the lentiviral expression vector pLenti6.2-GW/EmGFP containing Toso specific miRNA or control miRNA, which does not target any known vertebrate gene, was used. For the cDNA overexpression system, lentiviruses originating from the pLenti6/V5-DEST plasmid containing the Toso cDNA or the LacZ cDNA were generated. DNA-Lipofectamine™ 2000 complexes were prepared for all four transfection samples individually: 9 µg of ViraPower™ Packaging Mix and 3 µg of pLenti6/V5 expression construct were diluted in 1.5 ml of Opti-MEM® I medium without serum in a sterile 5-ml tube and mixed gently. In parallel, 36 µl of Lipofectamine™ 2000 was diluted in a separate 5 ml tube in 1.5 ml of Opti-MEM® I medium without serum, gently mixed, and incubated for 5 minutes at RT. After the 5-minute incubation, the diluted DNA and the diluted Lipofectamine™ 2000 were combined, mixed gently, and incubated for 20 minutes at RT. Meanwhile, HEK293FT cells were trypsinized, pelleted, and resuspended at a density of 1.2 x 10^6 cells/ml in Opti-MEM® I medium containing serum, yet without antibiotics. The DNA-Lipofectamine™ 2000 complexes were added into a T-75 tissue culture flask containing 5 ml of the growth medium containing serum without antibiotics. Next, 5 ml of the HEK293FT cell suspension were added into the flask, mixed gently, and

incubated overnight at 37°C in a CO_2 incubator. On the next day, the transfection medium was replaced by the normal growth medium containing 1 mM sodium pyruvate. The virus-containing supernatant was harvested 72-hour post-transfection by transferring the supernatant into a 15 mL falcon tube and pelleting the cell debris by centrifugation at 2,500 *g* for 5 minutes at 4°C. The supernatant was filtered through a 0.45 µm filter (Nalgene, NY, USA), aliquoted, and concentrated as described below (3.2.3.3).

3.2.3.3 Concentration of lentivirus via ultrafiltratoin

Lentivirus containing supernatant from HEK293FT cells was concentrated via ultrafiltration. Initially, a Centricon Plus-20 centrifugal filter device (Millipore, Ireland) was washed once with 20 ml of 70% EtOH following 2 times with 20 ml of sterile PBS. Up to 20 ml of 0.45 µm filtered lentiviral supernatant was transferred into a sample filter cup of the Centricon Plus-20 centrifugal filter device, following by centrifugation at 3500 *g* at 4^{o}C in a swinging bucket rotor until all supernatant has passed into the filtrate collection cup. The filter device was removed from the centrifuge and carefully separated sample filter cup from filtrate collection cup. Thereafter, the retentate cup was inserted into the sample filter cup, inverted and centrifuged at 1000 *g* for 2 minutes at 4^{o}C. The sample filter cup was carefully removed from retentate cup and concentrated sample was collected and measured the volume to calculate the concentrated ratio. Aliquots of concentrated sample were prepared in cryotubes and immediately used or stored at -80°C.

3.2.3.4 Lentiviral transfection of BJAB and Jurkat cells

BJAB and Jurkat cells were transfected with lentivirus for generating stable Toso knock-down or stable Toso overexpression cells. 10^6 cells were washed and suspended in 1 ml of the normal RPMI culture medium. This cell suspension was transferred into a 12-well plate and added with 0.5 ml of concentrated virus stock and 5 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 complete medium (final volume of 2 ml). To obtain better interactions of viral particles and cells, the plate was centrifuged without brake at 1500 *g* for 2 hours, and subsequently incubated overnight at 37°C in a CO_2 incubator. Next day, medium was replaced and blasticidin was added for the antibiotic selection (5 µg/ml or 10 µg/ml blasticidin for BJAB or Jurkat cells, respectively).

3.2.3.5 Lentiviral transfection of activated human primary T cells

For the lentiviral transfection of human T cells, 1 x 10^6 cells activated for 48 hours by the combination of anti-CD3 mAb and anti-CD28 mAb were used. Activated huamn T cells were transfected with the lentiviral supernatants produced by HEK293FT cells as described for BJAB and Jurkat cells. After 24 hours, cells were transfected once again under identical conditions to increase transfection efficiency, and then cultured in the RPMI 1640 complete medium supplemented with 20 ng/ml IL-2.

3.2.3.6 Generation of stable miRNA and cDNA expression cell clones

After 5 days cultured with blasticidin supplemented medium, cells were seeded in serial dilution on a 96-well plate containing selective medium (blasticidin supplemented) with each well containing less than 10 single cell clones, and incubated at 37^{o}C in the incubator. 30 days after plating, cells were analyzed for clonal outgrowth. Finally, cells from each outgrowth well were transferred and expanded in a cell culture flask, and then prepared for other analyses.

3.2.4 Biochemical methods

3.2.4.1 SDS-Polyacrylamide gel electrophoresis of proteins

After lyzed in ice-cold lysis buffer on ice, protein samples were denatured for electrophoresis by heating for 5 minutes at 99^{o}C in loading buffer. Protein separation was performed in NuPage 4-12% NOVEX® Bis-Tris gels (Invitrogen) in Xcell SureLock™ Mini-Cell (Invitrogen) containing Nupage MOPS SDS running buffer (Invitrogen). Gels were run at 200V constant voltage for 50 minutes.

3.2.4.2 Western blot analysis

SDS-PAGE gels (section 2.3.1) were equilibrated in the transfer buffer (10mM $NaHCO_3$, 3mM Na_2CO_3, 20% methanol) for 5 minutes. Proteins were transferred to PVDF transfer membrane (pre-wet with 100% methanol and equilibrated with transfer buffer) by electroblotting at 950 mA for 60 minutes in a XCell SureLock Mini-Cell and XCell II Blot Module (Invitrogen) containing NUPAGE Transfer buffer. Following that, the membrane was washed with TBST (150mM NaCl, 20mM Tris-HCl pH 7.5, 0.1% Tween 20) for 5 minutes to remove any residual acrylamide. The membrane was then blocked in 20 ml of Superblock T20 blocking buffer (Thermo scientific), for 1 hour with gentle rocking. After blocking, the membrane was incubated overnight at 4°C with diluted primary antibody, washed and incubated with appropriate horseradish peroxidase-conjugated antibody (1 hour, RT). Thereafter, the membrane was washed three times with TBST for 10 minutes each wash. The membrane was rinsed briefly with water, before covering with reagents from the ECL™ Western Blotting Analysis System (Amersham Biosciences). The blot was allowed to develop for 1 minute before the excess reagent was drained and the blot was exposed to High Performance Chemiluminescence film (Amersham Biosciences). Molecular masses of polypeptides were estimated by comparison to NOVEX MagicMark and NOVEX MultiMark protein markers.

3.2.4.3 Immunoprecipitation

For immunoprecipitation, cells were firstly lysed in the cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, phosphatase inhibitor cocktail and complete protease inhibitor cocktail) for 30 minutes, and then cell lysate was centrifuged at 12,000 g for 10 minutes at 4°C. The supernatants were collected for immunoprecipitation assay.

For immunoprecipitation of Flag-tagged protein, anti-Flag M2 agarose beads (Sigma) were added into the cell lysate (final concentration: 2% of the total reaction volume).

Alternatively, for immunoprecipitation of Fas surface protein, cell lysates were incubated with 1 μg of the anti-Fas antibody (C20, Santa Cruz) for 1 hour on ice. Thereafter, Protein A/G PLUS Agarose 25% (Santa Cruz) was added into the antibody-cell lysate (final concentration: 4% of the total reaction volume).

In both cases, the mixtures of agarose and cell lysate were mildly rotated overnight at 4°C. Thereafter, the agarose associated proteins were collected by centrifugation (5 minutes, 4°C and 2,000 *g*). The immunoprecipitates were then subjected to three consecutive washing and centrifugation steps (5 minutes, 4°C and 2,000 *g*). At the end of the immunoprecipitation procedure, 40-50 µl of 2X SDS reducing buffer was added to each tube and proteins were denatured by boiling at 95°C for 5 minutes. The tubes were centrifuged (5 minutes, RT and 12,000 *g*), and the supernatant was carefully transferred to a new tube. Immunoprecipitates were resolved by SDS-PAGE (3.2.4.1) and detected by western blotting (3.2.4.2) using different antibodies as indicated in each experiment.

3.2.4.4 Flow cytometry

For surface FACS staining, 2 x 10^5 to 5 x 10^5 cells were washed once with PBS, resuspended in 100 µl of FACS buffer and stained with Cy5, fluoresceinisothyocyanate-(FITC), phycoerythrin-(PE), or allophycocyanin-(APC) conjugated monoclonal antibodies in 5 ml FACS tubes. The antibodies used for surface FACS staining are shown in 3.1.9. Unstained cells or isotype-control stained cells served as a negative control, as indicated in each experiment. Unless otherwise specified, the antibodies for FACS staining were added to the cells at a concentration of 0.2 µg/ml. Cells were incubated for 30 minutes at 4°C in the dark and subsequently washed with FACS buffer.

In some experiments, biotin-labeled antibodies were used for surface FACS staining. These samples were stained with 0.2 µg/ml biotin-labeled antibodies for 30 minutes, at 4°C in the dark and subsequently washed with FACS buffer. Cells were then stained with a secondary antibody, Cy5- conjugated streptavidin, for 30 minutes at 4°C in the dark and finally washed with FACS buffer.

To detect Erk phosphorylation by flow cytometry, cell stimulation was stopped by adding ice-cold 2% paraformaldehyde, and then washed once with PBS. Cells were fixed in 2% paraformaldehyde at 37^{o}C for 10 minutes then chilled on ice for 1 minute and permeabilized with 95% MeOH on ice for 30 minutes. Cells were then blocked with 0.05% BSA at room temperature for 10 minutes following by incubation with anti-pERK rabbit polyclonal antibody (Cell Signaling Technology) for 1 hour at RT. Cells were washed twice with blocking buffer and stained with a secondary antibody, anti-rabbit IgG conjugated to Alexa-

647, at room temperature for 1 hour, and finally washed twice with FACS buffer and analyzed by flow cytometry.

For analysis of GFP expression, cells were washed once with PBS, resuspended in FACS buffer, and directly analyzed by flow cytometer.

All samples were measured by using a FACS Calibur flow cytometer (BD Biosciences) and subsequently analyzed by FlowJo software.

3.2.5 Apoptosis assay

Apoptosis was induced by treatment of cells with recombinant human CD95L (Apotech, Switzerland) or anti-hCD95 antibody (CH11, Beckman Coulter, France). The duration of stimulation as well as final concentration of stimuli are indicated in the Results section. Apoptosis was analyzed by AnnexinV staining (PharMingen). Briefly, cells were washed once with AnnexinV buffer, resuspended in 100 µl of AnnexinV buffer and stained with AnnexinV conjugated to allophycocyanine (APC) or fluoresceinisothyocyanate (FITC) for 30 minutes at 4°C in the dark. Subsequently, stained cells were washed twice with cold AnnexinV buffer, resuspended in this buffer, and directly quantified on a flow cytometer (FACS Calibur) and analyzed using FlowJo software. In transient transfection experiments (3.2.1.6), dead cells were removed (3.2.1.7) before apoptosis was induced and measured.

The percentage of apoptotic cells was calculated as follows: [(% experimental AnnexinV positive cells - % spontaneous AnnexinV positive cells)/(100 - % spontaneous AnnexinV positive cells)] x 100.

3.2.6 mAb blocking assay

For the antibody blocking assays, human T cells were pre-incubated with 2 µg/ml of anti-Toso mAbs, or control rat IgG (Jackson Laboratory) for 30 minutes on ice and then incubated for additional 30 minutes at 37°C. Subsequently, cells were washed twice with PBS, resuspended in the fresh medium and stimulated with FasL or TNFα.

3.2.7 Statistical analysis

All data obtained in the experiments are shown as means of at least two independent experiments ± SEM. Significance was analyzed using one-sided Student´s t test and the software package GraphPad Prism 4 (GraphPad Software, San Diego, CA). Significant differences were considered at * $p < 0.05$, ** $p < 0.01$.

4. Results

4.1 Establishing miRNA-mediated Toso gene knock-down

4.1.1 miRNA-mediated Toso gene knock-down

RNA interference (RNA*i*) is a process which leads to the degradation of endogenous mRNA via formation of duplexes with small non-coding RNAs. RNA*i* has become widely used as an efficient tool to down-regulate gene expression in mammalian cells. This enables phenotypical studies of 'loss-of-function' effects for specific genes. Several different strategies have been devised to achieve RNA interference in cells including synthetic short interfering RNAs (siRNA). Alternatively, transfection of plasmids encoding short hairpin RNAs (shRNAs), is commonly used. For permanent gene inactivation, it is necessary to stably integrate plasmids driving the expression of shRNAs into the genome of the respective target cell. Initially, RNA polymerase III promoters were used to mediate permanent shRNA expression. Recently, RNA polymerase II promoters have been employed to drive the expression of precursor microRNAs.

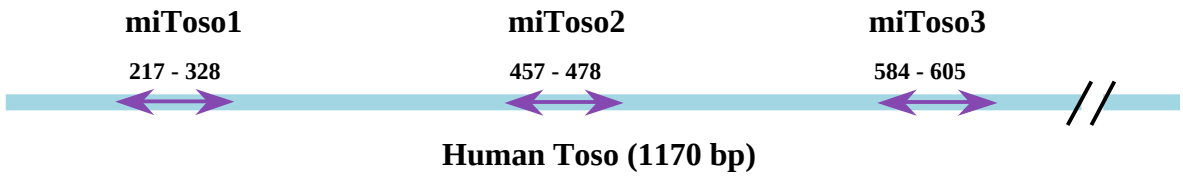
To investigate the biological role of Toso in Fas-mediated apoptosis, my studies have aimed at establishing transiently or stable and long-term Toso gene knock-down by employing RNA interference utilizing a lentiviral based miRNA vector system. miRNAs are processed from primary transcripts known as *pri-miRNA*, to short stem-loop structures called *pre-miRNA*, and finally to functional miRNA which regulates target mRNAs posttranscriptionally by directing cleavage and degradation. Toso specific miRNAs were transiently or stably introduced into cells utilizing a polymerase II based miRNA (*microRNA*) expression vector system (*BLOCK-iT-PoII system, Invitrogen),* which allowed me to achieve efficient Toso gene silencing in human lymphoma cell lines (BJAB and Jurkat), as well as in primary T lymphocytes.

Generation of Toso specific miRNA-constructs

Toso specific microRNAs were designed against three different regions of the human Toso cDNA sequence based on the sequence of the Genebank accession number AF057557 (Figure 4.1). A control miRNA which contains a random sequence and doesn't target any specific gene was also generated. These miRNAs were cloned into the miRNA expressing Gateway vector, pcDNA6.2-GW/EmGFP. For the lentiviral transfection, the inserted miRNA

cassettes were further transferred into lentiviral miRNA expressing vectors via homologous recombination using the Gateway technology (Invitrogen).

(A)



(B)

miToso1	: C TTC ATC AAG GCA GAA TAC AA
miToso2	: T TTG TTC CAG ATG CCT GCA TA
miToso3	: C GAG TGT CCA GAG CAT CTT CA
miNeg	: G TCT CCA CGC AGT ACA TTT CA

Figure 4.1: Design of 3 different miRNAs specific for different regions of Toso mRNA.

The targeting regions (A) and the sequences (B) of three different Toso specific miRNAs (miToso1, miToso2, and miToso3), as well as the sequence of the control miRNA are shown.

For the miRNA expression system, I utilized either the pcDNA6.2-GW/EmGFP vector for transient expression systems or the lentiviral expression vector pLenti6.2-GW/EmGFP for the generation of stable cell lines or for transient expression of miRNA in primary T cells. These miRNA expressing vectors are driven by an RNA polymerase II combined with a CMV promoter that enables co-cistronic expression of the EmGFP with miRNA. Utilizing this vector system, transfected cells can be readily identified by flow cytometry or fluorescence microscopy on the basis of their GFP expression.

As an initial test of the miRNA constructs, Jurkat cells were transiently transfected with Toso specific miRNA/GFP expressing constructs or control miRNA/GFP constructs in the miRNA-pcDNA6.2-GW/EmGFP vector using the Nucleofector transfection system (Amaxa). FACS analysis showed efficient transfection efficiency of these miRNA/GFP constructs by detecting more than 70% transfected (GFP positive) cells, as compared to untransfected cells (Figure 4.2A).

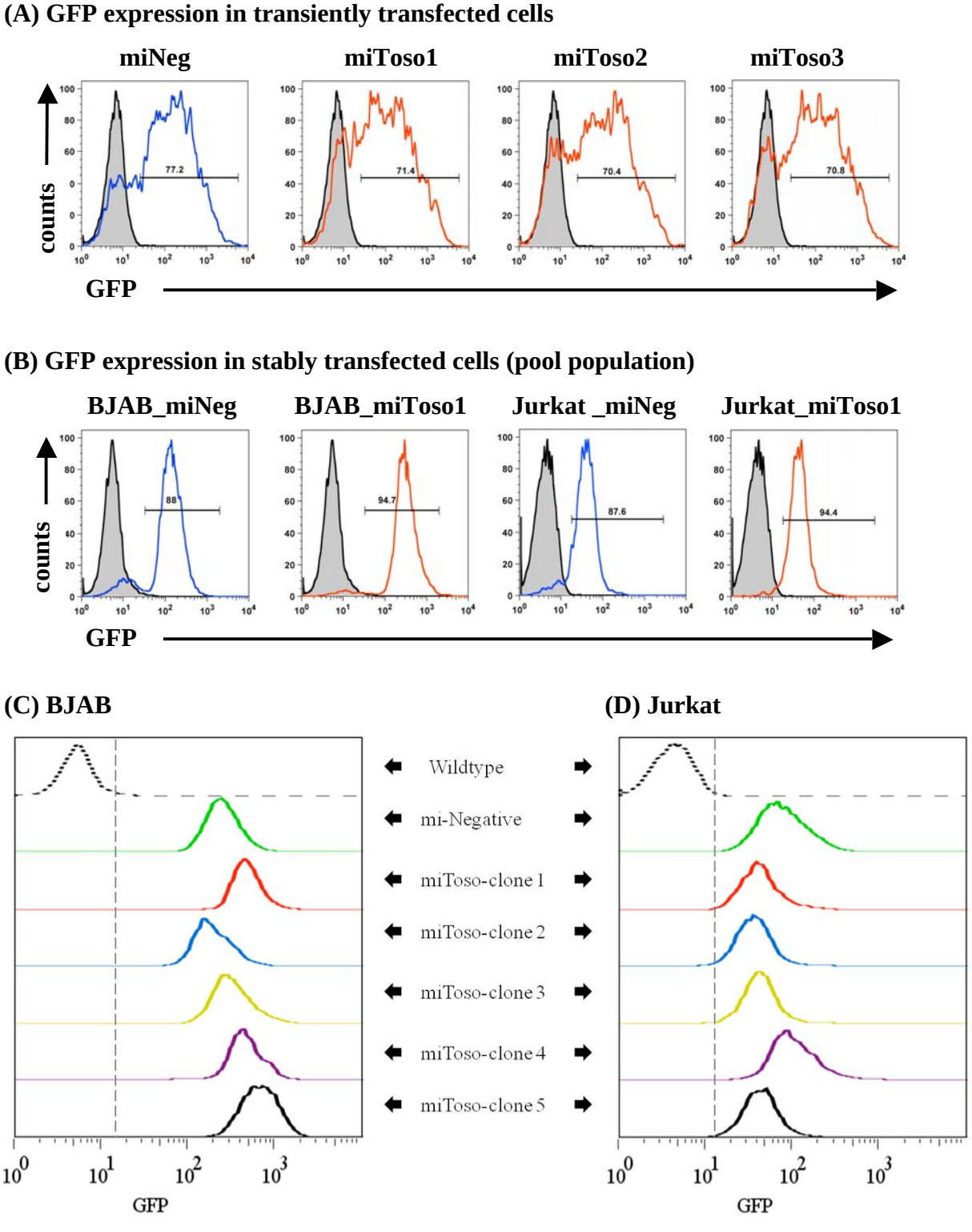


Figure 4.2: Transfection of Jurkat and BJAB cells with miRNA constructs.

(A) Jurkat cells were transiently transfected with three different Toso specific miRNA constructs (red) or control miRNA construct GFP (blue) constructs using the Amaxa nucleofection method. (B) Jurkat and BJAB cells (as indicated in the figure) were transduced with Toso miRNA-EmGFP-pLenti6 (red) or a control miRNA (blue, miNeg) using lentiviral delivery. The transfection (48h after nucleofection, A) or transduction (1 week

after antibiotic selection, B) efficiency was determined by FACS analysis (gated on viable cells) for GFP expression. Untransfected cells are shown as control (grey filled). The percentage of GFP positive cells is indicated in the histogram. GFP expression of Jurkat (C) and BJAB (D) wild type or single cell clones stably expressing Toso specific miRNA, or control miRNA (miNeg) was determined by FACS analysis (gated on viable cells). Data shown are representative of more than 3 independent experiments.

The Toso gene knock-down efficiency in cells expressing various Toso specific miRNAs or control miRNA was examined by using reverse transcriptase polymerase chain reaction (RT-PCR). As revealed by RT-PCR analysis (Figure 4.3A), Toso mRNA levels in cells expressing all three different Toso specific miRNAs showed significantly decreased Toso mRNA expression, as compared to control miRNA expressing cells. One of three different miRNAs against Toso, miToso1, which showed the highest knock-down efficiency, was selected and used for the generation of stable Toso knock-down cell lines and further functional experiments using these cells.

In further experiments, Jurkat and BJAB cell lines stably expressing Toso specific miRNA constructs or a control miRNA construct, were generated by utilizing a lentiviral miRNA vector system (3.2.3). The initial transfection using the lentiviral vector showed 20-30 % of GFP positive cells for Jurkat cells, and 30-40% of GFP positive cells for BJAB cells (data not shown). However, miRNA/GFP expressing populations were further selected and enriched by adding antibiotics (Blasticidin). Finally, stably miRNA expressing “pool-populations” which contain almost 100% GFP positive cells, were generated (Figure 4.2B). In addition, single cell clones of BJAB or Jurkat cells which express either Toso specific miRNA or control miRNA were also generated by a “limited dilution approach” after transfection (3.2.3.5). These stable miRNA expressing BJAB or Jurkat cells - pool populations or single cell clones - were subsequently used for functional assays to determine the role of Toso in Fas-mediated apoptosis.

Next, the selected single cell clones expressing Toso specific miRNAs or control miRNA (Figure 4.2C and D) were tested for their Toso knock-down efficiency utilizing quantitative real time PCR (3.2.2.3). This quantitative real-time PCR showed that the relative mRNA levels of Toso in 5 different stable clones of BJAB and Jurkat cells expressing Toso specific miRNAs was significantly reduced as compared to wild-type cells as well as cells expressing control miRNA (Figure 4.3B and C).

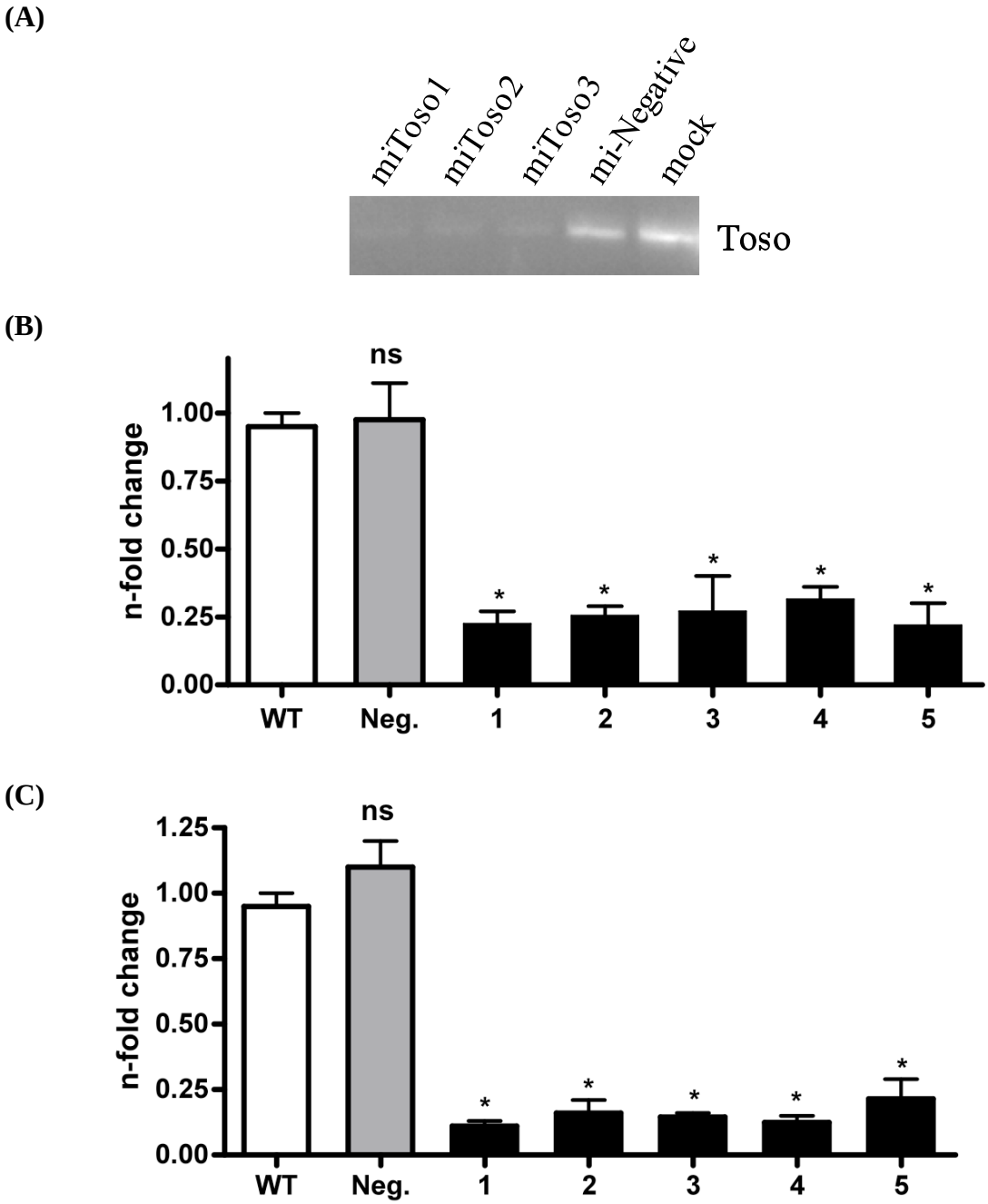


Figure 4.3: The Toso knock-down efficiency in cells expressing miRNA constructs.

(A) RT-PCR analysis of Toso expression in Jurkat cells untransfected (mock) or transiently transfected with 3 different Toso specific miRNAs (miToso1, miToso2, and miToso3) or negative miRNA (miNeg) using the Amaxa nucleofection method. 48-hour post-transfection, RNA was isolated and cDNA was synthesized. PCR analysis using Toso specific primers revealed the Toso mRNA expression. Toso mRNA expression data from quantitative real-time PCR analysis of BJAB (B) and Jurkat (C) cell clones stably expressing Toso miRNA (black bars), control miRNA (grey filled bar), or wild-type cells (white bar) are shown. Data shown are representative of 3 independent experiments and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test; ns, not significant).

Efficient miRNA-mediated Toso gene knock-down was further confirmed by the examination of Toso protein expression on the cell surface using a Toso specific antibody, which was generated by DNA immunization. Extracellular FACS analysis was shown significantly decreased Toso protein levels on the surface of Jurkat and BJAB cells stably expressing Toso specific miRNA, as compared to control miRNA expressing cells (Figure 4.4).

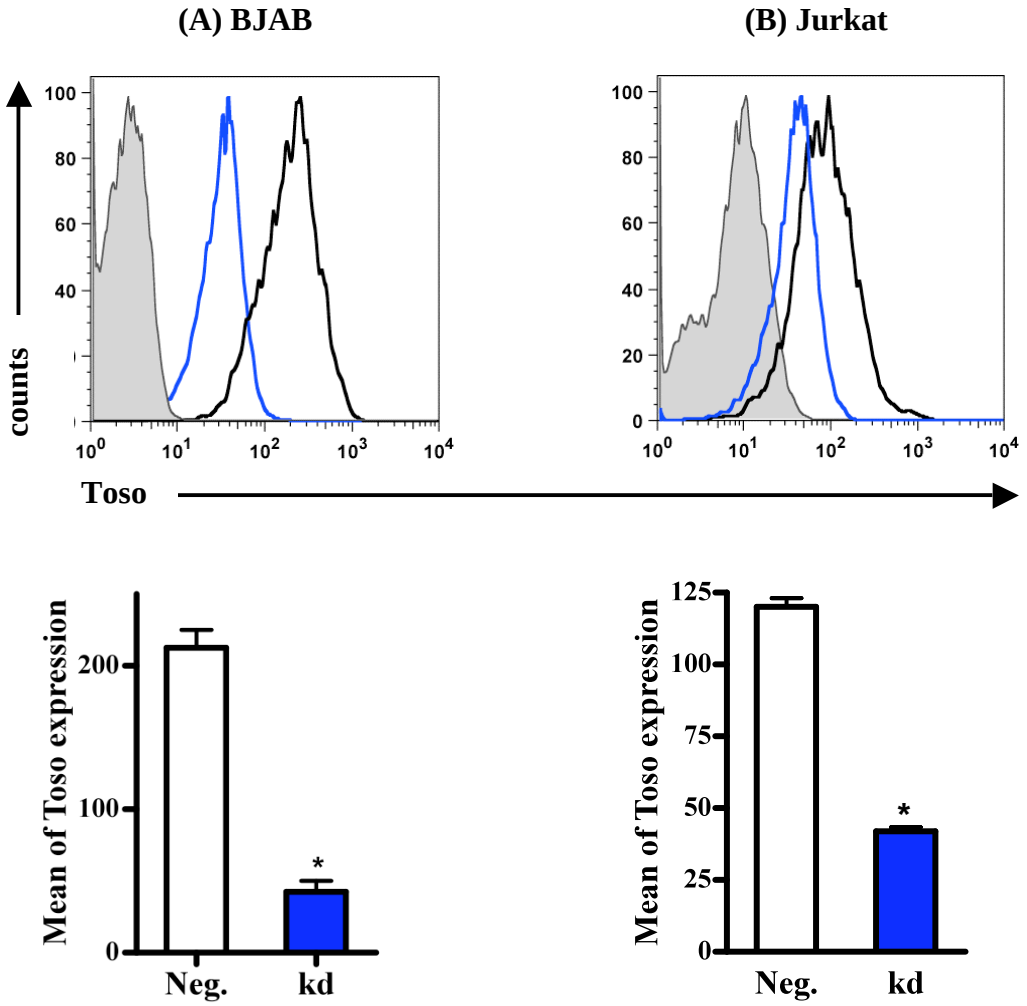
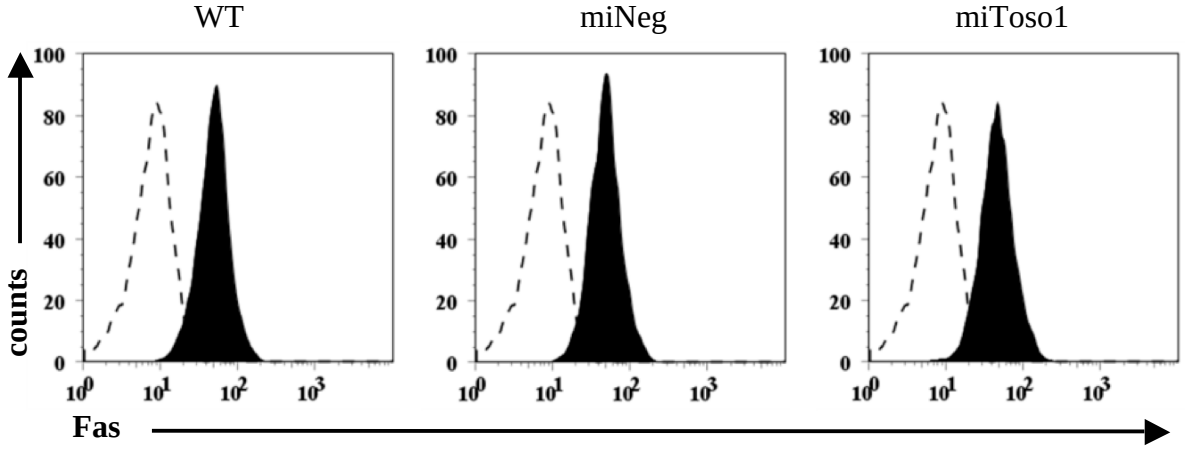


Figure 4.4: Toso surface expression in stable Toso knock-down Jurkat and BJAB cells.

The Toso surface expression of BJAB (A) and Jurkat (B) cells stably expressing Toso specific miRNA (blue) or control miRNA (black) was tested by FACS analysis after staining with a Toso specific antibody and secondary labeled goat anti-rat-Cy5 (gated on viable cells). Staining of the cells with rat-IgG antibody is shown as a control (grey filled). Comparison of the mean fluorescence intensity of Toso staining in the cells is shown in the lower panel. Data shown are representative of 3 independent experiments and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test).

Further studies examined whether the introduction of miRNAs might affect Fas surface expression. The cell surface expressions of Fas on cells stably expressing Toso specific miRNAs or control miRNA was evaluated by FACS analysis using a directly labeled antibody against the human Fas protein (3.2.4.4). The data showed that the expression of miRNAs did not change the Fas surface expression in both Jurkat and BJAB cells (Figure 4.5).

(A) Fas expression in stable Toso knock-down BJAB cells



(B) Fas expression in stable Toso knock-down Jurkat cells

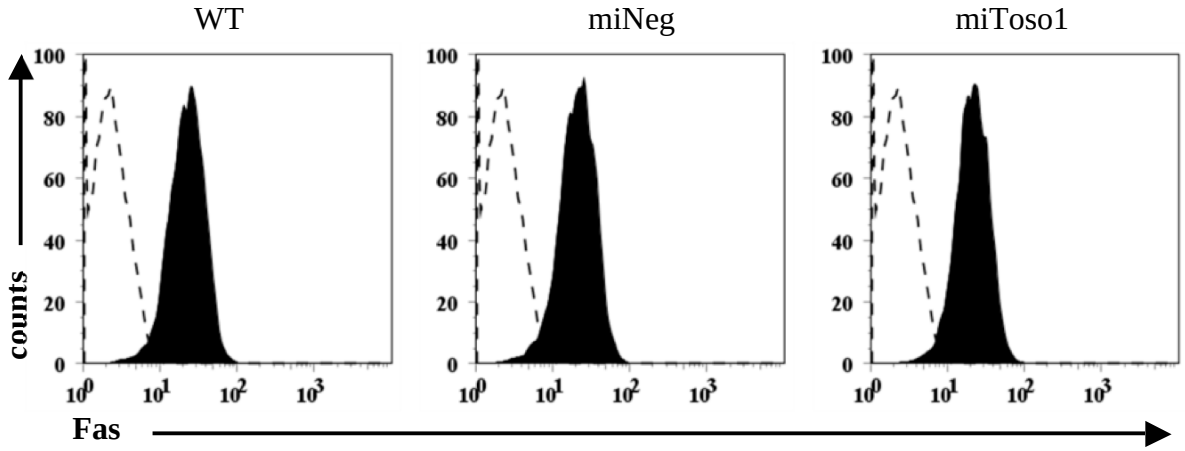


Figure 4.5: Fas surface expression in stable Toso knock-down BJAB and Jurkat cells was not changed in comparison to wild-type cells.

Wild type BJAB and Jurkat cells, and cells stably expressing control miRNA or Toso-specific miRNA were analyzed by FACS after staining with Fas specific antibody (gated on viable cells). Staining of the cells with isotype control antibody that has no relevant specificity with Fas is shown in dashed black lines. Data shown are representative of 4 independent experiments.

4.2 The effects of Toso knock-down on Fas-induced apoptosis in Type I and Type II tumor cells

Two different Fas-mediated apoptotic signaling pathways have been described. In Type I cells, death receptor-induced apoptosis depends on DISC formation and caspase-8 activation, while Type II cells are more dependent on a mitochondrial amplification loop and exhibit only poor, delayed DISC formation, and caspase-8 activation (Algeciras-Schimnich *et al.*, 2002; Barnhart *et al.*, 2003; Chaigne-Delalande *et al.*, 2008; Scaffidi *et al.*, 1998). To investigate the function of Toso in Fas-mediated apoptosis in these two different Fas signaling pathways, the Type I cell line BJAB, which is a Burkitt's lymphoma cell line, and the Type II cell line Jurkat, which is a T leukemia cell line, were used in my studies.

To investigate the role of Toso in Fas-mediated apoptosis of Type I cells, stable Toso knock-down BJAB cells or control cells were examined for both, dose dependence and kinetics of FasL-induced apoptosis by stimulation with different concentrations of FasL, or with a constant amount of FasL for different times. Early apoptotic cells were detected by staining with Cy5-labled AnnexinV and subsequent analysis by flow cytometry (3.2.5). AnnexinV binds specifically to phosphotidyl-serine (PS) (Koopman *et al.*, 1994) which is normally localized to the inner leaflet of the plasma membrane and translocates to the outer leaflet of the cell membrane in response to apoptotic signals (Martin *et al.*, 1995).

FACS analysis revealed an increased sensitivity to FasL-induced apoptosis in BJAB cells expressing Toso specific miRNA over time and over a wide range of FasL concentrations, when compared to control miRNA expressing cells (Figure 4.6). For example, following 50 ng/ml concentration of FasL treatment for 5 hours, 22% of apoptotic cells were detected in control BJAB cells while the percentage of apoptotic cells was increased to 58% in Toso knock-down cells (Figure 4.6A). Also, following 100ng/ml concentration of FasL treatment for 5 hours, 55% of apoptotic cells were detected in control BJAB cells, while the percentage of apoptotic cells was increased to 73% in Toso knock-down cells (Figure 4.6A). The dose responses shown in Figure 4-6C confirmed again the high sensitivity of Toso knock-down cells to Fas-mediated apoptosis. These data indicate that Toso negatively controls Fas-induced apoptosis.

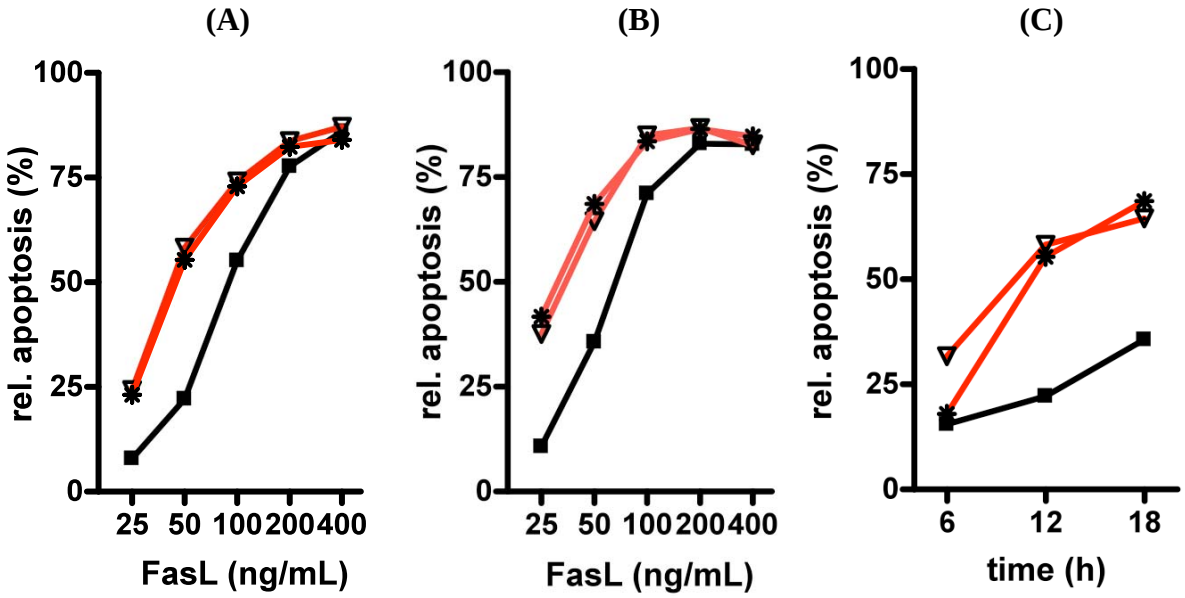


Figure 4.6: Toso knock-down results in an increased sensitivity to FasL-induced apoptosis in BJAB cells.

BJAB cells stably expressing control miRNA (black) or two different Toso specific miRNAs (red) were stimulated with the indicated concentrations of FasL for 5 hours (A) or 8 hours (B), or with 25 ng/ml FasL for the indicated times (C). Apoptotic cells were assessed by staining with AnnexinV, measured by FACS, and relative apoptosis rates (%) were quantified. Representative data from more than 10 independent experiments are shown.

Interestingly, the anti-apoptotic function of Toso in Fas signaling was not only observed in Type I BJAB cells, but also in Type II Jurkat cells. Indeed, similar to the effects observed in BJAB cells, specific knock-down of Toso in Jurkat cell rendered the cells more susceptible to Fas-mediated apoptosis. Experiments with Jurkat cells stably expressing Toso specific miRNA showed that FasL-induced apoptosis was also dependent on the expression level of Toso (Figure 4.7). For example, following stimulation with 50 ng/ml FasL, 40% of apoptotic cells were detected in control Jurkat cells, while the number of apoptotic cells was increased to 59% in Toso knock-down cells. Similarly, treatment of cells with 250 ng/ml FasL induced apoptosis in 55% of control cells, but in 77% of Toso knock-down Jurkat cells (Figure 4.7).

These findings suggest that the knock-down of Toso resulted in an increased sensitivity to Fas-induced apoptosis in both Type I BJAB and Type II Jurkat cells.

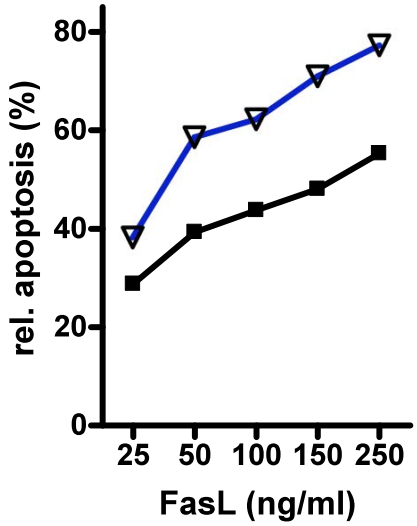


Figure 4.7: Toso knock-down results in an increased sensitivity to FasL-induced apoptosis in Jurkat cells.

Jurkat cells stably expressing specific Toso miRNA (blue line) or control miRNA (black line) were stimulated with the indicated concentrations of FasL for 6 hours. Apoptotic cells were assessed by staining with AnnexinV and analyzed by flow cytometry. Relative apoptosis rates (%) were quantified. Data shown is representative of 3 independent experiments.

4.3 The reconstitution of the Fas-induced apoptosis in Toso knock-down cells by utilizing "miRNA resistant" Toso cDNA construct

To ensure the specificity of Toso miRNA in Fas-induced apoptosis, a reconstitution experiment was performed by reintroducing Toso protein into stable Toso knock-down cells. To this end, a 'miRNA resistant' Toso cDNA containing silent mutations in the specific miRNA target site (Figure 4.8A) was generated (3.2.2.11). To generate this 'miRNA resistant' Toso cDNA, six nucleotides in the specific miRNA targeting site of the human Toso gene were substituted with alternative codons without changing the amino acid sequence. This Toso cDNA with silent mutations was then transiently transfected into BJAB cells stably expressing Toso specific miRNA, using the Amaxa nucleofection method (3.2.1.6). 48 hours after transfection with the Toso silent mutant in stable Toso knock-down BJAB cells, the re-expression of Toso on the surface was detected by FACS analysis (Figure 4.8B).

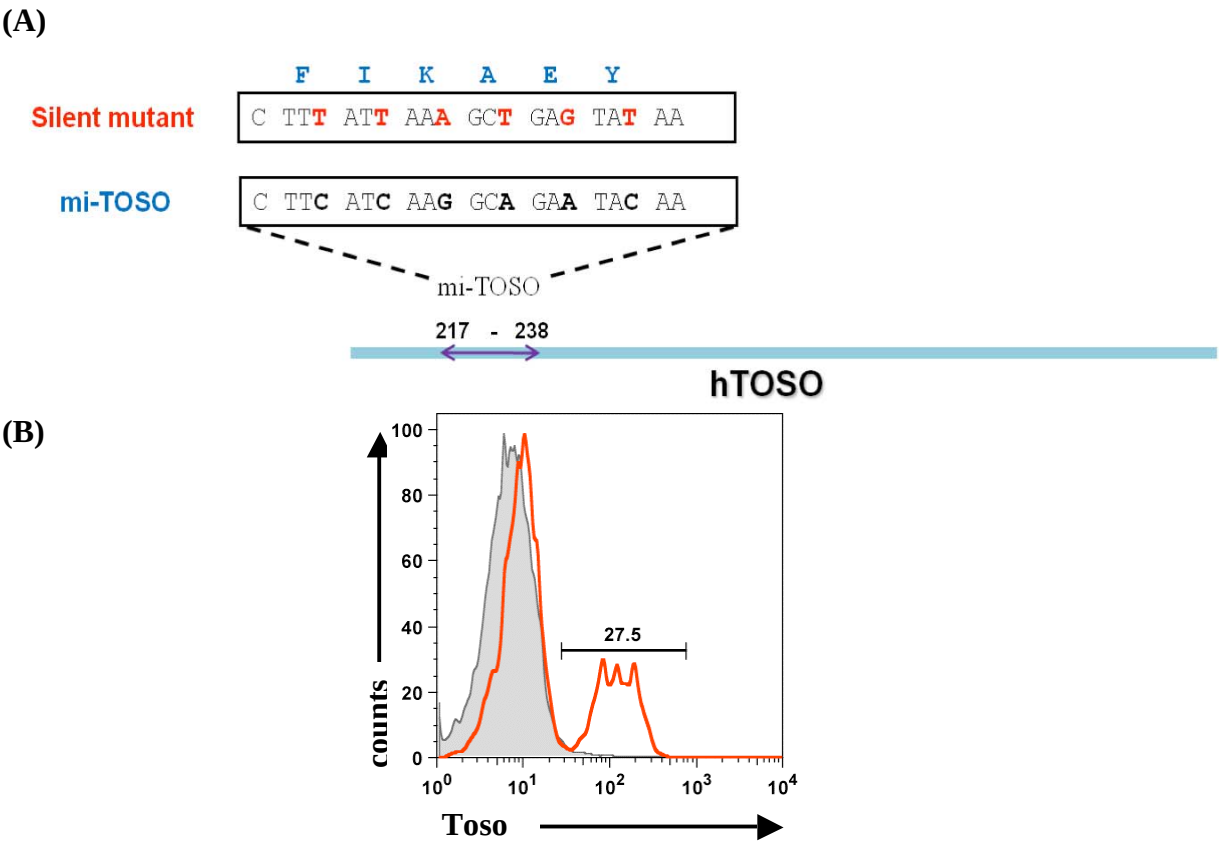


Figure 4.8: Reconstitution of Fas-induced apoptosis in Toso knock-down cells.

(A) A 'miRNA resistant' Toso cDNA containing silent mutations. Six nucleotides in the miRNA targeting site of the human Toso gene were substituted without changing the amino acid sequence. (B) Stable Toso knock-down BJAB cells were transiently transfected with the mutated Toso cDNA construct by using the Amaxa nucleofection method. The Toso surface expression of untransfected (grey filled) and transfected (red) cells was tested after 48 hours by staining with a specific Toso antibody and subsequent FACS analysis (gated on viable cells). The percentage of Toso expression is indicated in the histogram. Data shown are representative of 2 independent experiments.

The nucleofector system allows for efficient transfection of the Toso construct but this transfection method also results in the substantial amount of cell death during transfection procedures. Therefore, after Nucleofector transfection, dead cells were removed using the "Dead Cell Removal Kit" (Miltenyi Biotech) (3.2.1.7). To test the effect of Toso reconstitution in Toso knock-down cells on Fas-induced apoptosis, apoptosis was then induced by stimulating cells with FasL for 5 hours and cellular apoptosis was quantified by AnnexinV staining and FACS analysis (3.2.5). The increased sensitivity to Fas-induced apoptosis in Toso knock-down cells was clearly reversed by reintroducing a miRNA resistant Toso construct (Figure 4.9). Therefore, the specificity of Toso specific miRNAs for Fas-

induced apoptosis was confirmed by these reconstitution experiments using a Toso silent mutant construct.

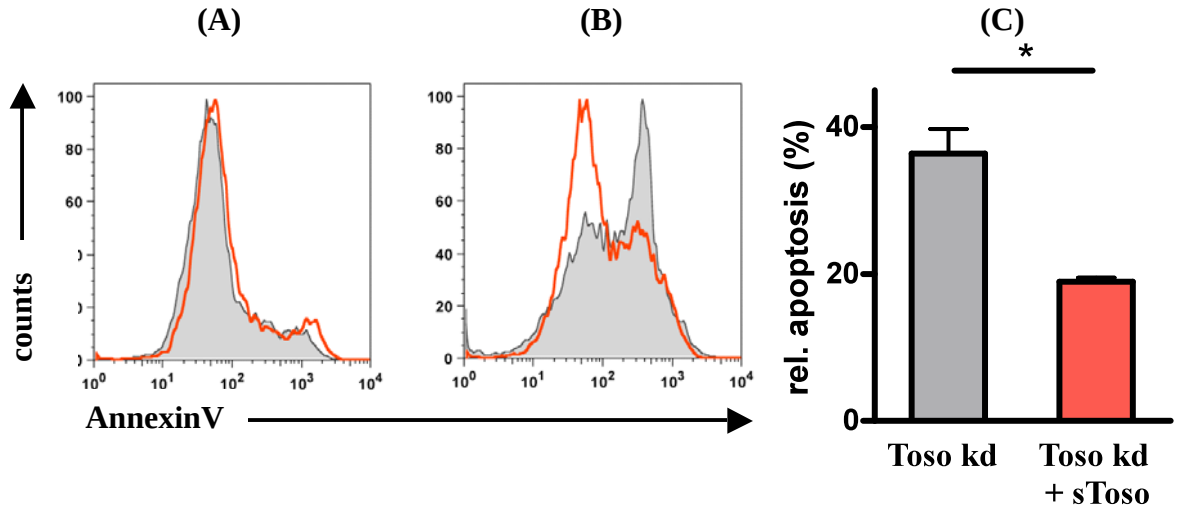


Figure 4.9: The increased sensitivity to Fas-induced apoptosis in stable Toso knock-down cells is reversed by reintroducing the "miRNA resistant" Toso cDNA.

Untransfected (grey filled) and transfected (red) stable Toso knock-down BJAB cells were unstimulated (A), or stimulated with 50 ng/ml FasL (B) for 5 hours. Apoptotic cells were assessed by FACS analysis after staining with AnnexinV. AnnexinV-positive cells from mock treated control cells (grey filled) or Toso silent mutant expressing cells (red) are shown in flow cytometric histograms. (C) Relative % of apoptosis is quantified. Data shown are representative of 2 experiments and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test).

4.4 The effect of Toso overexpression on Fas-mediated apoptosis

Since the knock-down of Toso resulted in an increased sensitivity to Fas-induced apoptosis, my studies further examined whether the overexpression of Toso could protect from Fas-mediated apoptosis. To this end, full-length human Toso cDNA was cloned and stable BJAB and Jurkat cell lines expressing the human Toso construct were generated.

Generation of stable Toso overexpressing BJAB and Jurkat cells

Full-length human Toso cDNA was successfully cloned from cDNAs isolated from human peripheral blood T lymphocytes (PBT) by reverse transcription PCR (3.2.2.3). The human Toso cDNA construct was cloned into the lentiviral pLenti6-V5 vector and subsequently this Toso construct was introduced into BJAB and Jurkat cell lines. Increased Toso mRNA levels in Toso cDNA transfected Jurkat or BJAB cells were confirmed by quantitative real-time PCR and increased Toso protein expression was detected by staining with Toso specific antibody and subsequent FACS analysis as shown in Figure 4.10 and 4.11, respectively.

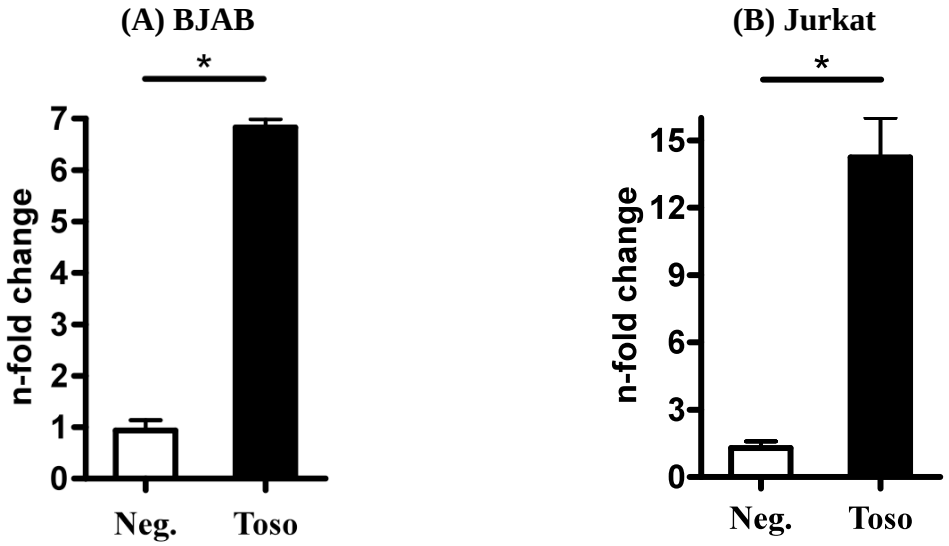


Figure 4.10: Toso mRNA levels in BJAB and Jurkat cells stably expressing Toso cDNA are significantly increased as compared to control cells.

Human Toso mRNA expression data from quantitative real-time-PCR analysis of BJAB (A) and Jurkat (B) cell clones stably expressing Toso cDNA (black bars), or control vector (white bar) are shown. Data shown are representative of 3 independent experiments and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test).

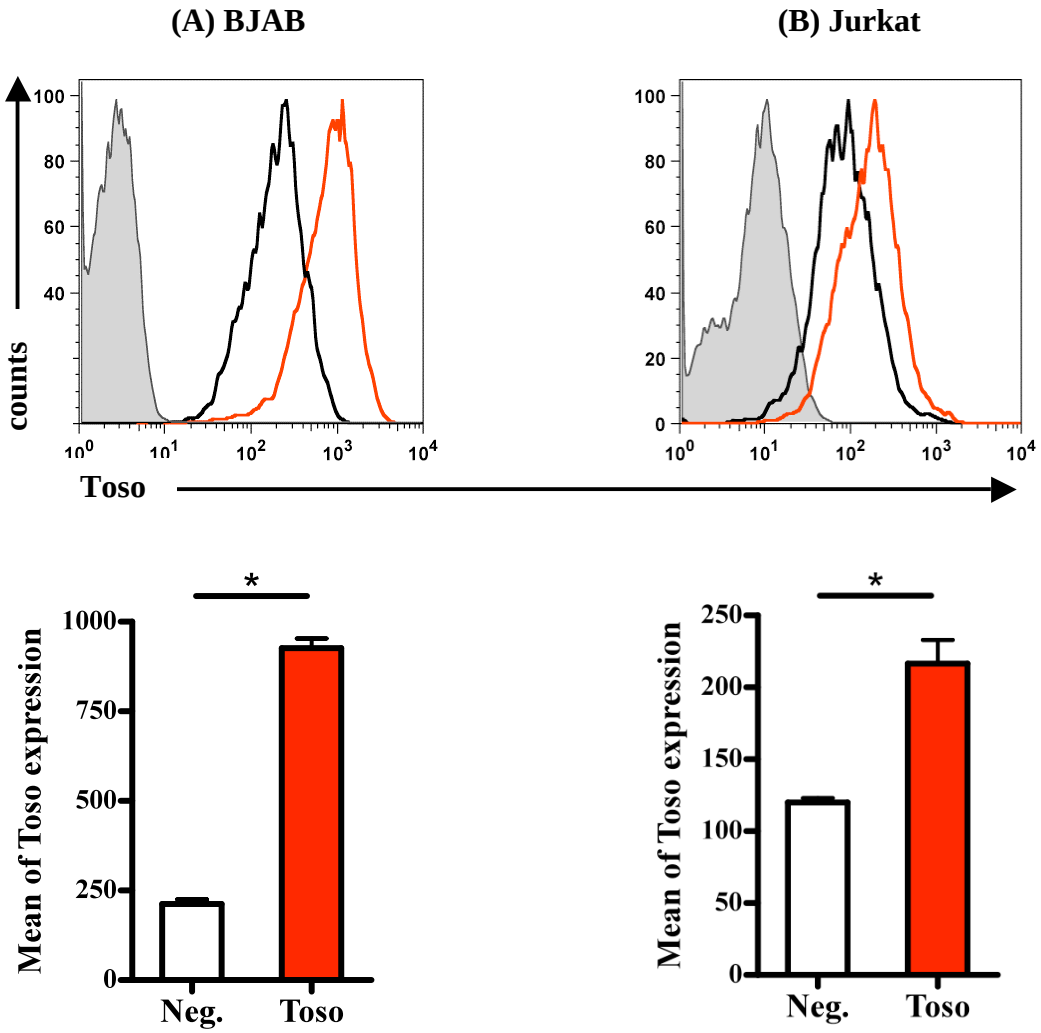


Figure 4.11: Toso surface expression of Jurkat and BJAB cells stably expressing Toso cDNA is significantly increased.

The Toso surface expression of BJAB (A) and Jurkat (B) cells stably expressing Toso cDNA (red), or control vector (black) was assessed by FACS analysis after extracellular staining using a Toso specific antibody and secondary labeled goat α-Rat-Cy5 (gated on viable cells). Staining of the cells with rat-IgG antibody is shown as a control (grey filled). A comparison of the mean fluorescence intensity of Toso expression on the cells is shown in lower panel. Data shown are representative of 3 independent experiments and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test).

Importantly, stable overexpression of Toso in BJAB and Jurkat cells did not change the level of Fas surface expression (Figure 4.12). My studies have further investigated the role of Toso overexpression in Fas-mediated apoptosis. To this end, control cells or cells stably expressing Toso cDNA were stimulated with different concentrations of FasL, or with a constant amount

of this ligand for different times. Apoptotic cells were then detected by staining with Cy5-labled AnnexinV and subsequent flow cytometric analysis (3.2.5).

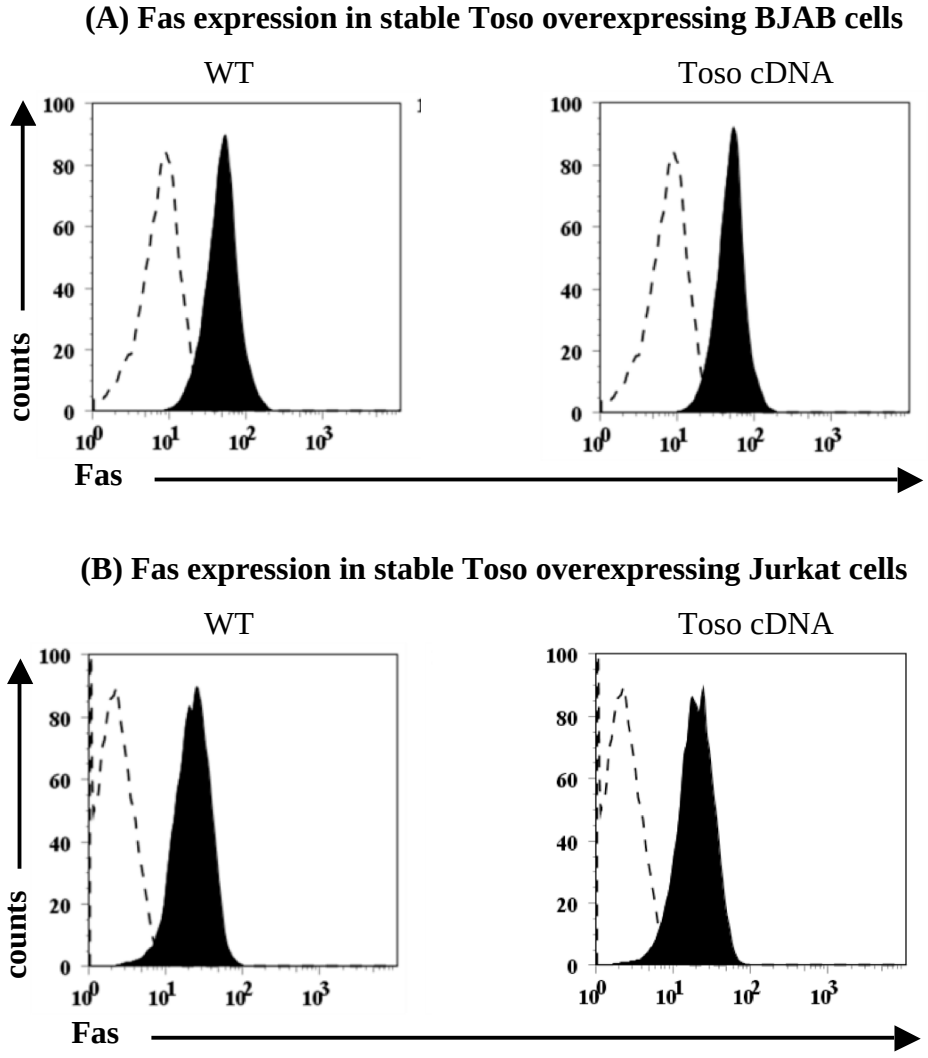


Figure 4.12: Fas surface expression in BJAB and Jurkat cells stably expressing Toso cDNA is not changed as compared to wild-type cells.

Wild-type BJAB and Jurkat cells, and cells stably expressing hToso cDNA were analyzed by FACS after staining for Fas specific antibody (gated on viable cells). Staining of the cells with isotype control antibody that has no relevant specificity with Fas is shown in dashed black lines. Data shown are representative of 4 independent experiments.

The experiments revealed that Toso overexpressing BJAB cells were significantly more resistant to Fas-induced apoptosis (Figure 4.13A). Following stimulation with 100 ng/ml FasL, 40% of apoptotic cells were detected in control BJAB cells, and the number of apoptotic cells was increased to 68% by 250 ng/ml of FasL treatment. However, in Toso

overexpressing cells, apoptosis reached only 15% in response to treatment with 100 ng/ml FasL, and 24% after treatment with 250 ng/ml FasL.

The overexpression of Toso in Jurkat cells also leads to a decrease of Fas-induced apoptosis (Figure 4.13B). Jurkat cells showed significantly reduced numbers of apoptotic cells after stimulation with 100 ng/ml FasL for 6 or 12 hours. Taken together, the overexpression of Toso in both, BJAB and Jurkat cells, had protective effects on Fas-mediated apoptosis.

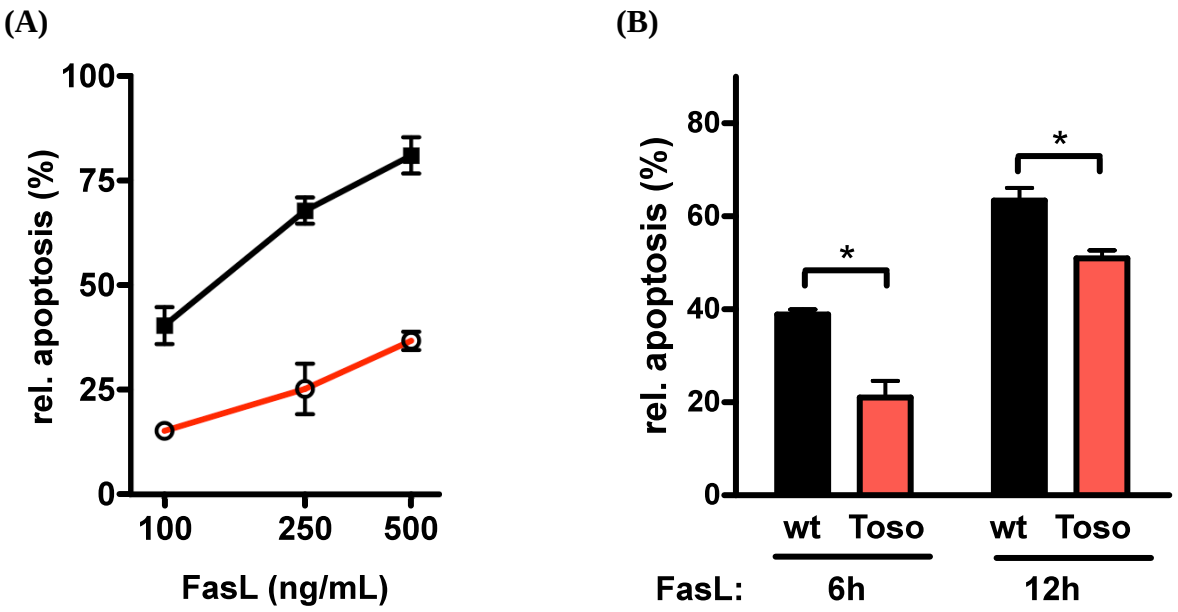


Figure 4.13: Toso overexpression protects cells from FasL-induced apoptosis.

(A) BJAB cells expressing either a control vector (black) or hToso cDNA (red) were stimulated with the indicated concentrations of FasL for 5 hours. (B) Jurkat cells expressing either a control vector (black bar) or hToso cDNA (red bar) were stimulated with 100 ng/ml FasL for the indicated times. Cells were stained with AnnexinV-Cy5, and analyzed by flow cytometry. The y-axis shows relative apoptosis rates (%). Representative data from more than 10 independent experiments are shown and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test).

4.5 Toso is a negative regulator of Fas-mediated apoptosis in primary human T cells

To extend my studies beyond transformed cell lines, the negative role of Toso in Fas-mediated apoptosis was also re-evaluated in human peripheral blood T lymphocytes (PBTs). In order to study Fas-induced apoptosis in primary cells, resting human primary T lymphocytes were isolated from peripheral blood of healthy donors (3.2.1.4). These primary T cells were then *in vitro* activated using immobilized anti-CD3 and anti-CD28 monoclonal antibodies to induce Fas surface expression on activated T lymphocytes. 24 hours after CD3/CD28 activation, the up-regulation of Fas on the surface of activated T cells was confirmed by FACS analysis (Figure 4.14). Expression of the T cells activation marker CD69 was also determined to confirm activation of the T cells.

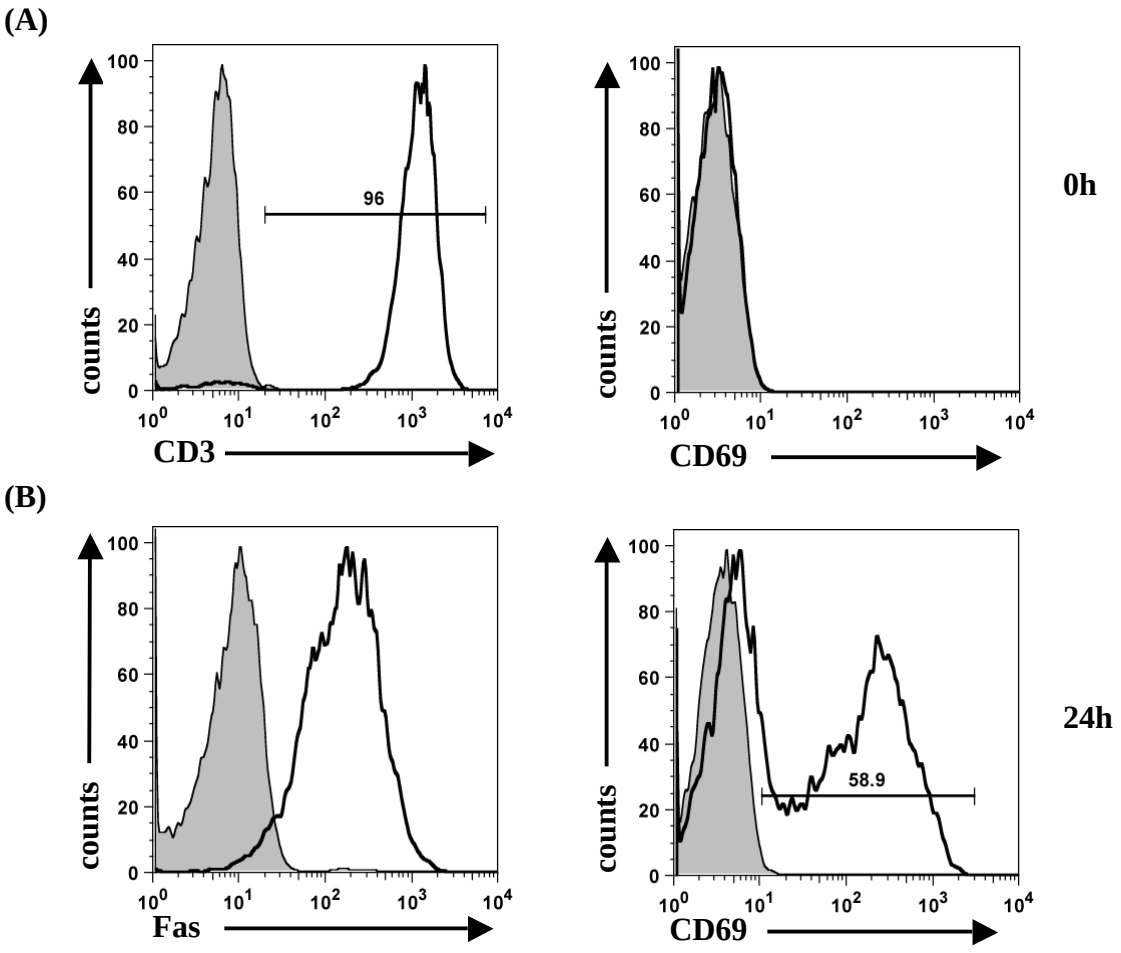


Figure 4.14: Purity and activation of human peripheral blood T cells.

(A) Freshly isolated human peripheral blood T cells were analyzed for surface expression of CD3 and CD69 by FACS (gated on viable cells). (B) Resting primary T cells were activated *in vitro* by using anti-CD3 and anti-CD28 antibodies. Following activation, cells were stained for CD69 and Fas, and analyzed by FACS. Staining

of the cells with isotype matched control antibodies of irrelevant specificity is shown in grey filled. Representative results of more than four independent experiments are shown.

Interestingly, Toso protein expression was observed to be regulated during T cell activation. Toso protein expression was rarely detectable in freshly isolated resting T cells; however, Toso expression was significantly up-regulated in activated T cells by stimulation with CD3/CD28. These data indicate that Toso expression is tightly controlled during T cell activation (Figure 4.15).

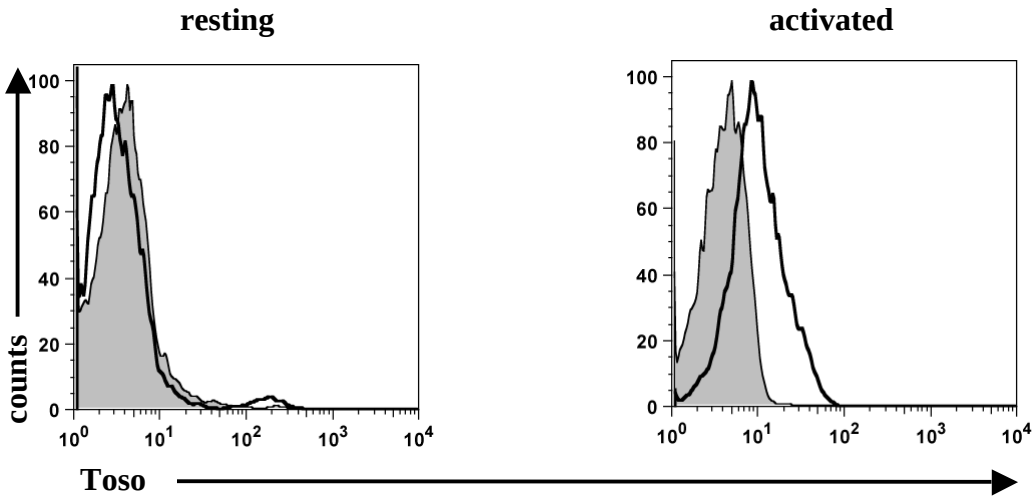


Figure 4.15: Toso surface expression is up-regulated following T cell activation.

Fresh isolated resting human peripheral blood T cells and T cells activated with CD3/CD28 for 2 days were analyzed by FACS after staining with a Toso specific antibody and secondary goat α-Rat-Cy5 antibody (gated on viable cells). As a control, staining of the cells with rat-IgG antibody is shown (grey filled). Data shown are representative of 5 independent experiments.

To confirm the anti-apoptotic function of Toso in primary cells, I have performed Toso knock-down experiments in human primary T cells using a lentiviral miRNA system (3.2.3). To this end, Toso specific miRNA/EmGFP and control constructs were lentivirally introduced into pre-activated primary human T lymphocytes. Two days after lentiviral transfection, the expression of Toso specific miRNA or control miRNA was detected by tracking GFP positive T cell populations (Figure 4.16A).

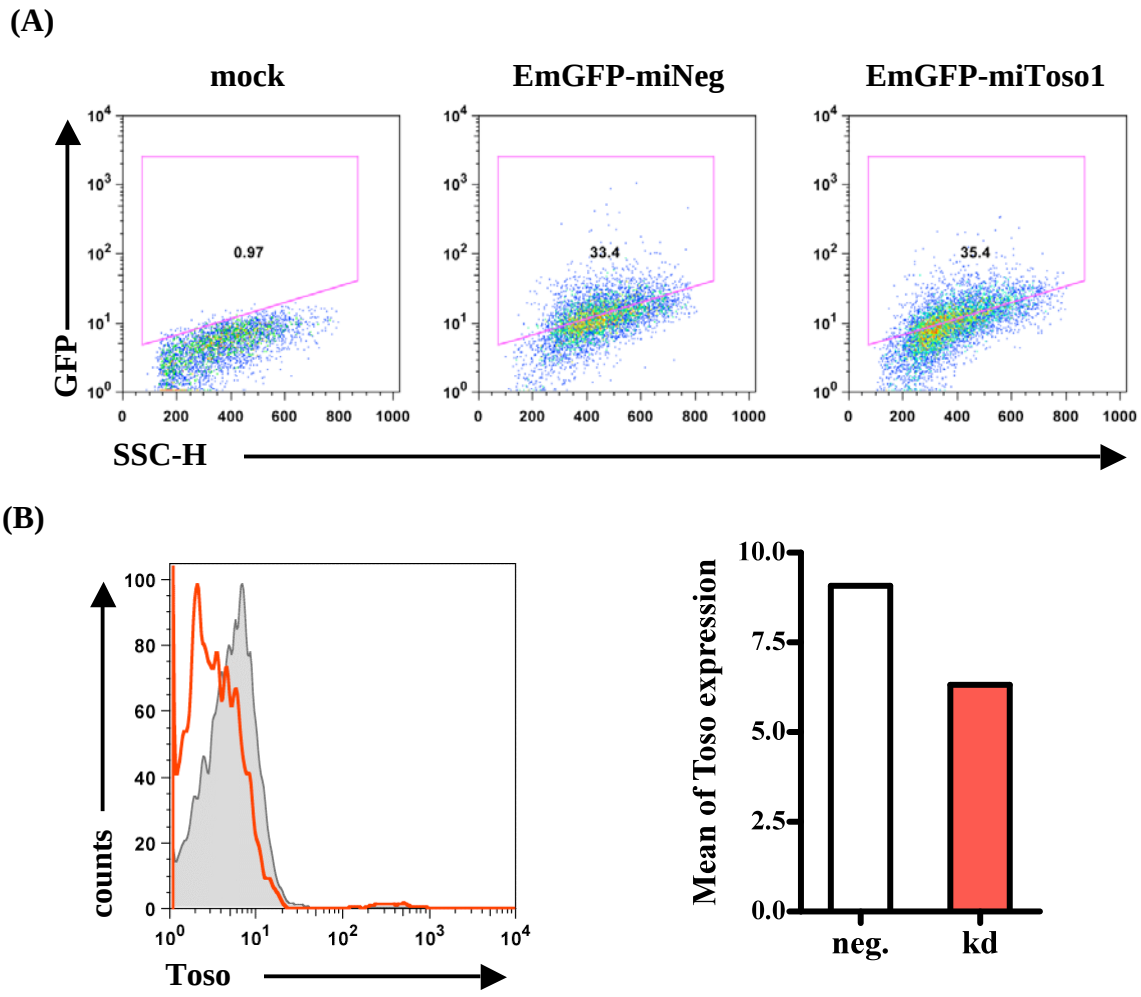


Figure 4.16: Toso surface expression in human peripheral blood T cells transfected with Toso miRNA is significantly decreased.

Activated human peripheral blood T cells were transfected with miNeg-EmGFP-pLenti6 (neg) or miToso1-EmGFP-pLenti6 (miToso1) vector using lentiviral delivery. (A) The transduction efficiency was determined 48 hours after transfection by FACS analysis (gated on viable cells) for GFP expression. The percentage of GFP positive cells is indicated in the dot blots. (B) The Toso surface expression on cells transduced with pLenti6-miNeg (grey filled) or pLenti6-miToso1 (red) was tested by FACS analysis after staining for Toso (gated on GFP positive cells). Comparison of mean GFP expression of the cells is shown on the right. Data shown are representative of 3 independent experiments.

After confirming the expression of the miRNA/EmGFP constructs in primary T cells by means of GFP expression, the knock-down efficiency of the Toso gene was determined by the level of Toso protein expression on the GFP positive populations. Toso specific miRNA expressing cells showed significantly reduced Toso surface expression as compared to control miRNA expressing cells (Figure 4.16B). To examine the Fas-induced apoptosis in

Toso miRNA expressing primary T cells, three days after lentiviral transfection, cells were stimulated with FasL for 5 hours and the relative rate of apoptosis was then determined by flow cytometric analysis of GFP positive populations (miRNA expressing cells) and comparison with the GFP negative population (miRNA non-expressing cells).

The data showed that the introduction of control miRNA did not exhibit any significant effects on Fas-induced apoptosis in primary T cells. However, primary T cells expressing Toso specific miRNA exhibited significantly higher rates of Fas-induced apoptosis as compared to non-transfected control cells (Figure 4.17, for stimulated cells).

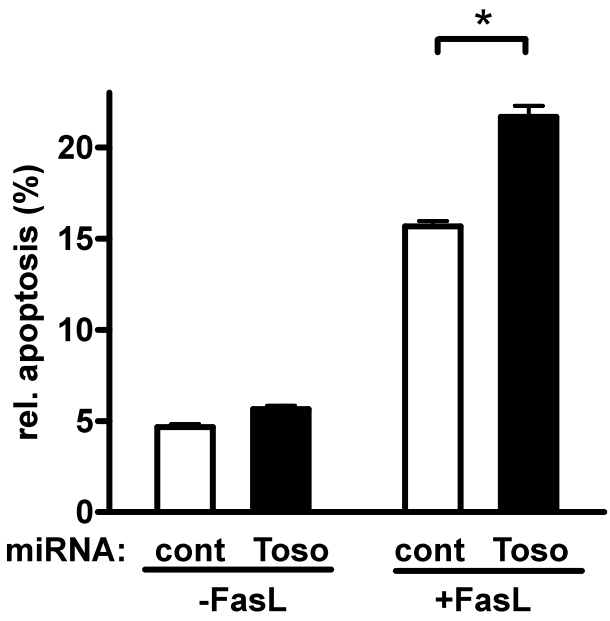


Figure 4.17: FasL-induced apoptosis in Toso knock-down primary T cells.

Activated human peripheral blood T cells were lentivirally transduced with Toso specific miRNA (black bars) or control miRNA (white bars). Cells were stimulated with FasL and apoptosis was assessed by staining with AnnexinV and measured by FACS. Relative apoptosis rates (%) were quantified. Data shown are representative of 2 experiments and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test).

To induce efficient overexpression of Toso in primary T cells, Toso cDNA was cloned into the pIRES/eGFP vector which contains an internal ribosome entry site (IRES) and allows the bicistronic expression of Toso and GFP protein in a ‘non-fusion’ form of the protein. This human Toso cDNA/IRES-GFP construct was introduced into activated primary T cells using

the nucleofector transfection system (3.2.1.6). 48h post-transfection, the cells were examined for GFP positive cells by flow cytometric analysis (Figure 4.18).

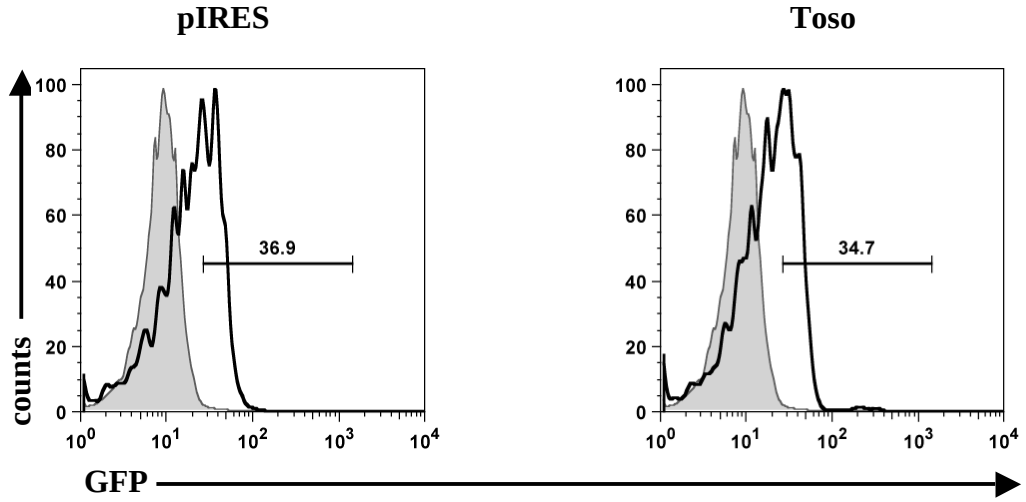


Figure 4.18: Transfection of activated human peripheral blood T cells with pIRES2/EGFP constructs.

Activated human peripheral blood T cells were transiently transfected with pIRES2/EGFP or Toso-EGFP-pIRES2 using the Amaxa nucleofection method. The transfection efficiency was determined by FACS analysis (gated on viable cells) for GFP expression 48 hours after transfection. The percentage of GFP expression is indicated in the histograms. Data shown are representative of 2 independent experiments.

After Nucleofector transfection, dead cells were removed using the "Dead Cell Removal Kit" (Miltenyi Biotech) (3.2.1.7). After the removal of dead cells, apoptosis was induced by stimulation with FasL. Cellular apoptosis was quantified by AnnexinV staining and FACS analysis of the GFP positive cell populations. The data showed that Toso overexpressing primary T cells (GFP positive population) were significantly less sensitive to Fas-induced apoptosis when compared to control transfected cells or GFP-negative (non-transfected) cells (Figure 4.19).

These results obtained with primary human T cells were consistent with the observations from experiments with BJAB and Jurkat cell lines. Therefore, my data clearly demonstrate an anti-apoptotic function of Toso in Fas-induced apoptosis both in transformed lymphoma cell lines and in primary T cells.

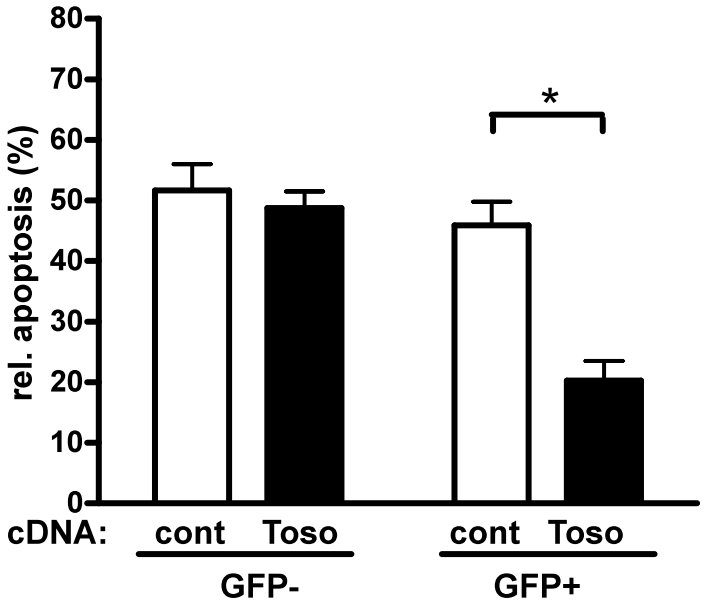


Figure 4.19: FasL-induced apoptosis in Toso overexpressing primary T cells.

Activated human peripheral blood T cells were transiently transfected with pIRES2/EGFP (white bars) or Toso-EGFP-pIRES2 (black bars) using Amaxa nucleofection. Cells were stimulated with FasL and apoptotic cells were determined by staining with AnnexinV and FACS analysis of GFP positive (transfected) and GFP negative (non-transfected) populations. Data shown are representative of two experiments and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test).

4.6 RIP1 is required for the anti-apoptotic function of Toso

To further explore the molecular mechanisms underlying the anti-apoptotic effects of Toso, I investigated whether RIP1 (receptor interacting protein 1), a protein involved in the activation of survival signaling pathways upon triggering of TNF-receptor family members (Ea *et al.*, 2006; Hsu *et al.*, 1996; Kelliher *et al.*, 1998; Ting *et al.*, 1996), is involved in Toso function. To this end, Toso was overexpressed in RIP1 deficient Jurkat cells and the anti-apoptotic function of Toso in Fas-induced apoptosis was compared to Toso overexpressing wild type Jurkat cells.

As previously shown (Figure 4.13A), overexpression of Toso clearly exhibits anti-apoptotic effects in wild-type Jurkat cells. However, Toso overexpression had no protective effects on Fas-induced apoptosis in RIP1 deficient Jurkat cells (Figure 4.20A). Moreover, RIP1 deficient Jurkat cells were much more sensitive to Fas-mediated apoptosis than wild-type Jurkat cells (Figure 4.20A). As control for RIP1 protein level, western blot analysis was

performed on cell lysates from wildtype or RIP1$^{-/-}$ Jurkat cells using RIP1 specific antibodies. Detection of β-actin was used to confirm the equal protein loading. As shown in Figure 4.20B, RIP1 was not detected in RIP1$^{-/-}$ Jurkat cells.

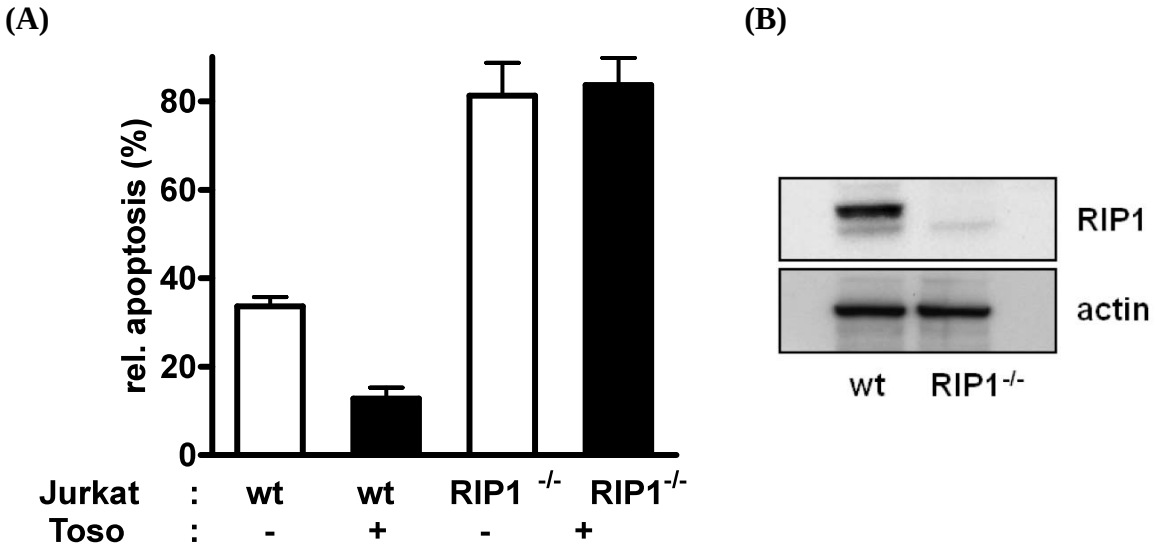


Figure 4.20: Toso overexpression does not show anti-apoptotic effects in RIP1 deficient Jurkat cells.

(A) Wild-type or RIP1 deficient Jurkat cells were transfected with a control or a Toso expressing construct. Cells were stimulated with 100 ng/ml FasL for 6 hours and apoptotic cells were analyzed by staining with AnnexinV. Relative apoptosis rates (%) were quantified. (B) RIP1 (top) and actin (bottom) expression levels in cell lysates of wild-type or RIP1 deficient Jurkat cells are shown by western blotting. Data are representative of 3 experiments and error bars represent the standard deviation of the mean.

While Toso overexpression showed no anti-apoptotic effects on Fas-induced apoptosis in RIP1 deficient Jurkat cells (Figure 4.20), my analysis of RIP1 on Toso function was further extended by performing Toso gene knock-down experiments using another human lymphoma cell line, BJAB cells. Employing shRNA-mediated gene knock-down, my studies also efficiently ablated RIP1 expression in BJAB cells stably expressing Toso. Toso overexpressing BJAB cells were transduced with lentiviral particles containing three different shRNA constructs specific for the RIP1 gene. Three days after lentiviral shRNA transfection, efficient RIP1 gene knock-down was confirmed by western blot analysis using RIP1 specific antibodies (Figure 4.21B). And then Fas-induced apoptosis was determined in RIP1 shRNA transfected cells or control cells. Similar to the results on RIP1 deficient Jurkat cells, Fas-induced apoptosis rates were significantly higher in Toso overexpressing and RIP depleted BJAB cells, as compared to Toso overexpressing control cells (Figure 4.21A). Together,

these data suggest that RIP1 is required for the anti-apoptotic function of Toso as revealed in both Jurkat and BJAB cell lines.

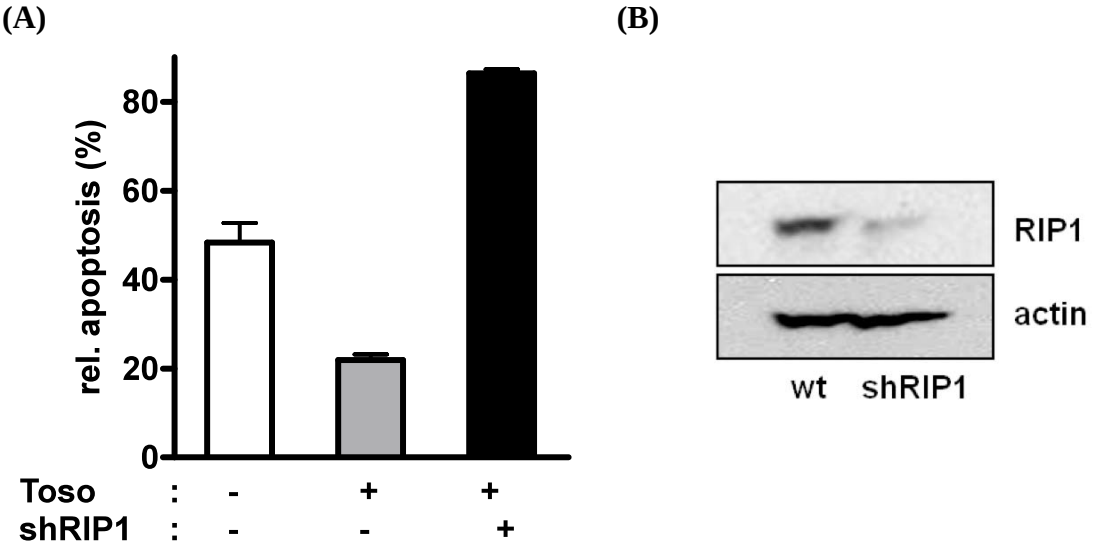


Figure 4.21: The knock-down of RIP1 in stable Toso overexpressing cells results in increased sensitivity to Fas-induced apoptosis.

(A) BJAB cells stably expressing Toso were transduced with lentiviruses expressing three different shRNAs specific for RIP1 or mock treated. Cells were stimulated with 200 ng/ml FasL for 5 hours and apoptotic cells were analyzed by staining with AnnexinV and FACS analysis. Relative apoptosis rates (%) were quantified. Data are representative of 2 experiments and error bars represent the standard deviation of the mean. (B) The RIP1 knock-down efficiency was monitored by immunoblotting for RIP1 (top). Actin staining was used as a control for protein loading (bottom).

Toso constitutively associates with RIP1

Since my studies have demonstrated that RIP1 is required for the anti-apoptotic function of Toso, my studies further examined whether RIP1 and Toso can either constitutively or inducibly associate with each other. In order to determine the interaction of Toso with RIP1, Toso immunoprecipitation was performed in Toso transiently overexpressing cells.

To this end, FLAG epitope-conjugated Toso protein was transiently expressed in BJAB or Jurkat cells and Toso protein and Toso associated proteins were isolated by immunoprecipitation using FLAG epitope specific antibody. Independently of cellular stimulation, RIP1 protein was specifically co-immunoprecipitated with Toso in Jurkat and BJAB tumor cells (Figure 4.22, lanes 1 and 2 and 7, 8, respectively). To control for the

specificity of the RIP1 detection, RIP1 deficient Jurkat cells were also included in this analysis (Figure 4.22, lane 3). Together these data indicate that Toso constitutively associates with RIP1. Thus, in BJAB and Jurkat cells, Toso is not only functionally, but also physically connected to RIP1.

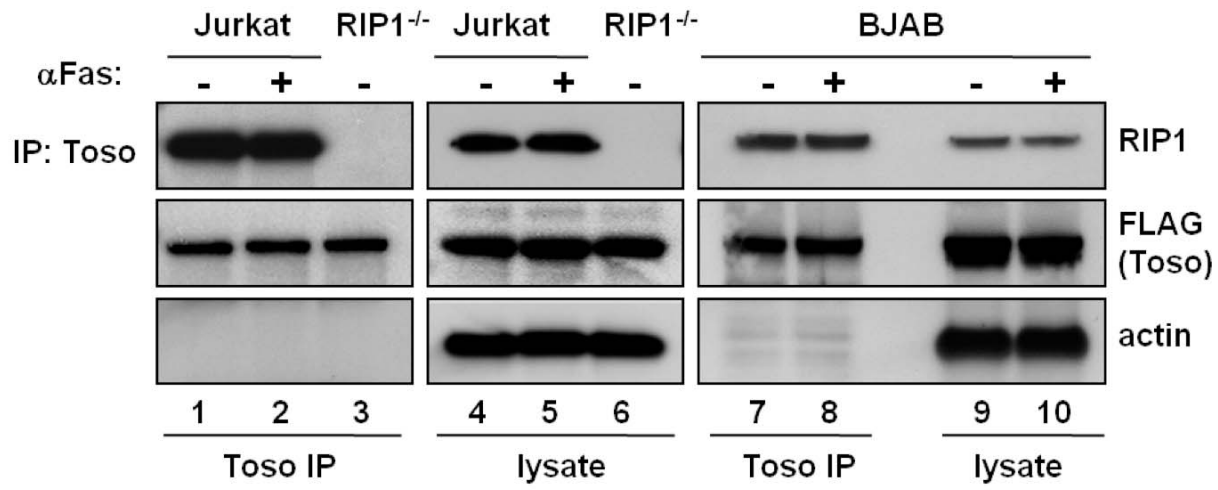


Figure 4.22: Toso constitutively associates with RIP1.

Wild-type (lanes 1, 2, 4, 5), RIP1 deficient (lanes 3, 6) Jurkat cells or wild-type BJAB cells (lanes 7-10) were transiently transfected with a Toso-FLAG construct. Cell lysates from anti-Fas (CH11) treated and untreated cells were immunoprecipitated (IP) using the anti-FLAG coupled agarose. Toso immunoprecipitates and corresponding total cell lysates were analyzed by western blotting for RIP1, FLAG, and actin. Representative data from 3 independent experiments are shown.

RIP1 ubiquitination is required for the anti-apoptotic function of Toso

As ubiquitination of RIP1 has been reported to be critical for the activation of NF-κB signaling pathways by TNFα (Bertrand *et al.*, 2008; Ea *et al.*, 2006; O'Donnell *et al.*, 2007) and RIP1 is also involved in the function of Toso, our hypothesis was that Toso might regulate RIP1 ubiquitination.

Site-specific ubiquitination of lysine 377 (K377) of RIP1 has recently been demonstrated to mediate efficient activation of the IKK complex by TNFα. To examine a potential involvement of K377 of RIP1 in the anti-apoptotic function of Toso, Toso overexpressing cells were transiently transfected with wild type RIP1 (RIP1-WT), or the RIP1-K377R

mutant, that does not undergo ligand-dependent ubiquitination and fails to activate IKK upon TNFα stimulation (Devin *et al.*, 2000; Ea *et al.*, 2006). Cells were then stimulated with FasL and apoptotic cell death was assessed (Figure 4.23). As seen before, reduced apoptosis was detected in Toso overexpressing cells as compared to control cells. Expression of RIP1-WT showed slightly protective effects at both concentrations of FasL stimulation. However, Toso overexpressing cells transfected with RIP1-K377R were significantly more susceptible to Fas-induced apoptosis than Toso overexpressing control cells or Toso overexpresssing cells transfected with RIP1-WT (Figure 4.23). Thus, Toso could not efficiently protect from Fas-mediated apoptosis in RIP1-K377R expressing cells.

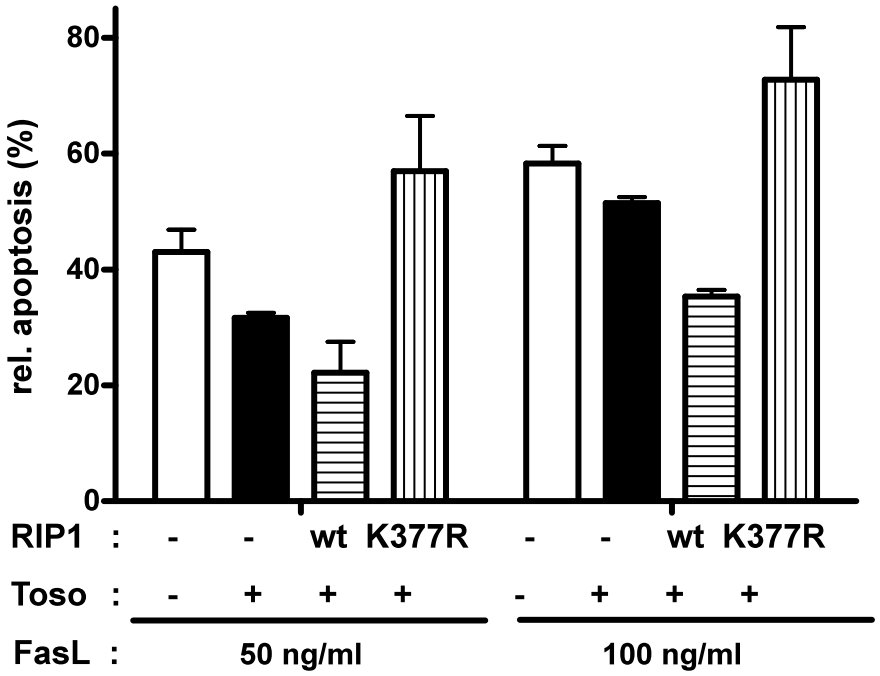


Figure 4.23: The polyubiquitination site at lysine 377 on RIP1 is required for the anti-apoptotic function of Toso.

Jurkat cells stably expressing Toso or wild-type Jurkat cells were transiently transfected with FLAG-RIP1 or FLAG-K377R RIP1 mutant. Cells were stimulated with the indicated concentrations of FasL for 12 hours and apoptosis was determined by staining with AnnexinV and FACS analysis. Relative apoptosis rates (%) were quantified. Representative data from 3 independent experiments are shown and error bars represent the standard deviation of the mean.

As a second independent approach, RIP1 deficient Jurkat cells were transiently transfected with control vector or cotransfected with RIP1-WT and Toso, or Rip1-K377R and Toso. To determine the transfection efficiency, transfected cells were lysed and analyzed for RIP1 and Toso expression by immunoblotting with respective specific antibodies. Data shown that no expression of RIP1 protein could be detected in RIP1 deficient Jurkat cells transfected with control vector (Figure 4-24A). In contrast, RIP1 and Toso were significantly overexpressed in cotransfected cells (Figure 4-24A). As shown before, Toso overexpression could not sufficiently protect from Fas-induced apoptosis in the absence of RIP1 (Figure 4.20A and 4.21A). When $RIP^{-/-}$ cells were transiently transfected with Toso and RIP1 expression constructs, Fas-induced apoptosis was significantly reduced (Figure 4.24B). However, Toso and RIP1-K377R expressing cells showed more sensitivity to Fas-induced apoptosis as compared to control cells (Figure 4.24B).

Together, our data suggest that Toso promotes the ubiquitination of RIP1 at K377 in response to Fas-triggering, and this ubiquitination step is an essential requirement for the anti-apoptotic function of Toso.

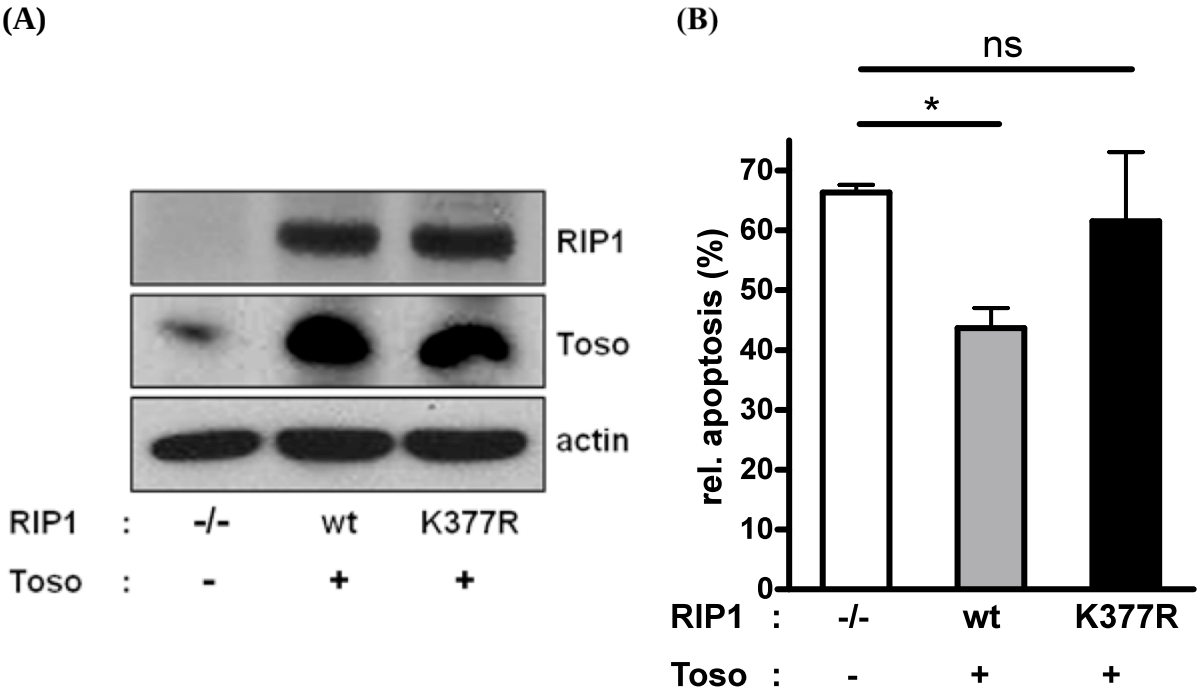


Figure 4.24: The overexpression of Toso does not block Fas-induced apoptosis in cells expressing RIP1 ubiquitination mutant, K377R-RIP1.

RIP1 deficient Jurkat cells were transfected with control vector, or cotransfected with Toso and wild-type RIP1 constructs, or Toso and K377R-RIP1 constructs. (A) Lysates from transfected cells were analyzed for RIP1 and Toso expression by immunoblotting with respective specific antibodies. (B) Apoptosis was induced by

stimulation with 50 ng/ml FasL for 6 hours and apoptosis was determined by staining with AnnexinV and subsequent FACS analysis. Relative apoptosis rates (%) were quantified. Representative data from 2 independent experiments are shown and error bars represent the standard deviation of the mean (*, $p < 0.05$ determined by *t*-test; ns, not significant).

Toso associates with FADD in a RIP1-dependent fashion

The C-terminus of the mouse Toso has been described to bind to FADD in *in vitro* binding assays (Song and Jacob, 2005). To investigate whether an association between Toso and FADD also exists *in vivo* and whether this association may be influenced by Fas-stimulation, Toso was immunoprecipitated from lysates of Fas-stimulated and unstimulated Jurkat cells. Precipitated proteins were then analyzed by immunoblotting using specific antibodies. As seen before (Figure 4.22), Toso was found to be constitutively associated with RIP1 (Figure 4.25, lanes 3 and 4). Moreover, FADD could be clearly detected in Toso immunoprecipitates from lysates of non-stimulated wild type Jurkat cells, and this association was significantly enhanced upon Fas-stimulation (Figure 4.25, lanes 3 and 4). Importantly, the association of Toso with FADD was almost completely abolished in RIP1 deficient Jurkat cells, independently of Fas-stimulation (Figure 4.25, lanes 1 and 2). Thus, *in vivo* the association of Toso with FADD surprisingly is dependent on RIP1.

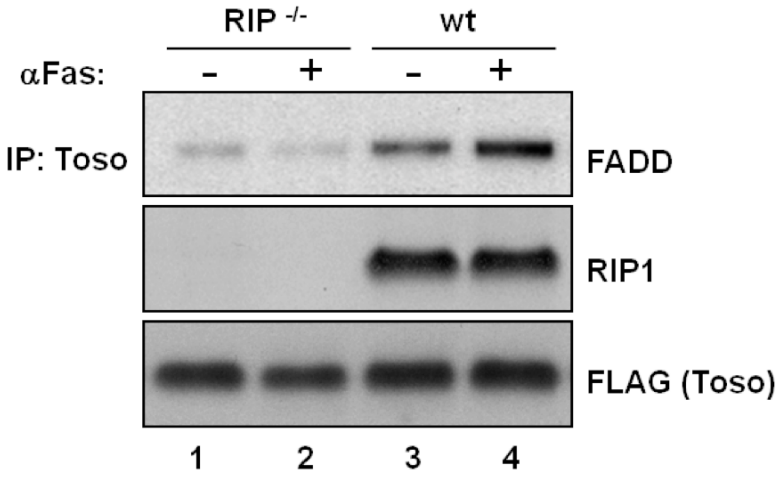


Figure 4.25: RIP1 is required for the association of Toso with FADD.
RIP1 deficient Jurkat cells (lanes 1-2) or wild-type Jurkat cells (3-4) were transfected with Toso-FLAG and stimulated with anti-Fas (CH11) for 15 minutes or left untreated. Cell lysates were immunoprecipitated with a FLAG-coupled agarose. Immunoprecipitates were analyzed by western blotting for RIP1, FLAG or FADD. Data are representative of 3 experiments.

The molecular interactions between RIP1, Toso and FADD were further examined in immunoprecipitation experiments, where I expressed Flag-tagged RIP1 either in control, Toso knock-down or Toso overexpressing Jurkat cells. After stimulation with anti-Fas for 0 or 15 minutes at 37 °C, the cells were lysed and subjected to immunoprecipitation using anti-Flag agarose. As shown in Figure 4.26 (lanes 3 and 4), low levels of RIP1 constitutively associate with FADD in non-stimulated Jurkat cells, and this association significantly increases upon Fas stimulation. Compared to normal Jurkat cells, the constitutive association of FADD with RIP1 was substantially higher in Toso overexpressing cells, where Fas-triggering however did not further enhance the association (Figure 4.26, lanes 5 and 6). Conversely, only low amounts of FADD were detected in RIP1 immunoprecipitates from Toso knock-down cells and in these cells, we also observed no further recruitment of FADD to RIP1 in response Fas-stimulation (Figure 4.26, lanes 1 and 2). Hence, the interaction between RIP1 and FADD is significantly influenced by the expression of Toso. Interestingly, we detected only a very weak association of FADD with the RIP1-K377R ubiquitination mutant in unstimulated Toso overexpressing cells and no further induction of FADD binding to RIP1-K377R could be observed upon Fas stimulation (Figure 4.26, lanes 7 and 8).

Together, the data suggest that in living cells Toso, RIP1 and FADD form a trimolecular protein complex. Deleting either Toso or RIP1 disrupts this protein complex, while Fas signaling seems to enhance the complex formation. Furthermore, the weak association of FADD with the RIP1-K377R mutant suggests, that RIP1 ubiquitination favors complex formation.

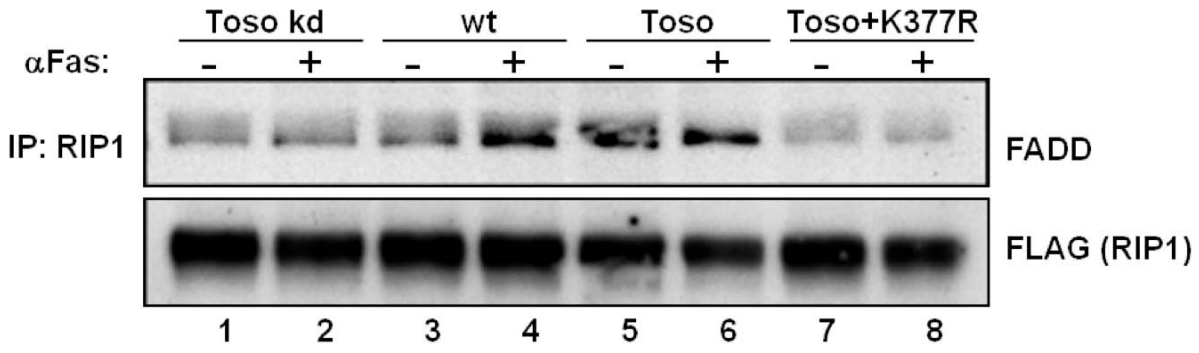


Figure 4.26: Toso and RIP1 ubiquitination promote the interaction of RIP1 with FADD.

Jurkat cells stably expressing Toso miRNA (lanes 1-2), wild-type (wt) Jurkat (lanes 3-4), or stable Toso overexpressing Jurkat cells (lanes 5-8) were transfected with FLAG-RIP1 (lanes 1-6) or FLAG-K377R RIP1 (lanes 7-8). Untreated cells (lanes 1, 3, 5, 7) or cells stimulated with anti-Fas (CH11) for 15 minutes (lanes 2, 4, 6, 8) were immunoprecipitated with anti-FLAG coupled agarose. Proteins bound to the RIP1

complexes were immunoblotted with antibodies against FADD (top) or FLAG (bottom). Data are representative of 2 experiments.

To further investigate the regulatory role of Toso for Fas-induced molecular signaling events Toso knock-down, Toso overexpressing or control Jurkat cells were stimulated with FasL. Fas was immunoprecipitated and subsequent immunoblotting for RIP1 revealed a constitutive association of RIP1 with Fas that was independent of Toso expression (Figure 4.27). Interestingly, in Toso overexpressing Jurkat cells Fas-stimulation induced a transient shift in the molecular weight of Fas associated RIP1 (Figure 4.27, lanes 7-9), indicative of posttranslational modifications of RIP1. This shift of RIP1 to a higher molecular weight was, however, not detectable in Fas immunoprecipitates of Toso knock-down cells or control Jurkat cells (Figure 4.27, lanes 1-6). Thus, in response to Fas-stimulation Toso overexpression promotes the posttranslational modification of RIP1.

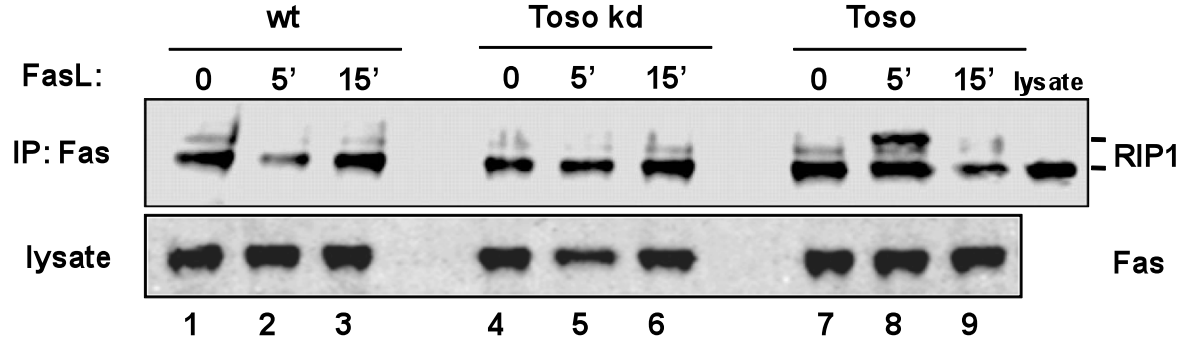


Figure 4.27: Toso overexpression facilitates the interaction of RIP1 with Fas.

Jurkat cells stably expressing control miRNA (lanes 1-3), Toso specific miRNA (lanes 4-6), or stable Toso overexpressing Jurkat cells (lanes7-9) were stimulated with FasL for the indicated times. Cell lysates were immunoprecipitated with an anti-Fas antibody (C20) and immunoprecipitates were immunoblotted for RIP1 (top). Fas protein levels in cell lysates are shown at the bottom. Data are representative of 3 experiments.

4.7 Toso mediates the activation of pro-survival signals

In an additional approach, I have attempted to interfere with the anti-apoptotic function of Toso by utilizing Toso-specific antibodies. By genetic immunization, we have developed monoclonal antibodies (mAb A36 and A38) that specifically recognize the extracellular portion of human Toso (Figure 4.28).

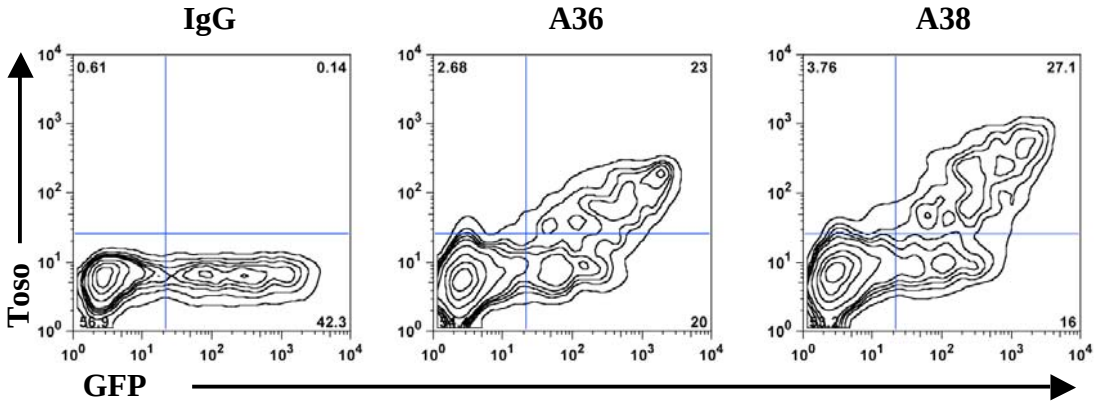


Figure 4.28: The specificity of anti-human Toso mAb A36 and A38.

Wild-type HEK cells were transiently transfected with pIRES2/EGFP or Toso-EGFP-pIRES2 by using lipofectamine 2000. The specificity of Toso mAb antibodies was assessed by analysis of GFP and Toso expression by FACS analysis after staining for anti-human Toso mAb A36, A38 or control rat IgG (gated on viable cells). Data shown are representative of 3 independent experiments.

In order to determine a potential blocking effect of Toso specific monoclonal antibodies on Fas-induced apoptosis in primary T cells, activated peripheral blood human T cells were pre-treated with the Toso specific antibodies A36, A38 or control rat IgG for 60 minutes. Apoptosis was induced by stimulation of cells with 100 ng/ml FasL for 5 hours. The relative apoptosis rate was determined by staining with AnnexinV and flow cytometric analysis.

The data showed that the anti-Toso mAb A36 had no effect on Fas-mediated apoptosis of human primary T lymphocytes; however, the anti-Toso mAb A38 significantly enhanced Fas-induced apoptosis as compared to control-Ig treated cells (Figure 4.29). Importantly, the addition of anti-Toso mAb A38 specifically increased Fas-mediated apoptosis, but did not induce apoptosis/cell death by its own. These results indicate that the Toso specific mAb A38 specifically block the anti-apoptotic function of Toso in response to Fas stimulation. However, the mechanism of action of this Toso blocking antibody (A38) is currently unclear and will be an interesting area of investigation for future studies.

Together, utilizing different experimental systems my studies clearly demonstrate that the cell surface protein Toso exhibits an inhibitory function on Fas-mediated apoptosis.

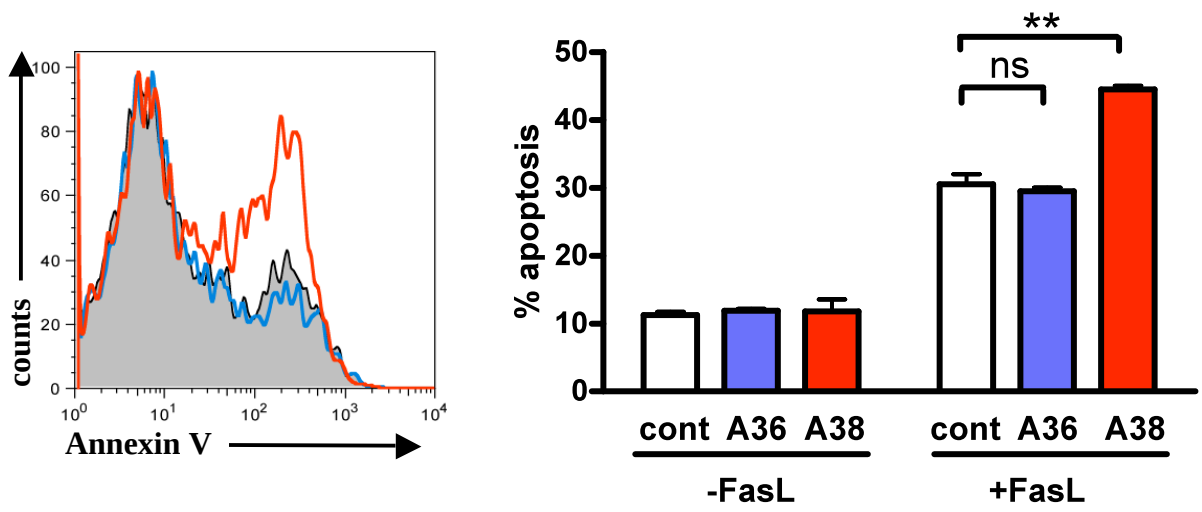


Figure 4.29: Anti-human Toso mAb A38 enhances Fas-mediated apoptosis.

Human primary T cells were pre-incubated for 60 minutes with 20 µg/ml of anti-human Toso mAb A36, A38 or control rat IgG. Cells were then stimulated for 16 hours with 100 ng/ml FasL and apoptosis was assessed by AnnexinV staining. Representative flow cytometric histograms from cells treated with control IgG (grey filled), anti-human Toso mAb A36 (blue) or anti-human Toso mAb A38 (red) are shown on the left. Relative % of apoptosis is quantified on the right. Data are representative of 4 experiments and error bars represent the standard deviation of the mean (**, $p < 0.01$ determined by *t*-test; ns, not significant).

RIP1 is involved in TNFα-induced NF-κB signaling and MAP kinase activation (Li *et al.*, 2006; Ting *et al.*, 1996). As RIP1 is also mediating the anti-apoptotic effects of Toso, I next investigated whether in response to TNFα stimulation, anti-Toso mAbs could also modulate cell survival signaling pathways, such as the activation of MAP kinase signaling pathways.

Human primary T lymphocytes were stimulated with TNFα and analyzed for Erk-activation. Freshly isolated human peripheral blood T cells were pre-treated with Toso specific monoclonal antibodies A36, A38, or rat control IgG for 60 minutes, and then cells were activated with 100 ng/ml TNFα for 10 minutes. Erk-activation was examined by intracellular flow cytometric analysis using a phospho-Erk specific antibody.

Addition of anti-TOSO mAb A36 did not influence TNFα-induced Erk-phosphorylation when compared to control-IgG treatment (Figure 4.30). However, pre-incubation of cells with anti-TOSO mAb A38 almost completely blocked TNFα-mediated Erk-activation in human T cells (Figure 4.30). Thus, anti-Toso mAb A38, but not mAb A36, specifically blocks the anti-

apoptotic function of Toso, as well as the TNFα-mediated activation of MAPK signaling pathways.

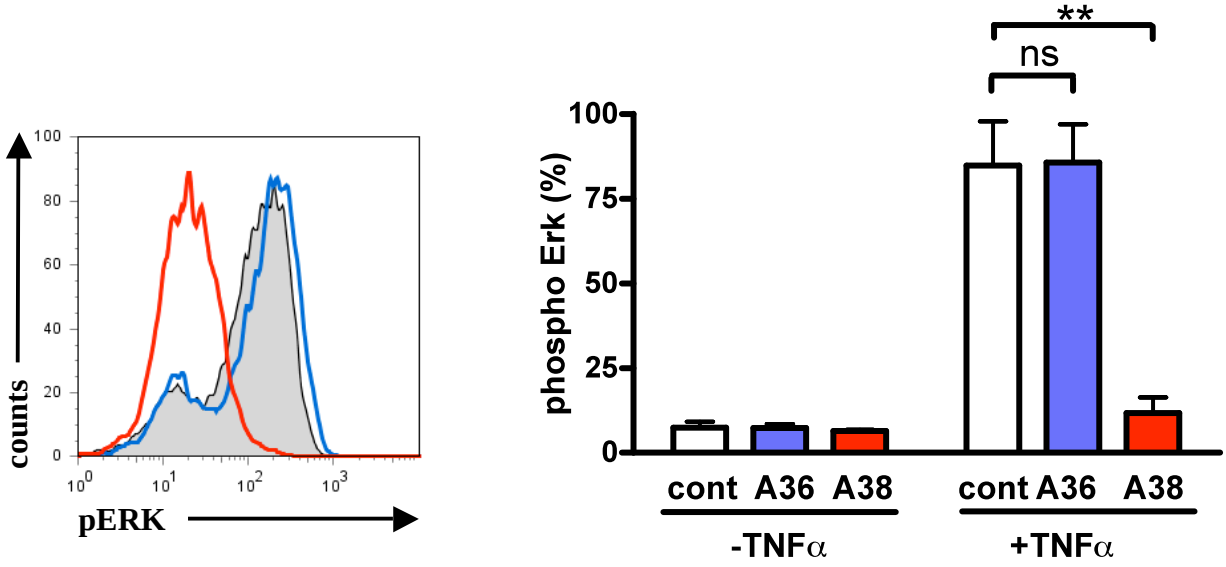


Figure 4.30: Anti-human Toso mAb A38 inhibits TNFα-mediated Erk-phosphorylation.

Human primary T cells were pre-incubated for 60 minutes with 20 µg/ml of human Toso mAb A36, A38 or control rat IgG. Cells were then stimulated for 10 minutes with 100 ng/ml TNFα. Erk-phosphorylation was examined by intracellular flow cytometric analysis using a phospho-Erk specific antibody. Representative flow cytometric histograms from cells treated with control IgG (grey filled), anti-human Toso mAb A36 (blue) or anti-human Toso mAb A38 (red) are shown on the left. Relative % of Erk-phosphorylation is quantified on the right. Data are representative of 3 experiments and error bars represent the standard deviation of the mean (**, $p < 0.01$ determined by *t*-test; ns, not significant).

These data indicate that the anti-apoptotic cell surface protein Toso is also able to promote TNF receptor-mediated activation of pro-survival signals, such as the activation of Erk signaling cascades.

Taken together, human Toso-specific mAbs significantly enhance sensitivity to death receptor-mediated apoptosis, and block TNFα-mediated Erk-signaling pathways in primary T cells. As Toso overexpression is frequently observed in autoimmune T cells and leukemias, results from antibody blocking experiments could be highly relevant for the development of novel clinical applications for the treatment of autoimmune disorders and cancer.

5. Discussion

Fas (CD95) is best characterized for its death inducing signaling activities. However, by mechanisms that are still poorly defined, Fas can also transduce pro-survival signals. Here my studies demonstrate that the transmembrane protein Toso acts as a novel regulator of Fas signaling by facilitating Fas-induced pro-survival signaling cascades. The anti-apoptotic function of Toso depends on RIP1 as an effector molecule. Toso is constitutively associated with RIP1 and, in response to Fas stimulation, the RIP1 ubiquitination is essentially required for the anti-apoptotic function of Toso. Ultimately, Toso mediates MAPK and thereby induces a shift in the balance of pro- to anti-apoptotic Fas-mediated signaling pathways.

5.1 Toso is a novel negative regulator for Fas-induced apoptosis

In this study, the expression levels of Toso have been modulated by specific gene knock-down and by overexpression. My studies reveled that the expression of Toso inhibits Fas-induced apoptosis in a dose dependent fashion. Together with previous studies that have reported protective functions of Toso in overexpression and transgenic systems (Hitoshi *et al.*, 1998; Song and Jacob, 2005), these data solidly establish an anti-apoptotic function of Toso in Fas signaling. Thus, Toso represents the unique case of a cell surface molecule that solely by cellular expression exhibits anti-apoptotic effects on the Fas-mediated signaling cascade.

The inhibitory effects of Toso on Fas-induced apoptosis were observed in Type I, as well as in Type II cells, that differ primarily in the level of DISC formation and in the production of active caspase-8. I also did not observe any significant effects on Fas-mediated capase-8 activation in Toso knock-down BJAB cells (Type I cells). Thus, Toso might exhibit its anti-apoptotic function by acting on signaling events that either lie downstream or in parallel to the activation of initiator caspase-8. This contrasts with a previous study reporting an inhibition of caspase-8 processing upon overexpression of Toso (Hitoshi *et al.*, 1998). This latter study, however, was performed on Jurkat cells, which, as Type II cells, only poorly activate caspase-8. Also, as the effects on caspase-8 cleavage were observed relatively late in the apoptotic program, they might have arisen from secondary effects of already activated downstream caspases. A caspase-independent mechanism of Toso function was further supported by our finding that Toso also inhibits caspase-independent necrotic forms of

TRAIL-R- and TNF-R-induced cell death (data not shown, Hitoshi *et al.*, 1998). Triggering of the Fas or tumor necrosis factor (TNF) family of death-domain (DD) receptors usually activates the canonical apoptotic pathway, which is initiated and executed by members of the caspase family. However, caspase inhibition does not always prevent cell death. Instead, it has revealed the existence of a necrotic signaling pathway leading to cell death. Therefore, it is tempting to speculate that Toso might be also involved in signaling pathway leading to caspase independent necrotic cell death.

5.2 RIP1 is required for Toso function

RIP1 was initially discovered in a yeast two-hybrid screen as an interaction partner of Fas (Stanger *et al.*, 1995) and also binds to other death receptors and death domain containing adaptors. As recent data demonstrate a requirement of the adaptor kinase RIP1 for caspase independent necrotic cell death induced by Fas (Holler *et al.*, 2000; Kawahara *et al.*, 1998), we hypothesized that RIP1 might be involved in Toso function. In agreement with this concept, Toso was constitutively associated with RIP1 in Toso overexpressing cells and the inhibitory activity of Toso was largely abolished in RIP1 knock-down or null mutant cells. Together, these data indicate that Toso utilizes RIP1 as a downstream effector molecule to mediate its anti-apoptotic function.

The role of RIP1 in Fas-mediated apoptosis is, however, still controversial (Ting *et al.*, 1996). RIP1 also binds to other death receptors and death domain containing adaptors and in TNF-receptor signaling has been demonstrated to be involved in the activation of NF-κB signaling pathways. Interestingly, the kinase domain of RIP1 is not required to mediate NF-κB activation by TNFα (Ting *et al.*, 1996). Utilizing a RIP1-K377R mutant, TNF-mediated activation of IKK has, however, recently been demonstrated to require site-specific ubiquitination of RIP1 at K377 (Ea *et al.*, 2006). The studies in our laboratory have demonstrated that Fas-stimulation also transiently induced RIP1 ubiquitination, which was dependent on Toso expression (data not shown). My studies have demonstrated that the RIP1-K377R mutant efficiently suppressed the anti-apoptotic function of Toso. Whether ubiquitinated RIP1 is subsequently targeted for proteosomal degradation is currently unclear. Together, our data suggest that Toso promotes the ubiquitination of RIP1 at K377 in response

to Fas-triggering, and this ubiquitination step is an essential requirement for the anti-apoptotic function of Toso.

5.3 Toso, RIP1 and FADD form a trimolecular complex

Furthermore, my studies indicate that upon Fas stimulation Toso, RIP1 and FADD form a trimolecular complex. This observation extends previous reports describing an interaction between the death domain of RIP1 and FADD (Vanden Berghe *et al.*, 2004), and an *in vitro* interaction between FADD and the C-terminus of Toso (Song and Jacob, 2005).

While Toso and RIP1 interact constitutively with each other, the association of FADD with the complex is stabilized by Fas-stimulation. My studies employing the RIP1-K377R ubiquitination mutant indicate that efficient recruitment of FADD also depends on RIP1 ubiquitination. Interestingly, Fas-stimulation induces the ubiquitination of RIP1 in Jurkat T cells and a posttranslationally modified, presumably ubiquitinated form of RIP1 transiently associates with FAS in response to FAS ligation. While the exact molecular mechanism that couples the Toso/RIP1 protein complex to the FAS signaling machinery is still not completely resolved, my current working hypothesis is, that Toso facilitates the ubiquitination of RIP1 on lysine 377 upon FAS stimulation.

Analogous to TNF receptor signaling, RIP1 ubiquitination may involve the E3 ligases cIAP1/2 and TRAF2 (Bertrand *et al.*, 2008; Lee *et al.*, 2004; Legler *et al.*, 2003; Wertz *et al.*, 2004). Ubiquitinated RIP1 can then recruit FADD to form a trimolecular protein complex consisting of Toso, RIP1 and FADD. The formation of this protein complex may serve a dual function. First, this complex may serve as a molecular sink for the death adaptor FADD, which then would not be available for efficient apoptotic signal propagation at activated FAS receptors, thereby decreasing apoptosis. In addition, the interaction between RIP1 and Toso with FADD may interfere with the recruitment of pro-caspase 8 to FADD molecule and/or its activation. Second, Toso and ubiquitinated RIP1 may exhibit an additional anti-apoptotic function by activating cellular pro-survival signals. Similar functions have been described for ubiquitinated RIP1 in the TNFα system. TNFα-induced ubiquitination of RIP1 at K377 prevents its association with caspase-8 and in a second function is involved in the activation of NF-κB signaling (O'Donnell *et al.*, 2007). It has been suggested that the second pro-

survival signal provided by the ubiquitination of RIP1 becomes effective via the up-regulation of NF-κB-dependent anti-apoptotic genes such as c-FLIP (O'Donnell *et al.*, 2007).

Interestingly, previous studies on Toso have proposed that Toso mediates its anti-apoptotic functions via increased transcription of the anti-apoptotic protein cFLIP and, in turn, inhibition of caspse-8 (Hitoshi *et al.*, 1998). My study could, however, not detect any significant changes in cFLIP mRNA levels upon overexpression of Toso in BJAB cells (data not shown), and also in other experimental systems increased Toso expression did not positively correlate with cFLIP expression (Proto-Siqueira *et al.*, 2008; Sigruener *et al.*, 2007). Furthermore, my studies indicate that Toso exhibits its protective effects on cell death independently of caspase-8 activation.

5.4 Toso mediates the activation of pro-survival signals

My studies and unpublished recent data from our laboratory demonstrate that Toso expression promotes the activation of death-receptor-mediated pro-survival signals. In my studies attempted to interfere with the anti-apoptotic function of Toso, as well as with the activation of cell survival pathways by utilizing Toso-specific antibodies. By genetic immunization, we have developed monoclonal antibodies (mAb A36 and A38) that specifically recognize the extracellular portion of human Toso. Anti-Toso mAb A36 had no effect on Fas-mediated apoptosis of human primary T lymphocytes; however, anti-Toso mAb A38 significantly enhanced Fas-induced apoptosis. Importantly, the addition of anti-Toso mAb A38 specifically increased Fas-mediated apoptosis, but did not induce apoptosis/cell death by its own. Moreover, addition of anti-TOSO mAb A38 almost completely blocked TNFα-mediated Erk-activation in human T cells. Thus, anti-Toso mAb A38 specifically blocks the anti-apoptotic function of Toso, as well as the TNFα-mediated activation of MAPK signaling pathways.

These observations suggest that Toso exhibits its anti-apoptotic effects by supporting Fas-mediated cellular activation pathways. Although the prototypic death receptor FAS is primarily recognized as a death-inducing receptor, accumulating evidence indicates that FAS ligation can also induce non-apoptotic signaling pathways (Algeciras-Schimnich *et al.*, 2003; Chen *et al.*, 2006; Lee *et al.*, 2006; Tamm *et al.*, 2004). Moreover, it has been proposed that

death receptor stimulation may induce apoptotic and non-apoptotic signals in all cells to some extent. The balance of pro- versus anti-apoptotic signals then defines the specific cellular response induced by death receptor stimulation. In the Fas system apoptotic signaling through caspases typically predominates. Only little is known about the molecular events that connect Fas signaling to the activation of pro-survival signals. My findings on the novel anti-apoptotic mechanism of Toso provide a molecular basis for Fas-induced activation of pro-survival signals.

Toso utilizes RIP1 as a downstream effector molecule. RIP1 can generate either pro-survival or pro-apoptotic signals depending on its ubiquitination state. Deubiqutinated RIP1 functions as a pro-apoptotic adaptor in response to TNFR1 (O'Donnell *et al.*, 2007), whereas polyubiquitinated RIP1 has anti-apoptotic properties and promotes cancer cell survival (Bertrand *et al.*, 2008; Devin *et al.*, 2003). Toso facilitates RIP1 ubiquitination in response to FAS ligation, which is an essential requirement for the anti-apoptotic function of Toso. An involvement of RIP1 has been reported for TNF- and TRAIL-mediated activation of NF-κB and MAP kinase signaling pathways (Devin *et al.*, 2000; Devin *et al.*, 2003; Ea *et al.*, 2006). It is therefore, not surprising that Toso not only exhibits anti-apoptotic effects on FAS-mediated apoptosis, but, according to my data and other reports (Hitoshi *et al.*, 1998; Shiiki *et al.*, 2000), also inhibits TNF- and TRAIL-mediated forms of cell death. Together, these observations support the idea that cells utilize common regulatory themes, such as the Toso/RIP1 pathway, to modulate death receptor signaling.

5.5 Potential role of Toso in autoimmunity

Apoptosis is tightly controlled in the immune system and even a small imbalance in the regulation of cell death and cell survival can result in disastrous pathological outcomes, such as autoimmunity or immune deficiency.

Gene expression studies have revealed that Toso mRNA levels are tightly regulated in during T cell activation. At early stages of T cell activation Toso gene expression is transiently increased, while Toso expression is hardly detectable during later stages of T cell activation (Abbas *et al.*, 2005, and data not shown; Hitoshi *et al.*, 1998). Given the anti-apoptotic function of Toso, coordinated expression of Toso during an immune response may therefore,

control survival of early effector T cells and permit apoptotic cell death during the contraction phase. A recent study has reported an overexpression of Toso in chronic lymphocytic leukemia (CLL), which is associated with progressive disease (Pallasch *et al.*, 2008; Proto-Siqueira *et al.*, 2008).

The overexpression of Toso is also associated with autoimmune disease as demonstrated by gene chip analysis. Toso has been identified as a significantly up-regulated gene in $CD4^+$ T cells of SLE patients, associated with the disease severe active state (Deng *et al.*, 2006). In addition, overexpression of Toso has also been observed in peripheral blood mononuclear cells from acute multiple sclerosis (MS) patients (Achiron *et al.*, 2007). It is tempting to speculate that the increased level of Toso expression in autoreactive T cells or leukemia cells renders these cells resistant to Fas-induced apoptosis. Whether Toso may promote non-apoptotic signal pathways through Fas under these particular pathological conditions is an exciting possibility that should be investigated.

Interestingly, the anti-apoptotic function of Toso, as well as TNFα-mediated activation of MAPKs could be blocked by a Toso specific monoclonal antibody (mAb A38). These findings may lead to the development of novel therapeutic applications for the treatment of autoimmune disorders and leukemias. By utilizing Toso blocking antibodies it may be possible to overcome resistance to death receptor-mediated apoptosis in Toso overexpressing autoreactive and/or leukemia cells. The mechanism of action of Toso blocking antibodies is currently unclear. The Toso-blocking antibody A38 is directed against the extracellular domain of Toso. Functional domain analysis has revealed a requirement of the extracellular immunoglobulin domain of Toso for its anti-apoptotic function (Hitoshi *et al.*, 1998). Amongst other possibilities, Toso blocking antibodies may interfere with immunoglobulin domain-mediated oligomerization of Toso or block the binding of so far unidentified *cis*- or *trans*-acting ligands for Toso.

5.6 Conclusion and perspectives

The recently identified transmembrane protein Toso has been implicated in the regulation of apoptosis in lymphocytes. Toso expression is restricted to the immune system and is associated with human autoimmune and neoplastic disorders.

This study firmly demonstrated that Toso represents the unique case of a cell surface molecule that solely by cellular expression protects from Fas-induced apoptosis. My data provide genetic evidence to establish a novel molecular mechanism for the anti-apoptotic function of Toso that involves the adaptor kinase RIP1 (Figure 5.1). The protective function of Toso requires RIP1 ubiquitination and involves the inducible formation of a trimolecular complex consisting of Toso, RIP1 and FADD. Furthermore, the study demonstrated that human Toso-specific mAbs significantly enhance sensitivity to death receptor-mediated apoptosis, and block TNFα-mediated Erk-signaling pathways in primary T cells (Figure 5.1). As Toso overexpression is frequently observed in autoimmune T cells and leukemias, results from our antibody blocking experiments could be highly relevant for the development of novel clinical applications for the treatment of autoimmune disorders and cancer.

This study provides strong evidence for non-apoptotic signaling of the prototypic death receptor Fas and provide a new molecular basis for how the execution of death receptor mediated pro-survival and pro-apoptotic signaling can be regulated in the immune system.

Cells likely utilize common regulatory themes, such as the Toso/RIP1 pathway, to control death receptor-mediated signal transduction. Thus, these data provide novel insight into how survival and death signals are regulated and integrated into cellular fate decisions. Such knowledge is also critical for our understanding of autoimmune disorders and may open up new prospects for potential new diagnostic and therapeutic applications.

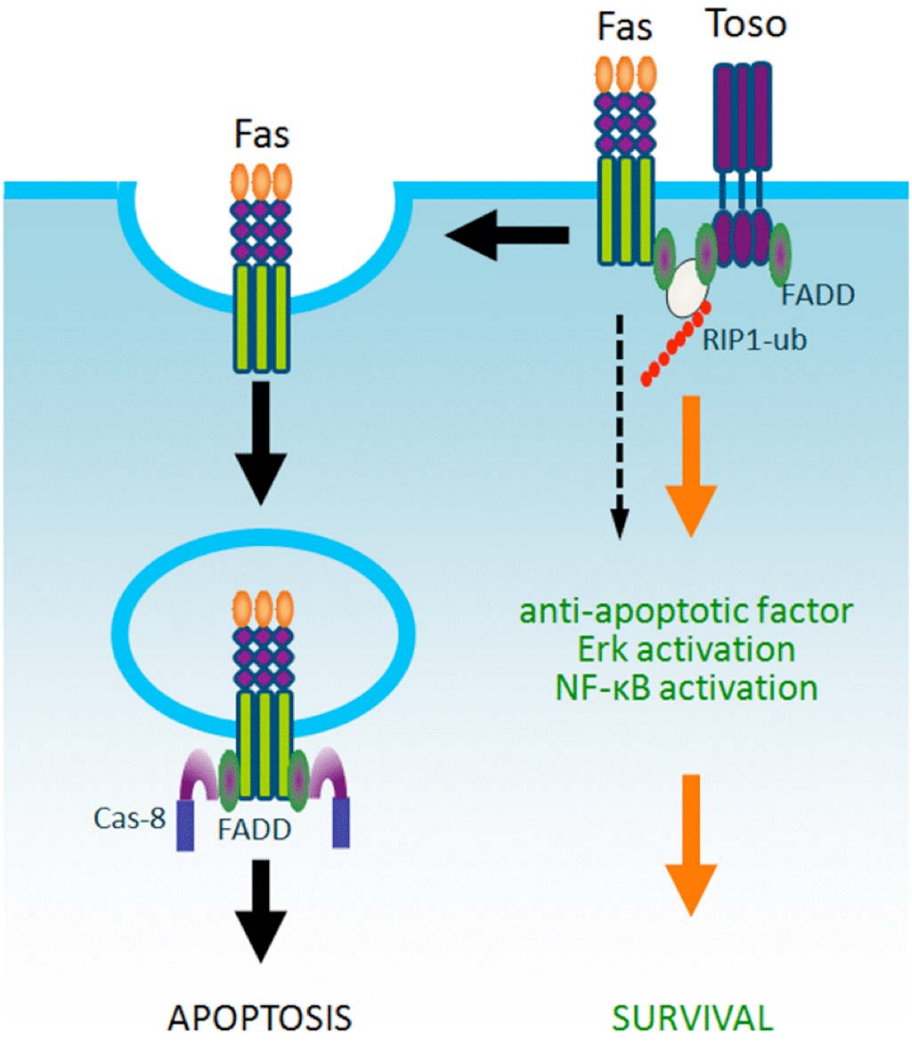


Figure 5.1: A model for the role of Toso in Fas-mediated apoptosis.

Fas mediated apoptotic signaling occurs at endosomal compartments after internalization. However, Fas ligation can also induce non-apoptotic signaling pathways at the plasma membrane in a particular cellular context. Toso is typically overexpressed under *in vivo* inflammatory conditions and CLL tumor cells. This Toso overexpression promotes the ubiquitination of RIP1 and the formation of trimolecular protein complex consisting RIP1, Toso and FADD. Ubiquitinated RIP1 subsequently induces the pro-survival signaling such as Erk and NF-κB pathways.

6. Summary

Toso, also known as Faim3, is a type I transmembrane protein belonging to the immunoglobulin gene superfamily. Toso was originally identified as a surface molecule acting as a potential negative regulator of lymphocyte apoptosis. Toso expression is restricted to the immune system and is associated with human autoimmune and neoplastic disorders. The biological function of Toso is however still elusive and the anti-apoptotic mechanism of Toso is only insufficiently understood.

The present study investigated how Toso participates in the regulation of apoptotic and survival signaling pathways in response to Fas in lymphocytes. Employing miRNA mediated specific Toso gene knock-down, my study firmly demonstrates that Toso represents the unique case of a cell surface molecule that solely by cellular expression protects from Fas-induced apoptosis. The study further provides genetic evidence to establish a novel molecular mechanism for the anti-apoptotic function of Toso that involves the adaptor kinase RIP1. Toso constitutively associates with RIP1 and the anti-apoptotic mechanism of Toso function involves ubiquitination of RIP1 at K377.

My study revealed that the activation of Fas induces the formation of a trimolecular complex consisting of Toso, RIP1 and FADD, which probably promotes the activation of pro-survival signaling mediated by Fas. Moreover, I could demonstrate that human Toso-specific mAbs significantly enhance sensitivity to death receptor-mediated apoptosis, and block TNFα-mediated Erk-signaling pathways in primary T cells. As Toso overexpression is frequently observed in autoimmune T cells and leukemias, results from antibody blocking experiments could be highly relevant for the development of novel clinical applications for the treatment of autoimmune disorders and cancer.

The present study provides strong evidence for non-apoptotic signaling of the prototypic death receptor Fas. Furthermore, the Toso/RIP1 pathway provides a new molecular basis for how the execution of Fas mediated pro-survival and pro-apoptotic signaling can be regulated in the immune system.

7. Zusammenfassung

Toso, auch Faim3 genannt, ist ein Typ I Transmembranprotein, das zur Superfamilie der Immunglobuline gehört. Ursprünglich wurde Toso als ein Zelloberflächenprotein identifiziert, welches eine potentiell negative regulatorische Funktion bei der Apoptose von Lymphozyten ausübt. Die Expression von Toso ist beschränkt auf Zellen des Immunsystems. Beim Menschen ist eine erhöhte Expression von Toso mit Autoimmunstörungen und neoplastischen Erkrankungen assoziiert. Die biologische Funktion von Toso ist jedoch immer noch nicht hinreichend charakterisiert und unser Wissen über den anti-apoptotischen Mechanismus von Toso ist noch äußerst lückenhaft.

In der vorgelegten Studie wurden die molekularen Mechanismen untersucht, über die Toso an der Regulation von Fas-induzierten pro- und anti-apoptotischen Signalübertragungs-wegen in Lymphozyten beteiligt ist. Mittels miRNA vermitteltem spezifischen ‚knock-down' von Toso, konnte in der vorliegenden Arbeit klar aufgezeigt werden, dass allein durch die Expression von Toso auf der Zelloberfläche ein signifikanter Schutz vor Fas-induzierter Apoptose ausgeübt wird. Darüber hinaus erbringt die Studie den genetischen Nachweis für einen neuartigen Mechanismus der anti-apoptotischen Funktion von Toso, welcher die Adaptorkinase RIP1 mit einbezieht. Toso bindet konstitutiv an RIP1, und der anti-apoptotische Mechanismus von Toso verläuft über die Ubiquitinierung von RIP1 an Lysin 377 (K377).

In meiner Studie konnte weiterhin gezeigt werden, dass die Stimulation von Fas die Bildung eines trimolekularen Komplexes, bestehend aus Toso, Rip1 und FADD, induziert. Die vorgelegten Daten unterstützen die Arbeitshypothese, dass dieser Proteinkomplex die Aktivierung von Fas-induzierten ‚pro-survival' Signalen vermittelt. Des Weiteren konnte ich in meiner Arbeit aufzeigen, dass spezifische monoklonale Antikörper gegen humanes Toso eine Verstärkung der Todesrezeptor (‚death receptor')-vermittelten Apoptose bewirken, als auch TNFα-vermittelte Erk-Signalübertragungswege blockieren. Da in autoimmunen T Lymphozyten und bei Leukämien häufig eine Überexpression von Toso beobachtet wird, könnten die Ergebnisse meiner Antikörper-Blockierungsstudien weitreichende Bedeutung für die Entwicklung neuer innovativer Behandlungsmethoden gegen Autoimmunerkrankungen und Krebsleiden haben.

Die vorgestellten Ergebnisse erbringen einen klaren Nachweis für die Aktivierung von nicht-apoptotischen Signalen über den prototypischen Todesrezeptor Fas. Außerdem stellt der hier identifizierte Toso/RIP1 Signalübertragungsweg eine neue, bislang unbekannte molekulare Basis für die Regulation von Fas-vermittelten ‚pro-survival' und ‚pro-apoptotischen' Signalen im Immunsystem dar.

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List of own publications

Papers

Xuan-Hung Nguyen**,** Philipp A. Lang, Gulnar Fattakhova, Dieter Adam, Niko Föger, Md. Azhar Kamal, Patricia Prilla, Sabine Mathieu, Christina Wagner, Karl S. Lang, Tak Mak, Andrew C. Chan and Kyeong-Hee Lee. Toso regulates the balance between apoptotic and non-apoptotic death receptor signalling by facilitating RIP1 ubiquitination. *Under revision: Nature Medicine***.**

Selected oral presentations

Xuan-Hung Nguyen, Gulnar Fattakhova, Kyeong-Hee Lee. 2008. Toso: A novel regulator of signal transduction for survival and apoptosis in lymphocytes. 31st North German Immunology workshop. November. Borstel: Germany.

Selected poster presentations

Xuan- Hung Nguyen, Kyeong-Hee Lee. 2008. Toso: A novel regulator of signal transduction for survival and apoptosis in T lymphocytes. Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI). Vienna: Austria. September 3th-6th.

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Erklärung

Die vorliegende Arbeit wurde von Juni 2007 bis Februar 2010 unter der Betreuung von Frau Dr. Kyeong-Hee Lee, am Forschungszentrum Borstel in der Laborgruppe Molekulare Immunologie angefertigt.

Ich versichere, dass ich die vorliegende Dissertation ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Hilfsmittel verwendet habe. Weder vor noch gleichzeitig habe ich andernorts einen Zulassungsantrag gestellt oder diese Dissertation vorgelegt. Ich habe mich bisher noch keinem Promotionsverfahren unterzogen.

Lübeck, den 15.03.2010