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# CD95-RESISTANCE IN LONG-TERM-ACTIVATED CYTOTOXIC T LYMPHOCYTES

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# Contents

**Abbreviations** ..... **III**

**1. Introduction** ..... **1**

1.1 Cell death ..... 1

1.2 Function of T lymphocytes and their subsets ..... 8

1.3 T cell signaling ..... 13

1.4 NF-κB ..... 18

1.5 Aim of the study ..... 23

**2. Material & Methods** ..... **24**

2.1 Material ..... 24

2.2 Methods ..... 32

2.2.1 Cell culture ..... 32

2.2.2 Generation of alloreactive long-term-activated, CD95-resistant T cells ..... 32

2.2.3 Western Blot ..... 34

2.2.4 Lipid Rafts ..... 35

2.2.5 Electrophoretic Mobility Shift Assay (EMSA) ..... 37

2.2.6 Gene Chips ..... 38

2.2.7 2D-Difference-In-Gel-Electrophoresis (2D-DIGE) ..... 38

**3. Results** ..... **42**

3.1 Part I: Relevance of NF-κB for CD95 resistance in long-term-activated T cells ..... 42

3.1.1 Repeated TCR stimulation induces CD95 resistance in long-term-activated T cells ..... 42

3.1.2 Both, short- and long-term-activated T cells, display constitutive caspase activation ..... 44

3.1.3 Expression of NF-κB signaling mediators differs between short- and long-term-activated T cells ..... 45

3.1.4 NF-κB is persistently active in short- and long-term-activated T cells ..... 47

3.1.5 Inhibition of NF-κB at different sites did not revert CD95 resistance of long-term-activated T cells ..... 48

3.1.6 NF-κB signaling mediators are recruited into lipid rafts after TCR activation ..... 50

3.1.7 Mediators of TCR-, NF-κB- and CD95 signaling are constitutively associated with lipid rafts in apoptosis-sensitive and -resistant T cells ..... 50

3.1.8 Screening by 2D-DIGE revealed no proteins known to be directly involved in regulation of apoptosis ..... 53

3.2 Part II: CD95 resistance in Jurkat cell lines ..... 56

3.2.1 The Jurkat cell lines JM301 and JM319 are CD95- resistant despite receptor surface expression ..... 56

3.2.2 The gene expression profile of Je6.1, JM, and JM301 and JM319 correlates with CD95 sensitivity ..... 57

3.2.3 Gene chip analysis revealed 276 differentially regulated genes between CD95-sensitive and -resistant Jurkat cell lines ..... 58

3.2.4 Gene chip analysis revealed differences in the Rho/ROCK-signaling pathway, which is required for CD95 signaling ..... 59

3.2.5 Inhibition of ROCK1 renders CD95-sensitive Jurkat cells apoptosis-resistant ..... 60

3.2.6 A total of 95 differentially regulated proteins were identified by 2D-DIGE of Jurkats .... 62
3.2.7 The intersection of 2D-DIGE analysis of Jurkats and CTLs lowered the amount of candidate proteins for further analysis .... 63
3.2.8 Hsp90 is not involved in CD95 resistance .... 64
3.2.9 Downregulation of Grp78 by deoxynivalenol sensitizes to CD95-induced apoptosis .... 65
3.2.10 Upregulation of Grp78 by valproic acid increased apoptosis in CD95-sensitive Jurkat cells .... 66
3.2.11 Detection of a hypothetical protein with a known CD95 binding site by 2D-DIGE .... 68

**4. Discussion .... 70**
4.1 Constitutive alloantigen stimulation of human T cells induces CD95 resistance .... 70
4.2 Short-and long-term-activated T cells show no difference in NF-κB activity .... 71
4.3 Differences in the phosphorylation status of Bcl-10 could be relevant in the regulation of cytoskeletal rearrangements, which are important for the induction of apoptosis .... 71
4.4 The NF-κB signaling mediators TRAF2 and TRAF6 can also be associated to apoptosis signaling and were both differentially recruited into lipid rafts of short- and long-term-activated T cells .... 72
4.5 Reduced constitutive lipid raft recruitment of Zap-70, PLC-γ and PKC-θ suggests reduced $Ca^{2+}$ influx in long-term-activated T cells protecting them from apoptosis .... 73
4.6 Reduced CD95 recruitment to lipid rafts might be due to constitutive PI3K activity in long-term-activated T cells .... 74
4.7 Overexpression of CD95L and complete localization into lipid rafts may inhibit CD95-induced apoptosis by blocking the binding site of CD95 .... 75
4.8 CD95-sensitive and -resistant Jurkat cells as model system for the study of long-term-activated T cells .... 75
4.9 The Rho/ROCK signaling pathway is important for the induction of apoptosis in response to CD95 signaling .... 76
4.10 2D-DIGE identified a hypothetical protein of the ezrin-radixin-moesin (ERM) family with a known CD95 binding site .... 76
4.11 Grp78 protects from CD95-induced apoptosis .... 77
4.12 The proteasome and Annexin A1 play a role in regulation of apoptosis .... 78

**5. Summary .... 79**

**6. References .... 81**

**7. Appendix .... 103**
7.1 Gene chip analysis of Jurkats .... 103
7.2 Identified protein spots in 2D-DIGE analysis of Jurkats .... 105
7.3 Identified protein spots in 2D-DIGE analysis of short- and long-term activated T cells .... 109

**Statutory Declaration .... 111**

# Abbreviations

17-AAG: 17-allylamino-17-demethoxygeldamycin

2D-DIGE: 2 dimensional Difference In-Gel Electrophoresis

ALL: Acute Lymphoblastic Leukemia

AP: activator protein

Apaf-1: apoptosome-associated factor-1

APC: antigen presenting cell

BAFF: B cell-activating factor

Bak: Bcl-2 homologous antagonist/killer

Bax: Bcl-2-associated X protein

Bcl: B cell lymphoma

BH: Bcl-2 homology domain

Bid: BH3-interacting domain death antagonist

CaMKII: Calcium/calmodulin-dependent protein kinase II

cAMP: Cyclic adenosine monophospate

CAPE: caffeic acid phenetyl ester

CARD: caspase recruitment domain

CARMA: CARD-containing signaling adaptor protein

Caspases: Cysteine-dependent aspartate-specific proteases

CD: cluster of differentiation

CICD: Caspase-independent cell death

Csk: C-terminal Src kinase

CTLs: cytotoxic T lymphocytes

DAG: diacylglycerol

DD: death domain

DED: death effector domain

DISC: death inducing signaling complex

DMSO: Dimethyl sulfoxide

DNA: deoxyribonucleic acid

DON: deoxynivalenol

DR: death receptors

DTT: dithiothreitol

ECL: enhanced chemiluminescence

Elk: Ets-like gene

EMSA: Electrophoretic Mobility Shift Assays

EndoG: Endonuclease G

ER: endoplasmic reticulum

Ets: E-twenty six

FADD: Fas-Associated protein with death domain

FITC: fluorescein

Fos: FBJ murine osteosarcoma viral oncogene

FoxP3: Forkheadbox P3

GADS: Grb-2 realted adaptor downstream of Shc

GAP: GTPase activating protein

GDI: GDP-dissociation inhibitor

GEF: guanine-nucleotide exchange factor

GEM: glycolipid enriched membrane

GPI: glycosylphosphatidylinositol

Grb: growth factor receptor-bound protein

Grp78: 78-kDa glucose-regulated protein

GTP: guanosine triphosphate

HLA: human leucocyte antigen

Hsp: Heat-shock protein

HRP: Horseraddish peroxidase

IAP: inhibtors of apoptosis proteins

ICAD: inhibitor of the caspase-activated DNase

ICAM: intracellular adhesion molecule

IFN: interferon

IKK: IκB kinase

IAP:  inhibitors of apoptosis proteins

IκB: inhibitors of NF-κB

IPG: immobilized pH gradient

ITAM: immunotyrosine-based activation motif

Jnk: c-Jun N-terminal kinase

LAT: linker of activated T cells

LFA: lymphocyte functional antigen

MALT: mucosa-associated lymphoid tissue

MAP: mitogen-activated protein

MHC: major histocompatibility complex

MS: mass spectromety

NEMO: NF-κB essential modulator

NES: nuclear export sequence

NF-κB: nuclear factor κB

NFAT: nuclear factor of activated T cells

NLS: nuclear localization signal

PBL: peripheral blood lymphocytes

PBS: phosphate buffered saline

PE: phycoerythrin

PEA: Phosphoprotein enriched in astrocytes

PI3K: Phosphatidylinositol-3-kinase

PIPKI: type I phosphatidiylinositol-phasphate kinase

PKB: proteinkinase B

PKC: protein kinase C

PLA2: Phospholipase A2

PMA: Phorbol 12-myristate 13-acetate

PMSF: phenylmethylsulfonylfluorid

PTEN: phosphatase and tensin homologue

PTP: permeability transition pore

Ras: rat sarcoma

Rho: Ras homologue

RHD: Rel-homology domain

RIP: receptor-interacting protein

RNA: ribonucleic acid

ROCK: Rho-associated coiled coil-containing protein kinase

ROS: reactive oxygen species

SDS: sodium dodecyl sulfate

SH: Src homology domain

Shc: Src homology 2 domain-containing protein

SLP-76: SH2 domain containing leukocyte protein of 76kDa

Smac: second mitochondrial activator of caspases

SOS: son of sevenless

Src: sarcoma

TAB: TAK1-binding protein

TAD: C terminal transcriptional activation domain

TAK: transforming-growth-factor-β-activated kinase

tBid: truncated Bid

TCA: trichloroacetic acid

TCR: T cell receptor

$T_H$: T helper cell

TNF: tumor necrosis factor

TNFR: TNF receptor

TRADD: TNFR-associated death domain protein

TRAF: TNF receptor associated factor

TRAIL: TNF related apoptosis inducing ligand

$T_{reg}$: T regulatory cells

UGB: upper gel buffer

VDAC: voltage-dependent anion channel

XIAP: X-linked inhibitor of apoptosis proteins

Zap: zetta-chain-associated protein

# 1. Introduction

## 1.1 Cell death

There are many types of cell deaths defined by morphological or biochemical behavior of the cell. Apoptosis is defined by its morphological criteria, including cell shrinkage, nuclear deoxyribonucleic acid (DNA) fragmentation and membrane blebbing.[76] Phosphatidylserine exposure on apoptotic cells facilitates phagocytic uptake mainly by macrophages leading to programmed cell death without causing an immune response.[47] In contrast to apoptosis, in necrosis the disruption of the cell membrane leads to a release of cytoplasm contents into the surrounding environment, finally resulting in inflammation.[135] Necroptosis is a programmed cell death leading to cell swelling and membrane breakdown.[53] Autophagy, or autophagocytosis, is also a strongly regulated process that plays a role in development, and homeostasis. It involves the formation of a membrane around the cellular components and fusion with lysosomes, which subsequently degrades the contents.[86]

### 1.1.1 Caspases are the main initiators of apoptosis

Cysteine-dependent aspartate-specific proteases (caspases) have a central role in the regulation and execution of most types of apoptosis. Recently, it has become clear that caspases which were initially considered to be involved only in apoptosis are also responsible for various other functions, including activation, differentiation, proliferation and survival of T cells.[75,110] Caspases are a family of highly conserved cytosolic proteases that contain a cysteine residue in the active site and that cleave their substrate after an aspartatic-acid residue. All caspases are produced by cells as catalytically inactive precursors that contain an amino-terminal pro-domain that keeps the protease in an inactive state. They can be divided into initiator and effector caspases. Initiator caspases are typically activated in response to particular stimuli, such as caspase-8 after death receptor ligation, caspase-9 after apoptosome formation and caspase-2 after DNA damage. Effector caspases such as caspase-3, -

6 and -7 are activated by initiator caspases and are responsible for the cleavage of many cell death substrates.[36] Nuclear shrinkage and budding is caused by caspase-mediated cleavage of nuclear lamins and cleavage of cytoskeletal proteins, such as fodrin and gelsolin.[81] The cleavage of rat sarcoma (Ras) protein homologue (Rho) -associated coiled coil-containing protein kinase (ROCK) -1 generates a truncated kinase with increased intrinsic activity resulting in cell fragmentation, blebbing, and the destruction of the cell by phosphorylation and consequently stabilization of filamentous actin, myosin light chains, and coupling of actin-myosin filaments to the plasma membrane.[27] Another important caspase target is the inhibitor of the caspase-activated DNase (ICAD). The cleavage of the inhibitory part leads to the release of the endonuclease CAD and therefore fragmentation of the DNA.[114] Cleavage of chromosomal DNA into oligonucleosomal size fragments is an integral part in apoptosis.[174]

Caspases can be activated via two different pathways, depending on the initial signal. The extrinsic pathway is initiated upon death ligand binding to death receptors and the intrinsic pathway is activated by cellular stress, such as cytotoxic drugs, DNA damage and growth factor withdrawl. A crosstalk exists at multiple levels.[140]

### 1.1.2 Caspase-independent cell death

Caspase-independent cell death (CICD) usually occurs when caspase inhibitors are present or the pathway is genetically disrupted. CICD involves receptor-interacting protein (RIP) and phospholipase A2 (PLA2) leading to the production of toxic levels of reactive oxygen species (ROS) upon death receptor binding, which can also kill neighbor cells. The strong production of ROS is usually reduced by the presence of activated caspases and the cleavage of RIP and PLA2. Other forms of CICD include the lysosomes. Lysosomal proteases, such as cathepsin D can be activated by proteolytic processing and their release into the cytosol finally results in cell death. This mechanism plays a role in glucocorticoid-mediated cell death in thymic T cells.[18,166]

### 1.1.3 The extrinsic pathway

In the extrinsic pathway, death receptors (DR) which belong to the tumor necrosis factor receptor (TNFR) gene superfamily such as TNFR1, CD95 (CD: cluster of differentiation), and TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 usually cluster and trimerize upon binding of the corresponding ligands. Upon ligand binding, adaptor molecules are recruited to the intracellular death domain (DD) of the DR, and initiate the formation of the death-inducing signaling complex (DISC), which includes the ligand, DR, adaptor and the initiator caspase. The adaptor protein Fas-associated protein with death domain (FADD) binds to CD95 or TRAILR1 and –R2 and recruits via its death effector domain (DED) the inactive initiator caspase-8. Procaspase-8 is activated upon autocatalytical proteolysis and initiates apoptosis by direct cleavage of downstream effector caspases such as caspase-3, -6 and -7, where the extrinsic and intrinsic pathway converge. TNFR1 signaling involves a different set of adaptor proteins, including receptor-inactivating protein (RIP) and TNFR-associated death domain protein (TRADD). TNFR signaling can mediate both, pro- and anti-apoptotic signals.[88]

### 1.1.4 The intrinsic pathway

The intrinsic pathway is activated upon cellular stress induced by cytotoxic drugs, DNA damage or oxidative stress and hinges on the balance between pro- and anti-apoptotic members of the B cell lymphoma-2 (Bcl-2) superfamily of proteins.[34,82] Bcl-2 family members have up to four conserved Bcl-2 homology (BH) domains and many of them also contain a carboxy-terminal hydrophobic domain, which is essential for their targeting to the mitochondrial outer membrane. The members of the Bcl-2 family regulate the permeability of the mitochondrial membrane, leading to the release of cytochrome c, apoptosis-inducing factor (AIF), Endonuclease G (EndoG) and second mitochondrial activator of caspases (Smac) in response to cell death-inducing signals.[34,56] Once in the cytoplasm, cytochrome c complexes with apoptosome-associated factor-1 (Apaf-1) and procaspase-9 to form the apoptosome, which leads to activation of caspase-9 and subsequent cleavage of caspase-3. Permeabilization of the outer mitochondrial membrane can be achieved

by Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) by forming a pore.[94] The anti-apoptotic proteins Bcl-2 and B cell lymphoma extra-large (Bcl-XL) inhibit the pore formation and subsequent cytochrome c release by interacting with Bax and Bak. By caspase-8 mediated cleavage of the pro-apoptotic BH3-interacting domain death agonist (Bid) to truncated Bid (tBid), the extrinsic pathway can be linked to the intrinsic pathway.[94,168] The Bcl-2-associated death promoter (Bad) is a pro-apoptotic member of the BH3-only family, a subfamily of the Bcl-2 family, and does not contain a C-terminal transmembrane domain, unlike most other members of the Bcl-2 family. Dephosphorylation of Bad e.g. by $Ca^{2+}$-activated calcineurin leads to heterodimer formation with anti-apoptotic proteins Bcl-2 and Bcl-$X_L$, thus allowing Bax/Bak-triggered apoptosis.[167] Phosphatidylinositol-3-kinase (PI3K) activity-triggered, protein kinase B (PKB)/Akt-dependent phosphorylation of Bad leads to homodimer formation of Bad and is thus anti-apoptotic.[80,128] Smac promotes apoptosis by directly interacting with inhibitors of apoptosis proteins (IAPs) and disrupting their ability to inactivate the caspase enzymes.[40] The release of AIF and EndoG from mitochondria leads to their translocation to the nucleus, where they cause DNA fragmentation and chromatin condensation.[95] The permeability of the mitochondrial membrane can also be regulated by $Ca^{2+}$, through inner membrane permeability transition involving the opening of a permeability transition pore (PTP).[16] The PTP consists of voltage-dependent anion channels (VDAC), cyclophilin D and several other proteins. High $Ca^{2+}$ levels in the mitochondrial matrix lead to a voltage-dependent opening and the release of $Ca^{2+}$ together with cytochrome c, Smac, AIF and EndoG to translocate into the cytosol. [16]

### 1.1.5 Type I + II cells

Two types of CD95-mediated extrinsic apoptotic signaling pathways have been identified.[131] Type I cells are characterized by high levels of CD95, DISC formation and high amounts of active caspase-8. In these cells, caspase-8 directly activates the downstream effectors caspase-3, and -7. In type II cells, less DISC formation leads to lower levels of active caspase-8. Therefore these cells require an additional amplification loop that involves the cleavage of Bid to its truncated form tBid, which induces the pro-apoptotic functions of the mitochondria.[94,101] A number of studies

have indicated that CD95 signaling in T cells is mediated by the type-I-cell-mechanism since T cells from Bcl-2 transgenic mice[127], as well as that T cells from mice deficient in both Bax and Bak[169] are not protected from CD95-mediated apoptosis.

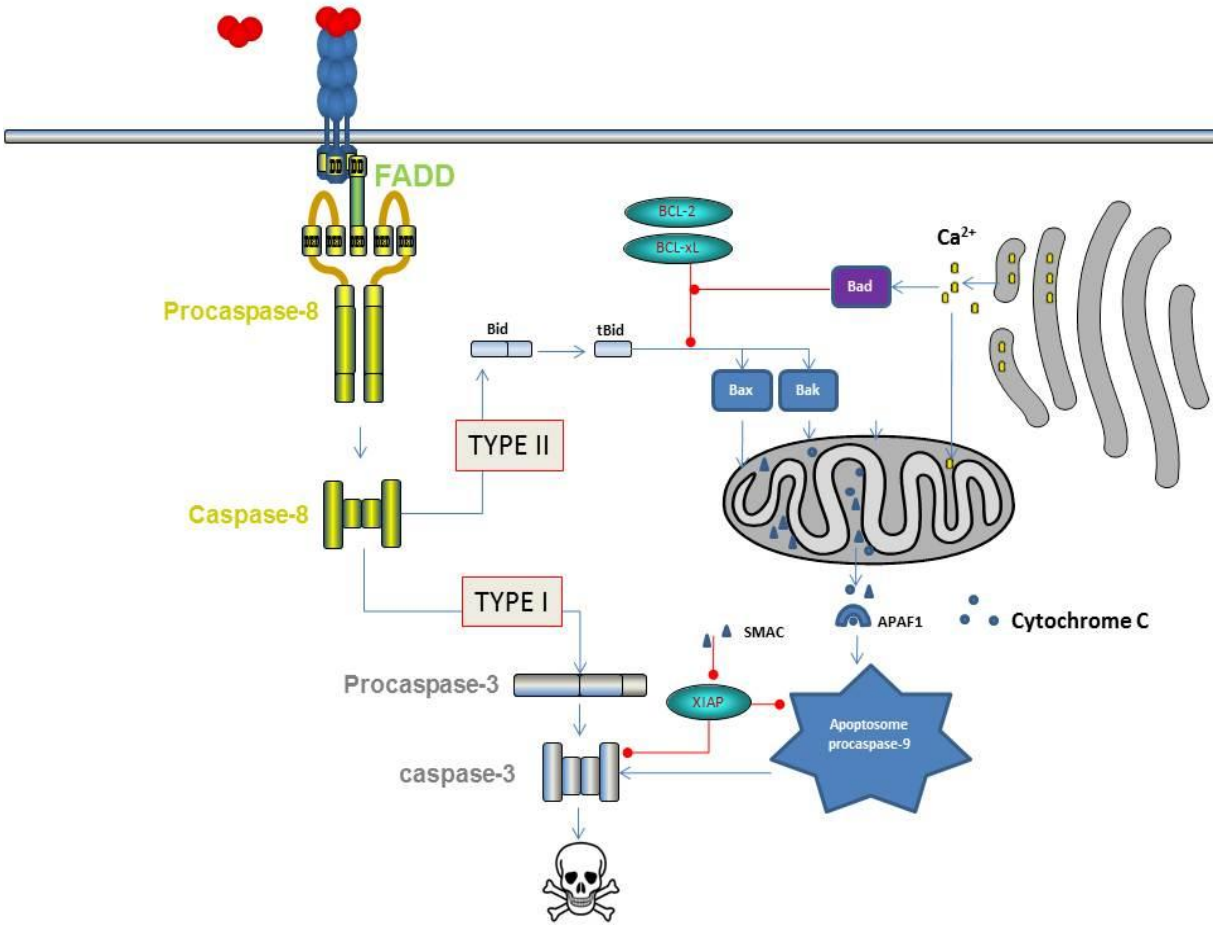



**Figure 1: CD95 signaling in type I and type II cells**

The binding of soluble or membrane-bound CD95L (sCD95L/mCD95L) leads to trimerization of CD95 and subsequent recruitment of the adaptor protein FADD and procaspase-8. Procaspase-8 is activated upon autocatalytical cleavage. Unlike type I cells, in type II cells DISC formation is not sufficient for apoptosis, but a mitochondrial feedback loop is required in which caspase-8 cleaves Bid to tBid, which in turn activates Bax and Bak, which form pores in the outer mitochondrial membrane to release apoptogeneic factors such as cytochrome c, Smac, AIF and EndoG into the cytosol. Bcl-2 and Bcl-$X_L$ inhibit pore formation by interacting with Bax and Bak. Cytochrome c activates Apaf-1 to form with caspase-9 the apoptosome. Activated caspase-9 leads to subsequent cleavage and activation of caspase-3. The X-linked inhibitor of apoptosis protein (XIAP) inhibits the apoptosome and caspase 3 but is itself inhibited by Smac released from the mitochondria. Cytochrome c release can also be triggered by increased $Ca^{2+}$ levels in the inner matrix leading to opening of the voltage-dependent permeability transition pore. Calcium activates also calcineurin to dephosphorylate Bad. Dephosphorylated, active Bad inhibits the anti-apoptotic function of Bcl- $X_L$ and Bcl-2.

### 1.1.6 Receptor internalization is required for efficient DISC formation in CD95 type I cells

Especially in type I cells, CD95 receptor complex internalization is necessary for sufficient DISC formation. This process requires cytoskeletal rearrangements to allow CD95 clustering and cellular polarization.[3,90] For this purpose, CD95 must be localized within lipid rafts, which will be described later in further detail1.3.2.[44] Only palmitoylated CD95 can be found in lipid rafts leading to high molecular weight death-inducing signaling complexes and the process of palmitoylation is reversible.[49] Ezrin (vilin-2 or cytovillin) is a member of the Ezrin-Radixin-Moesin (ERM) protein family, which all serve as mediators between the plasma membrane and the actin cytoskeleton. Ezrin has a CD95 binding site for linking the CD95 receptor complex to the cytoskeleton. Phosphorylation of ezrin by ROCK1 is required for linking the CD95 receptor complex to actin.[121] ROCK1 is activated by the Rho signaling pathway and plays a main role in regulating the shape of the cytoskeleton (Figure 3).[64] Ezrin is associated to the membrane by binding to $PIP_2$ and binds only to palmitoylated CD95.[99] Phosphorylated ezrin can also bind Rho-GDP dissociation inhibitor (GDI), which is usually bound to Rho, masking its palmitoylation. The release of Rho leads to its lipid raft localization and contact to a Rho-guanine nucleotide exchange factor (GEF).[154] The activation of actin polymerization leads to the association of neighboring lipid raft microdomains and finally to clathrin-dependent endocytosis and traffic converging into the golgi region.[35] In the process of clathrin-dependent endocytosis, adaptor protein 2 (AP2) is recruited to the membrane by binding to phosphatidylinositol-bisphosphate ($PIP_2$). High $PIP_2$ levels are therefore required for CD95 linkage to the cytoskeleton and the process of CD95 receptor complex formation. $PIP_2$ is generated by the activity of type I phosphatidiylinositol-phosphate kinases (PIPKIs) which use phosphatidylinositol-4-phosphate and phosphatidylinositol-5-phosphate as substrates.[39] $PIP_2$ levels are mostly regulated by phosphatase and tensin homologue (PTEN) and PI3K by changing $PIP_2$/$PIP_3$ levels. ROCK also activates PTEN. PTEN increases $PIP_2$ levels by dephosphorylation of $PIP_3$ and PI3K activity leads to decreased $PIP_2$ levels.[22] PI3K can also activate Rho-GTPase-activating proteins (GAPs), thereby inhibiting the activation and phosphorylation of PTEN and Ezrin.[83]

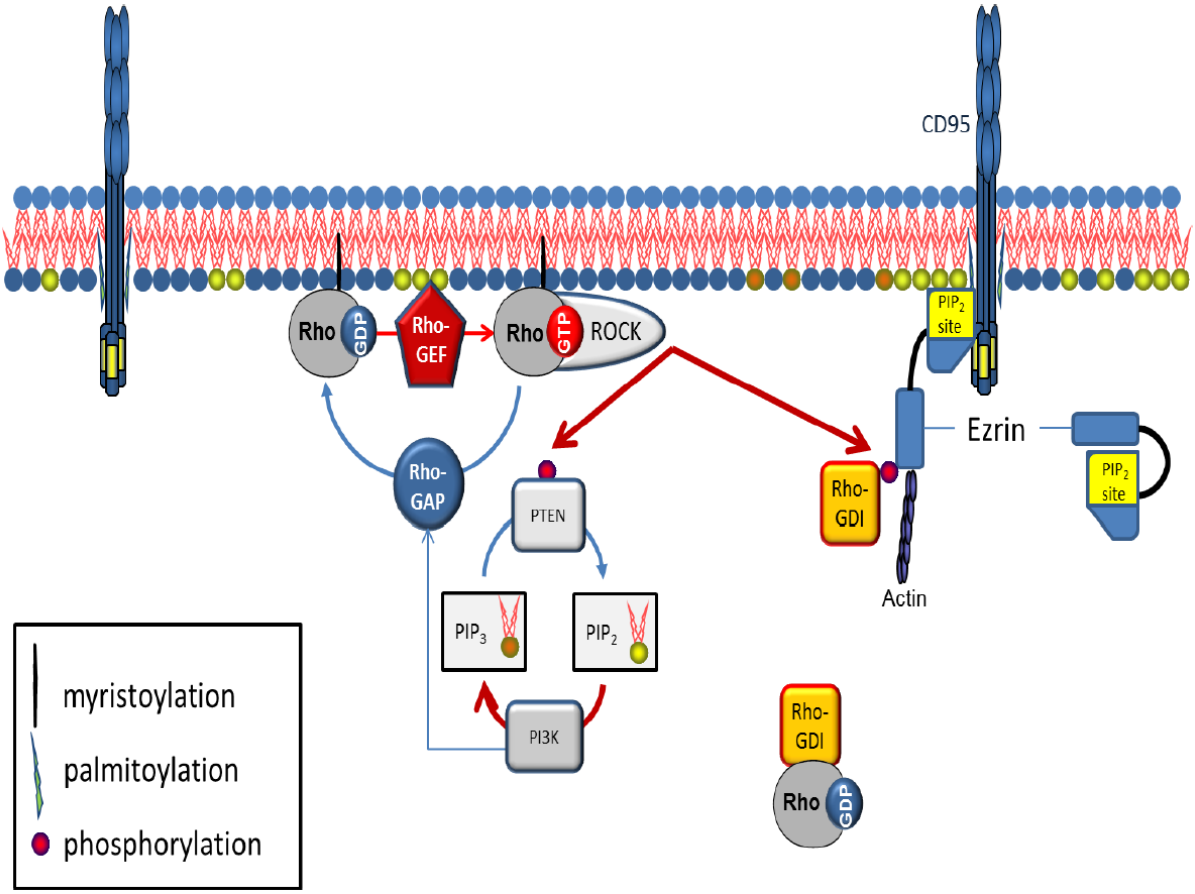



**Figure 2: The Rho/ROCK pathway is required for CD95 mediated apoptosis**

Rho is a small G-protein which is localized in lipid rafts by myristoylation. Rho-GEFs have a Pleckstrin homology (PH) domain and bind to PIP2in the membrane. The transition of GDP to GTP bound to Rho mediated by Rho-GEFs leads to binding and activation of ROCK. ROCK phosphorylates cytosolic ezrin leading to a conformational change and its binding to PIP2in the membrane. Membrane-bound ezrin associates with palmitoylated CD95 and links the receptor complex to the cytoskeleton via actin. ROCK phosphorylates also PTEN leading to increased $PIP_2$ levels. Rho-GAPs catalyze the exchange from GTP to GDP bound to Rho, thus, inactivating the process. GDIs bind to Rho, thereby masking its myristoylation. GDIs bind to phosphorylated ezrin,thereby,releasing Rho, which translocates to the membrane. PI3K phosphorylates $PIP_2$ to $PIP_3$. PI3K can also activate Rho-GAPs.

### 1.1.7 Regulation of the CD95 signaling pathway

There are several checkpoints to control the outcome of CD95 signaling. The process of CD95 palmitoylation is reversible, and only palmitoylated CD95 associated to lipid rafts has the potential to induce apoptosis, at least in CD95 type I cells.[14] Upregulation of PI3K and subsequent activation of PKB/Akt also contribute to CD95 resistance and prevent efficient DISC formation. PI3K activity is associated with

depalmitoylation and lower levels of CD95 localized to lipid rafts.[14] Cellular FADD-like IL-1beta-converting enzyme (FLICE, caspase-8) inhibitory protein (c-FLIP) can block death receptor signaling and caspase-8 activation by competing with caspase-8 recruitment to FADD. cFLIP/caspase-8 heterodimers support the subsequent activation of NF-κB and mitogen-activated protein kinase (MAPK) acting as mediators of cell survival. Impaired DISC assembly in CD95-resistant T cells can also be induced by increased recruitment of FLIP short ($FLIP_s$) to the DISC. The inhibition of CD95 induced apoptosis on DISC level by FLIP is mostly regulated by overexpression.[66,132] Phosphoprotein enriched in astrocytes (PEA) -15 is a death effector domain-containing phosphoprotein that binds extracellular signal-regulated kinase (Erk) and restricts it to the cytoplasm. Phosphorylated PEA-15 also binds to FADD and, thus, blocks apoptosis induced by death receptors.[46,124] Decoy receptor 3 (DcR3) is a soluble receptor member of the TNF superfamily that binds to CD95L and and functions to prevent CD95-CD95L interactions by blocking the binding-site.[177]

### 1.1.8 CD95-resistant T cells in human diseases

The existence of CD95-resistant T cells could be shown in a variety of autoimmune diseases, such as rheumatoid arthiritis (RA) and multiple sclerosis (MS), which can be explained by changes in the expression of pro- and anti-apototototic proteins or defects in the signaling pathway. The apoptosis inhibitors FLIP and survivin were found to be upregulated in CD95-resistant T cells from patients with MS[136,137], whereas CD95 resistance in T cells of RA patients could be associated with elevated Bcl-2 levels[133]. CD95 resistant T cells are thought to develop in these diseases by continuous stimulation with self-antigen.[148]

## 1.2 Function of T lymphocytes and their subsets

T lymphocytes are a class of white blood cells which are derived from pluripotent hematopoietic bone marrow stem cells and bear variable T cell receptors (TCR) for recognizing antigens.[2] The TCR recognizes antigen bound to a major histocompatibility complex (MHC) molecule I or II.[32] MHC-I presents peptides

generated during the degradation of intracellular proteins by the ubiquitin proteasome system and is present on all nucleated cells.[132] Peptides presented by MHC-II are generated in acidified endocytic vesicles and are only present on a few antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages and B cells.[152] T cells can be divided into different subpopulations, depending on the expression of the co-receptors CD4 or CD8 and secreted cytokines. While CD4 has a MHC-II binding site, CD8 binds to MHC-I.[89,130]

### 1.2.1 From T cell progenitor to effector

The earliest T cell progenitors are found in the bone marrow and are derived from pluripotent bone marrow stem cells.[2] The molecular crosstalk between stem cells and cellular constituents of the bone marrow microenvironment, known as hematopoietic stem cell (HSC) niches is thought to control the balance between HSC self-renewal and differentiation.[171] Stromal cells in bone marrow secrete cytokines, including stem cell factor (SCF) and interleukin- (IL-)7, which is required for the early development of pre-T cells. C-Kit (CD117) is the receptor for SCF, which is present on T and B cell precursors, and disappears when the lymphocyte precursors start to recombine their antigen receptor genes in the thymus.[8] The whole further education to naïve T lymphocytes occurs in the thymus.[109] The most immature T cell precursor is $CD4^-CD8^-CD25^-CD44^+$.[55] CD44 functions as a thymus homing molecule.[33] CD25 is the $\gamma$-chain of the IL-2 receptor (IL-2R) for high affinity binding, which is required for cell proliferation. Only the cells that succeed in in-frame rearrangement of the gene encoding the TCR $\beta$–chain are selected for further differentiation.[58] The initial development is promoted by the Notch pathway and is supported by IL-7 derived from cortical thymic epithelial cells (cTECs).[104] The expressed $\beta$-chain then pairs with the surrogate pT$\alpha$–chain to form the pre-TCR complex.[163] The successful expression of the pre-TCR complex on the cell surface triggers rapid cell proliferation, which causes the cessation of $\beta$-chain gene rearrangement. Delta-Notch interaction, initiates the signals for further development into a $CD4^+CD8^+$ double positive (DP) population and the initiation of $\alpha$-chain gene rearrangement.[25] The cortical DP-thymocyte population contains the unselected

repertoire of T cells, which undergo positive and negative selection.[146] T lymphocytes are positively selected by low-avidity interaction with MHC-I and MHC-II on thymic epithelial cells and differentiate into single positive (SP) $CD8^+$ or $CD4^+$ thymocytes. Thymocytes which fail to recognize MHC molecules undergo apoptosis. The process of negative selection contributes to the deletion of self-reactive T cells. High-avidity interactions elicit signals that lead to the deletion of thymocytes. Failure of negative selection can lead to autoimmune diseases such as RA and MS. Most DP thymocytes fail to receive the correct TCR signals and, therefore, only 3-5% of developing thymocytes survive this checkpoint of T cell development.[146] Mature SP thymocytes express the sphingosine-1-phosphate receptor 1 (S1P1), which regulates the egress of mature T cells from the thymus into blood vessels, where the concentration of S1P is higher.[107]

Mature naïve T cells seed the secondary lymphoid tissues, the spleen, lymph nodes and mucosa-associated lymphoid tissues (MALT) and circulate through the lymphatic system. The expression of C-C chemokine receptor type 7 (CCR7) controls the homing to secondary lymphoid organs. T lymphocytes leave the circulation by traversing the high endothelial venules (HEV) in the lymph nodes and MALT. They re-circulate via the lymphatic system, through chains of lymph nodes, which gives them the opportunity to contact their antigen.[111] Once activated, T lymphocytes differentiate into effector cells and undergo extensive clonal expansion.[38] Effector T cells migrate to sites of infection by the expression of adhesion molecules and chemokine receptors, such as CXC chemokine receptor type 3 (CXCR3) and CCR5. T cells are retained at sites of infection but dependent on chemokines.[26,176]

### 1.2.2 T cell subsets

#### 1.2.2.1 T helper cells

T helper ($T_H$) cells express CD4 and recognize antigen presented by MHC class II on the surface of APCs, such as DCs, macrophages and B lymphocytes. APCs are functionally defined cells which take up antigens and present them to lymphocytes. $T_H$ cells were originally distinguished according to the blends of cytokines they produce, such as $T_{H1}$, $T_{H2}$, $T_{H3}$ and $T_{H17}$. $T_{H1}$ cells secrete interferon-$\gamma$ (IFN-$\gamma$) and activate macrophages to destroy intracellular bacteria, especially mycobacteria. [113]$T_{H2}$ cells produce IL-4, -5, -6 and -13 and are required for B cell differentiation.[113,120] $T_{H17}$ cells have been classified by the secretion of IL-17 and play a significant role in the defence against extracellular bacteria and some fungi.[118] $T_{H3}$ cells secrete transforming growth factor-$\beta$ (TGF-$\beta$) and IL-10 and are involved in protecting mucosal surfaces in the gut from non-pathogenic non-self-antigen.[170] Follicular helper T cells or $T_{FH}$ are classified by their constitutive expression of the B cell follicle homing receptor CXCR5 and mediate B cell activation through expression of CD40 ligand (CD40L) and the secretion of IL-21 and IL-4, which triggers germinal center formation.[83] Another claimed T cell subset are IL-9-producing $T_{H9}$ cells focused on defending against helminth infections.[31,161]

#### 1.2.2.2 T regulatory cells

Another important T cell subset are $CD4^+CD25^+FoxP3^+$ (Forkheadbox P3) regulatory T ($T_{reg}$) cells which can be defined as T cells that can functionally suppress an immune response by down-modulating the activity of effector cells.[51]

#### 1.2.2.3 Cytotoxic T lymphocytes

$CD8^+$ T cells recognize antigen presented by MHC class I molecules, which is expressed on all nucleated cells. Naïve $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTLs) after stimulation with antigen and co-stimulation by APCs (00).

The most characteristic feature of CTL differentiation is the development of intracellular granules that contain cytotoxic proteins, such as perforin, granzymes and granulysin. The principal mechanism of cytotoxic T cell action is the calcium-dependent release of these lytic granules.[73] Perforin polymerizes in the target membrane to form a pore through which the granzymes can enter. Granzymes are serine proteases and activate apoptosis in the cytoplasm of the target cell. Activated T cells also express death ligands, such as CD95L and TRAIL, which can initiate apoptosis independent of perforin and granzyme. CTLs also release IFN-$\gamma$, TNF-$\alpha$ and -$\beta$. TNF-$\alpha$ and -$\beta$ can kill several target cells through interaction with TNF receptor (TNFR) -I.[12]

### 1.2.3 Apoptosis in T cells

Apoptosis plays a key role in the development, morphogenesis, deletion of cancerous or infected cells and control of lymphocyte homeostasis, which is essential for the normal function of the immune system.[60] In the process of T cell development in the thymus only T cells with a successful receptor gene rearrangement survive. During positive selection, T cells which fail to recognize self-MHC undergo apoptosis. In negative selection, T cells which are reactive to self-peptides presented on MHC molecules are eliminated, which guarantees the deletion of auto-reactive T cells. Apoptosis is not only important during T cell development, but also for the elimination of expanded activated T cells in the periphery to shut down the immune response by which only a few memory cells survive to ensure an immediate and effective response after repeated antigen recognition.[84,144] Activated T cells require survival signals otherwise they are eliminated by activated cell autonomous death (ACAD). Repeated strong TCR signaling leads to activation-induced cell death (AICD) usually by enhanced production of CD95L.[30]

## 1.3 T cell signaling

### 1.3.1 The T cell receptor complex

The TCR α:β- or γ:δ- heterodimer is associated with a complex of five other chains: two ε, δ, γ and a ξ:ξ homodimer as well as ξ:η heterodimer in mice, collectively called CD3, which are required for signaling. Each signaling chain has one immunoreceptor tyrosine-based activation motif (ITAM), whereas each ξ-chain has three, which are becoming phosphorylated upon activation and initiate further signaling (1.3.31.3.3). The TCR has negative charges within the membrane spanning domain, whereas CD3 molecules have positive charges. Antigen binding leads to TCR dimerization and TCR-CD3-complex formation, which balances the positive and negative charges, thus stabilizing the complex. TCR-CD3-complex formation leads to localization into lipid rafts within the membrane.[19,103]

### 1.3.2 Lipid rafts function as signaling domains in TCR signaling and many other processes in T cells

Lipid rafts are defined as detergent-resistant membrane microdomains of specific lipid and protein composition. They are enriched in sphingolipids, cholesterol and glycosylphosphatidylinositol- (GPI-) anchored proteins and play essential roles in TCR signaling.[103,141] Lipid rafts accumulate also several cytoplasmic proteins important for signal transduction, including the sarcoma (Src) family kinases, such as lymphocyte-specific protein tyrosine kinase (Lck), as well as transmembrane proteins such as the co-receptors CD4 and CD8 and the transmembrane adapter linker of activated T cells (LAT).[6,52,125,155] Lipid rafts play an essential role for either initiation or amplification of the signaling process. Usually membrane proteins that are post-translationally modified by S-palmitoylation or *N*−myristoylation can be found constitutively in lipid rafts, for example N-myristoylated Lck and S-palmitoylated CD4 or CD8. Proteins that have been modified by unsaturated fatty acids or prenyl groups are excluded from lipid rafts, such as transferrin receptor 1 (TfR1).[6,43,52,155] Other inter-membrane proteins, such as the TCR, are only associated to lipid rafts after antigen recognition.[103] Other cytoplasmic proteins are only recruited to lipid

rafts after signaling, such as phospholipase C (PLC)-γ by LAT or ξ-chain-associated protein (Zap) -70 by the TCR-CD3-complex. Various T cell subpopulations differ in the use of membrane rafts. Besides TCR signaling, lipid rafts are also involved in the initiation of signal transduction of many other pathways and processes, such as cytokine and chemokine receptor signaling, adhesion molecules, fast antigen presentation, establishing cell polarity and in the first steps of nuclear factor-κB (NF-κB) and CD95 signaling.[5,44,110,141]

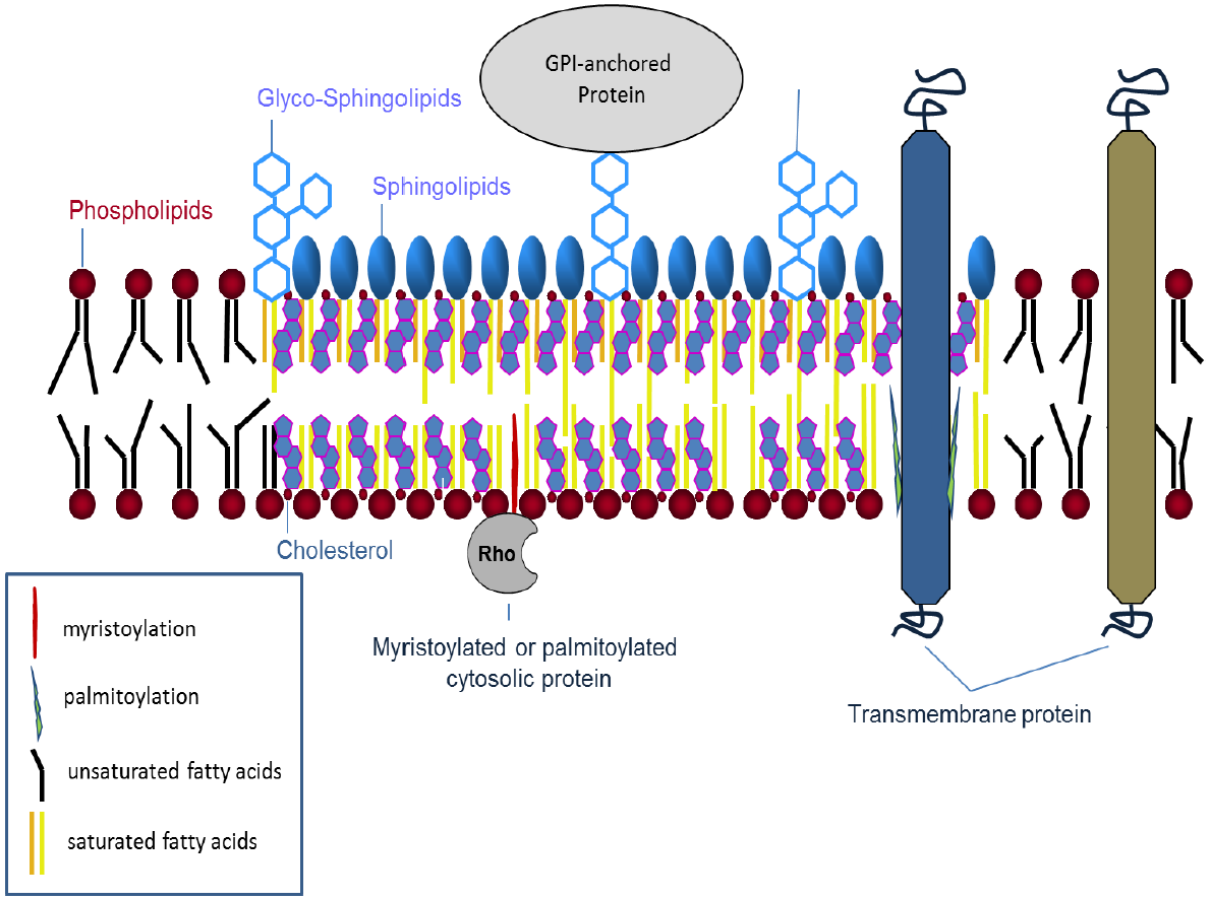



**Figure 3: Structure of lipid rafts**

Lipid rafts consist of sphingo- and phospholipids carrying unsaturated fatty acids. High amounts of cholesterol are swimming inside the membrane between unsaturated phospholipids. The outer membrane within lipid rafts is made of a high amount of sphingolipids and glycosphingolipids. GPI-anchored proteins are constitutively localized within lipid rafts. On the inner side of the membrane of lipid rafts are palmitoylated and myristoylated proteins such as Rho and Src family kinases, which are constitutively localized within lipid rafts. Transmembrane proteins are also only localized within lipid rafts by palmitoylation or myristoylation, such as CD95.

### 1.3.3 TCR-signaling

The TCR localization into lipid rafts upon stimulation and TCR-CD3-complex formation brings the ITAMs into the direct neighbourhood of Src family kinases for phosphorylation. Constitutively lipid raft-associated Src family kinases, such as Lck, contain two tyrosine residues. One stimulates kinase activity and phosphorylation of the second tyrosine residue leads to a conformational change that blocks the active site.[43] CD45, a transmembrane protein tyrosine phosphatase, activates the Src kinase activity after removing the inhibitory phosphate.[157] Activated Src kinases phosphorylate the ITAMs of the CD3 molecules. Zap-70 binds to the phosphorylated ITAMs and subsequently activates LAT and Src homology 2- (SH2-) domain containing leukocyte protein of 76kDa (SLP-76).[160] Phosphorylated SLP-76 binds to cytosolic PLC-$\gamma$. Tec kinases receptor-like kinase (Rlk) and IL-2-inducible T cell kinase (Itk) have a PH domain, that allows them to interact with phosphorylated lipids on the inner face of the cell membrane. They also interact via their SH2 and SH3 domains with the phosphorylated adaptor proteins LAT and SLP-76.[91] The SH2 domain can bind to phosphotyrosine residues and the SH3 domain is involved in interactions with proline-rich regions. Itk phosphorylates PLC-$\gamma$ bound to SLP-76 for activation. PLC-$\gamma$ cleaves inositol phospholipids to generate the intracellular secondary messenger diacylglycerol (DAG) and inositol trisphosphate ($IP_3$).[78] Protein kinase C-$\theta$ (PKC-$\theta$) binds to DAG and initiates several signaling pathways resulting in nuclear localization of transcription factors, such as NF-$\kappa$B.[67] $IP_3$ binds to $IP_3$-receptors ($IP_3R$) inducing the opening of calcium channels in the cell membrane and preferentially the ER, which causes the release of $Ca^{2+}$ into the cytosol. $Ca^{2+}$ binds to calcineurin. Calcineurin is a phosphatase which dephosphorylates cytosolic nuclear factor of activated T cells (NFAT) leading to its activation. Active NFAT translocates to the nucleus and activates transcription until it becomes phosphorylated in the nucleus and transported back to cytosol.[68] (**Fehler! Verweisquelle konnte nicht gefunden werden.**)

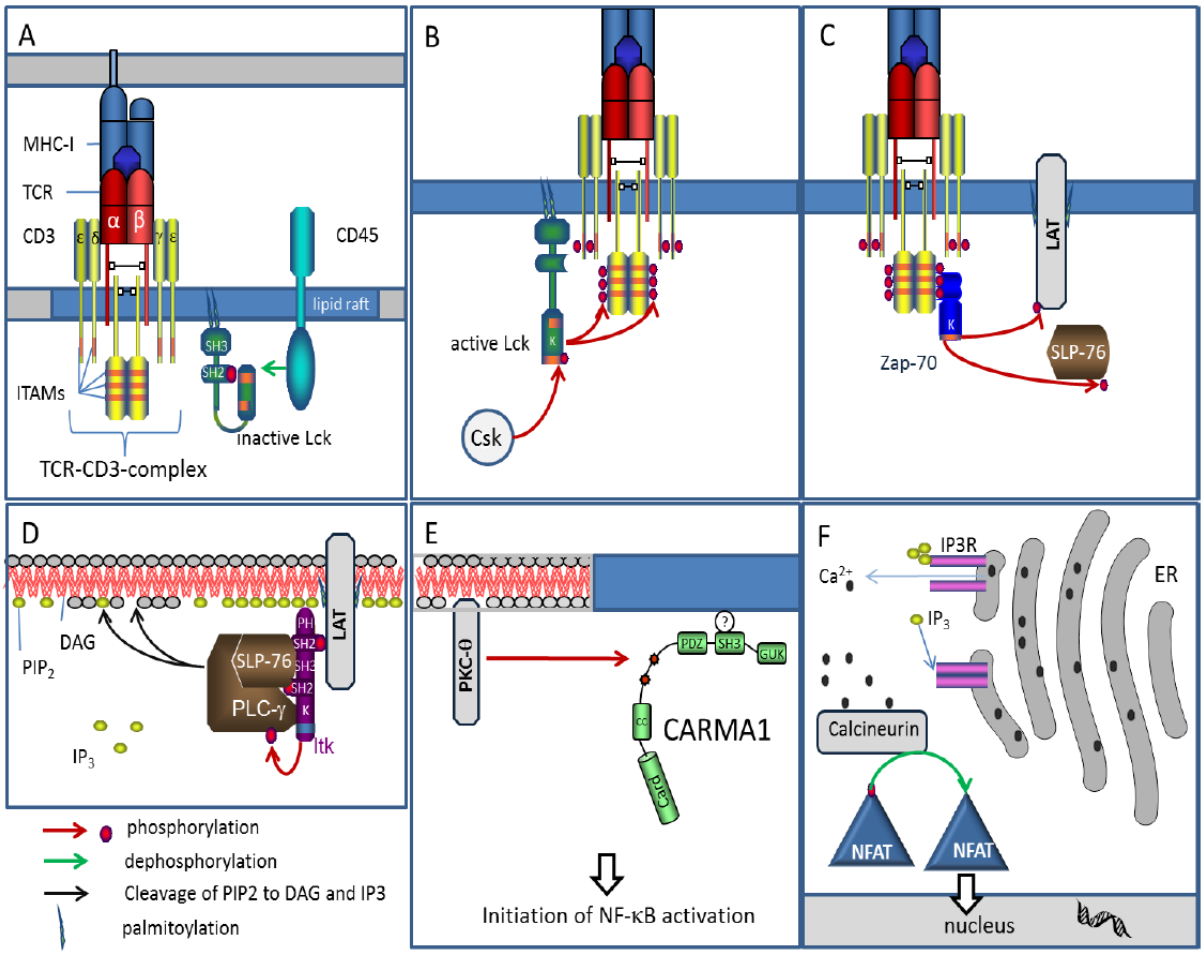



**Figure 4: T cell receptor signaling**

**(A):** The formation of TCR:CD3 complex leads to lipid raft localization. Dephosphorylation of Lck by CD45 on its inhibitory tyrosine residue activates them. **(B):** Active Lck phosphorylates the ITAMs of CD3 molecules. Phosphorylation by Csk inactivates Lck. Thus, the balance between Csk and CD45 determines the signaling activity of Lck in response to receptor aggregation. **(C):** ZAP-70 binds to the ITAMs and phosphorylates SLP-76 and LAT. **(D)**: The Tec kinase Itk bind to phosphorylated LAT and have also a PH domain to get associated with the cell membrane by its own. SLP-76 phosphorylated by ZAP-70 forms a complex with PLC-□ and can be found in a complex with Itk and LAT. Itk activates PLC-□ to cleave $PIP_2$ into $IP_3$ and DAG. **(E):** PKC-γ gets activated by binding to DAG and phosphorylates CARMA1, which finally results in the initiation of NF-□B activation. **(F):** $IP_3$ binds to IP3R to allow $Ca^{2+}$ entry from the ER. $Ca^{2+}$ activates calcineurin, but is also a seond messenger for many other proteins. Calcineurin dephosphorylates NFAT, which moves into the nucleus to regulate gene transcription.

Another signaling pathway starts with the growth factor receptor-bound protein (Grb) -2-related adaptor downstream of SH2 domain-containing protein (Shc) (GADS). GADS is recruited by phosphorylated LAT. GADS recruits the rat sarcoma- (Ras-)

GEF son of sevenless (SOS), and the small G protein Ras. Ras binds to guanosine diphosphate (GDP). SOS exchanges GDP with guanosine triphosphate (GTP) to activate Ras. Ras activates a MAPK kaskade and finally activates the transcription factor E-twenty six (Ets) -like gene (Elk), which initiates the FBJ murine osteosarcoma viral oncogene homologue (Fos) gene.[98] (Figure 5)

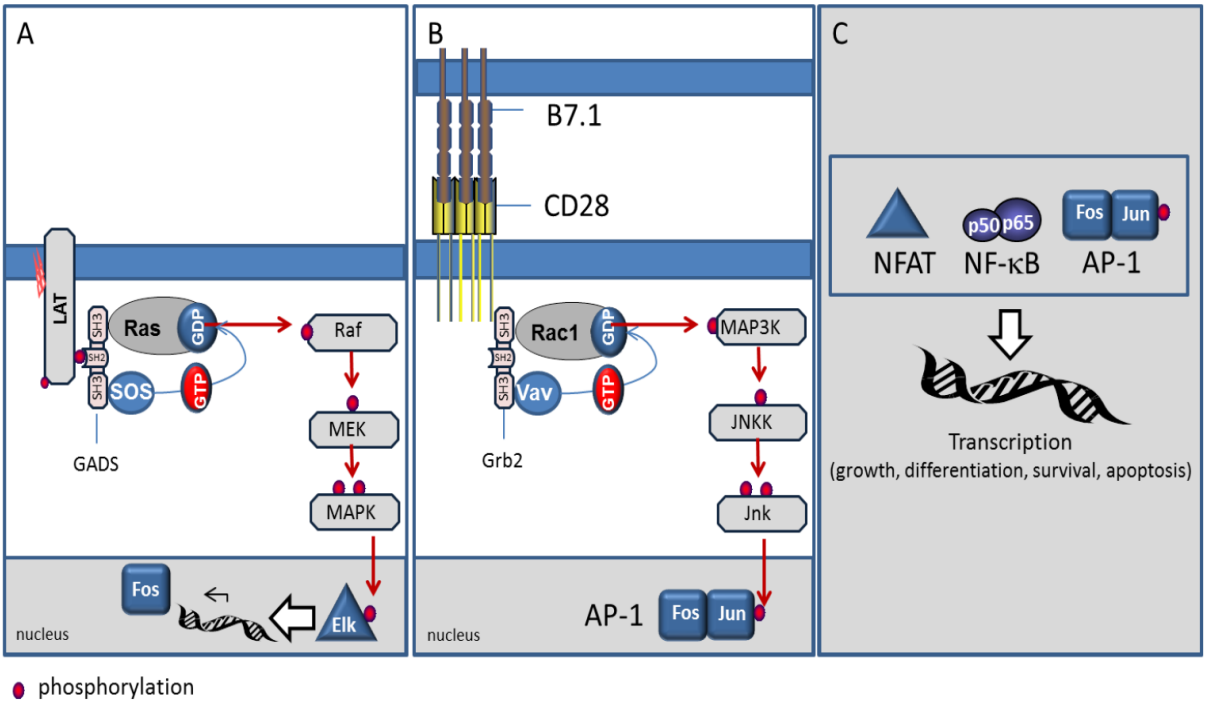



**Figure 5: TCR signaling and co-stimulation**

**(A):** The adaptor protein GADS binds to phosphorylated LAT and recruits the GEF SOS and the small G protein Ras. SOS exchanges GDP with GTP bound to Ras, which activates a MAPK cascade. The MAPKKK rapidly growing fibrosarcoma (Raf) phosphorylates MAPK/ERK kinase (MEK). MEK phosphorylates MAPK twice, which translocates to the nucleus and activates the transcription factor Elk leading to gene transcription of the AP-1 subunit Fos. **(B):** Co-Stimulation of CD28 by B7.1 activates another MAPK cascade via Rac-1, MAPKKK1, JNKK and Jnk finally resulting in Jun phosphorylation in the nucleus. Fos and Jun then form the transcription factor AP-1. **(C):** The transcription factors NFAT, NF-κB and AP-1 interact leading to gene transcription important for growth, differentiation, survival and apoptosis.

### 1.3.3 Co-stimulation of T cells

T cells require three types of signal for full activation: antigenic peptide presented on a MHC molecule, costimulatory signals transduced via CD28 and signaling by specific cytokines such as IL-2, whereas cytokine signaling is more involved in differentiation of $T_H$ cells, e.g. IL-17 to $T_{H17}$ cells.[1] The principal co-stimulatory

molecules expressed on APCs are B7.1 and B7.2 (CD80 or CD86) molecules, which bind to CD28 on T cells. Crosslinking of CD28 delivers the co-stimulatory signal. The requirement for the co-stimulatory signal ensures that T cells are not unspecifically activated by self-antigens. Antigen recognition without co-stimulation induces anergy. The co-stimulatory signal of CD28 activates another MAPK cascade, which activates c-Jun N-terminal kinase (Jnk) to move in the nucleus to phosphorylate Jun.[1] Fos and Jun form the activator protein (AP) -1.[22] The transcription factors NF-κB, NFAT and AP-1 interact to induce specific gene transcription, such as of IL-2, leading to cell proliferation and differentiation.[102] In recent years, it was discovered, that death receptors, such as CD95, can also have a costimulatory function and interfere with TCR signaling.[149]

### 1.3.4 CD95 as a non-apoptotic costimulatory signal in T cells

CD95 signaling has been shown not only to be involved in apoptosis but can also induce anti-apoptotic signals depending on the cell type, microenvironment and signal strength.[147] Membrane bound CD95L (mCD95L) has been shown to be essential for triggering apoptosis, whereas non-apoptotic activities are promoted by soluble CD95L (sCD95L) proteolytically cleaved by matrix metalloproteinases (MMPs) from the membrane-bound form.[117] Low doses of CD95L lead to increased activation and proliferation following TCR signaling in naïve T cells, whereas high concentrations of CD95L silence T cells.[149]

## 1.4 NF-κB

The NF-κB family of transcription factors consists of five members, p50/p105, p52/p100, p65, RelB and c-Rel which form different NF-κB complexes by homo- and heterodimers.[54,162] All family members share an N-terminal Rel-homology domain (RHD) which is responsible for dimerization and DNA binding. In addition, Rel-subfamily members p65, RelB and c-Rel contain unrelated C-terminal

transcriptional activation domains (TADs), necessary for the positive regulation of gene expression. Due to the lack of TAD in p50 and p52, they repress transcription, unless they are associated with a TAD-containing NF-κB family member or another co-activator. The p50/p65 dimer represents the major NF-κB-complex in T cells and plays a significant role for cell death and normal function of the immune system. Disregulated NF-κB expression has been linked to cancer, inflammatory and autoimmune diseases and viral infection.[10] Constitutively activated NF-κB could be observed in Hodgkin's lymphoma[11,15], Multiple myeloma[15] and Acute Lymphoblastic Leukemia (ALL)[79]. NF-κB inhibits apoptosis by altering the expression of many genes associated with tumor growth and survival.[9] It can both, induce and repress gene expression by binding to discrete DNA sequences, known as κB elements, within promoters and enhancers. It thereby regulates cellular responses to stimuli such as stress, cytokines, ultraviolet irradiation, free radicals and bacterial or viral antigens.[71]

### 1.4.1 Activation of NF-κB

NF-κB complexes are retained in the cytoplasm by inhibitors of NF-κB (IκBs), which include IκBα, IκBβ, IκBγ, IκBε, the p105 and p100 precursors of p50 and p52, and the atypical members Bcl-3, IκBξ and IκBNS (IκBδ).Typical IκB proteins inhibit transcription by masking a conserved nuclear localization signal (NLS) found in the RHD of NF-κB proteins. Due to a nuclear export sequence (NES) present in IκB and p65 proteins, p50/p65-IκBα complexes permanently shuttle between nucleus and cytoplasm in unstimulated cells. Because of a more efficient nuclear export, steady state localization is mostly cytosolic.[61] Activation of NF-κB is controlled by the IκB kinase (IKK) complex, which consists of three catalytic subunits — IKKα, IKKβ and IKKγ. The IKK complex phosphorylates IκB proteins on specific serine residues, which induces their subsequent polyubiquitination and proteasomal degradation, allowing NF-κB dimers to translocate freely to the nucleus and activate gene transcription.[72] There are two main pathways, the canonical and the non-canonical pathway, as well as several alternative pathways leading to the activation of NF-κB.

### 1.4.2 The canonical pathway in T cells

In most cell types the canonical or classical activation pathway starts in response to inflammatory mediators such as TNFα and IL-1. The IKK complex phosphorylates IκBα, leading to its ubiquitination and degradation, allowing p65/p50 to translocate to the nucleus. T cells require the caspase recruitment domain (CARD)-containing signaling adaptor protein (CARMA; CARD11), Bcl-10 and the paracaspase mucosa-associated lymphoid tissue protein (MALT) 1 to form the so called CBM-complex, which is specific for T cells and not needed in other cell types.[161] TCR triggering leads to the activation of the serine-threonine kinase PKC-θ, which phosphorylates CARMA.[108,142] Phosphorylation induces a conformational change and oligomerization. Activated trimerized CARMA binds Bcl-10, which in turn binds MALT1. Bcl-10 and CARMA proteins interact via their CARD domains[17], whereas the association between Bcl-10 and MALT1 depends on amino acids (aa) 106-120 of Bcl-10 and the Ig-like domains of MALT1.[100,159] Trimerized MALT1 is able to activate TNFR-associated factor (TRAF) -6 and -2 by stabilizing their trimerization or by inducing a conformational change of existing TRAF trimers.[119] MALT1 mediates also rapid proteolytic cleavage and inactivation of the NF-κB inhibitor A20 after TCR stimulation.[29] TRAF2 targets RIP for K63-linked polyubiquitination, whereas TRAF6 catalyzes K63-linked autopolyubiquitination.[13] A20 inhibits the activation process by its deubiquitinase activity.[143] K63-linked polyubiquitination leads in both cases to recruitment of IKK–γ, also known as NF-κB essential modulator (NEMO), which trimerizes over its NEMO ubiquitin-binding domain triggering its own K63-linked polyubiquitination at the C-terminus.[42] TGF-β-activated kinase- (TAK-) 1-binding protein (TAB) 2 or 3 of the TAK-1 complex binds to polyubiquitinated IKK-γ and brings TAK-1 in direct proximity to IKK-β which is bound to the N-terminus of IKK-γ. Phosphorylation of IKK-β by TAK-1 is the final crucial event in NF-κB activation and leads to phosphorylation of IκB on $Ser^{32}$ and $Ser^{36}$**,** targeting it for degradation.[24] This results in nuclear translocation of NF-κB by opening its NLS, where it binds to κB binding sites leading to transcriptional activation of genes important for immune response, cell proliferation and suppression of apoptosis.[105,145,165] The canonical pathway can be terminated for example by induction of IκBα gene

expression targeted by activated NF-κB in an auto-regulatory feedback loop. Newly synthesized IκBα can enter the nucleus, bind to NF-κB and shuttle it back to the cytoplasm.[69]

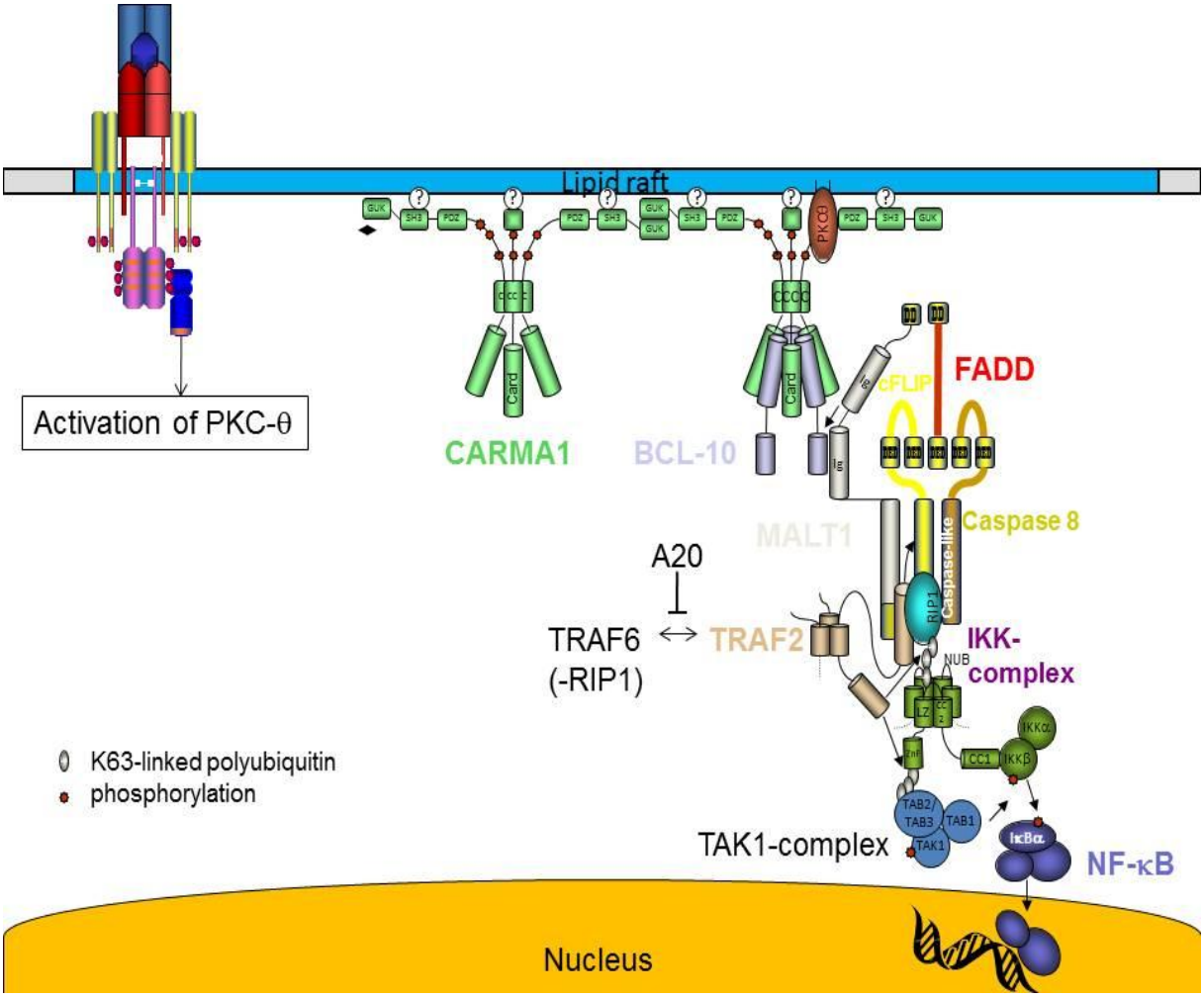



**Figure 6: NF-κB signaling in T cells – The CARMA1 signalosome**

TCR signaling leads to the activation of PKC-θ. PKC-θ phosphorylates CARMA1 molecules, which trimerize via their CC-domain and oligomerize via their guanylate kinase-like (GUK) domain. CARMA1 is constitutively localized in lipid rafts over an unknown binding partner. Trimerized CARMA1 recruits Bcl-10 via its CARD domain, which in turn recruits MALT to form the CBM-complex. MALT1 binds to Bcl-10 over their immunoglobuline (Ig) domains and also to cFLIP and caspase-8 via their caspase-like domain. TRAF2 trimers are then recruited, which ubiquitinate RIP. The IKK complex binds to ubiquitinated RIP or autoubiquitinated TRAF6 and becomes itself ubiquitinated at its zinc finger domain. This results in the recruitment of the TAK1 complex consisting of TAK1, TAB1, and TAB2/3, and phosphorylation of IKKβ. Activated IKKβ in turn phosphorylates IκBα which is subsequently proteasomally degraded, thereby making the hidden NLS of NF-κB accessible.

### 1.4.3 The non-canonical pathway

The non-canonical (alternative) NF-κB pathway is activated by receptors that are involved in lymphoid tissue organogenesis and lymphocyte development, such as the lymphotoxin-β receptor and the receptor for B cell-activating factor (BAFF).[37] Upon stimulation of the non-canonical pathway, NF-κB-inducing kinase (NIK) is activated leading to the phosphorylation of the p100 subunit. p100 is proteolytically processed to p52 resulting the activation of p52-containing NF-κB complexes, mainly consisting of p52/RelB heterodimers. Cellular inhibitor of apoptosis proteins (c-IAPs) were found to function as regulators of non-canonical NF-κB signaling by ubiquitination of NIK which promotes its proteasomal degradation.[59] RIP1 acts as a negative regulator of the non-canonical NF-κB pathway by stabilization of TRAF2. Loss of TRAF2, TRAF3, cIAP1 and/or cIAP2 leads to stabilization of NIK and activation of the non-canonical pathway.[77]

### 1.4.4 Dual role of NF-κB signaling in apoptosis

NF-κB can promote or inhibit apoptosis in certain cell types and in response to certain stimuli. Protective activities of NF-κB are due to an activated transcription of anti-apoptotic genes, including the Bcl-2 family members Bcl-2 and Bcl-$X_L$, XIAP, cIAP-1 and cIAP-2, cFLIP, and decoy death receptor TRAIL-R.[85] It has also been shown that p65 can actively suppress caspase-8.[23] But especially NF-κB activation can also lead to cell death by upregulation of its pro-apoptotic target genes TRAIL, TRAIL-R1 and-R2 , CD95L, CD95, Bax, Bcl-$X_S$, p53, and c-myc or by repression of anti-apoptotic proteins such as Bcl-$X_L$, XIAP, and A20.[85]

## 1.5 Aim of the study

Our group showed that long-term-activated T cells after repetitive alloantigen stimulation exhibit an effector/memory phenotype and are resistant to CD95 stimulation, while short-term-activated T cells are CD95-sensitive.[148] Long-term-activated T cells also show constitutive caspase cleavage and higher caspase activity, usually a sign for apoptosis. Instead, these cells were resistant to CD95 stimulation and displayed a strong permanent association between CD95 and caspase-8. After CD95 triggering, short-term-activated T cells recruited caspase-8, caspase-10, and FADD to the DISC, whereas long-term-activated T cells showed impaired DISC formation. However, caspases which were, for a long time, thought to be involved solely in apoptosis also contribute to specific aspects of immune cell development and differentiation, activation and proliferation. Caspase-8 inhibition by Z-VAD.fmk prevents T cell activation, and caspase-8 deficient patients exhibit decreased proliferation of T, B and NK cells after antigen stimulation due to defects in NF-κB activation.[48] It has been shown that caspase-8 in T cells is required for NF-κB signaling, which has pro- and anti-apoptotic functions, and that caspase-8 and c-FLIP associate in lipid rafts with NF-κB adaptors, where also CD95 signaling takes place.[5,44,110] Furthermore, it has been reported that CD95 can induce NF-κB signaling. Therefore, this thesis aims at characterizing the role of NF-κB in the regulation of CD95 signaling in long-term-activated T cells and the role of lipid rafts for these signaling pathways. For this purpose, the NF-κB activity of short- and long-term- activated T cells before and after CD95 stimulation was analyzed by electro-mobility shift assays and by the use of different NF-κB inhibitors to define the importance of NF-κB signaling for CD95 sensitivity. The composition of CD95- and NF-κB signaling molecules was analyzed by isolation and western blot analysis of lipid rafts. Furthermore, sensitive and resistant Jurkat cell lines were used for the analysis of the mechanisms leading to CD95 resistance by genomic and proteomic screening, using gene chip analysis and two-dimensional-difference-in-gel electrophoresis (2D-DIGE). A better understanding of the molecular mechanisms leading to CD95 resistance in long-term-activated T cells might help to identify new molecular targets for the therapy of autoimmune diseases, such as multiple sclerosis, hematologic cytopenias and rheumatoid arthritis.[28,179]

# 2. Material & Methods

## 2.1 Material

### 2.1.1 Consumable supplies

**Table 1: Consumable supplies**

| Consumable supply | Company |
|---|---|
| 10 ml plastic pipette | Costar |
| 2 ml plastic pipette | BD Falcon |
| 25 ml plastic pipette | Costar |
| 5 ml plastic pipette | Costar |
| 96 well plate flat-bottom | Greiner-bio-one |
| 96 well plate round-bottom | Greiner-bio-one |
| Centrifuge tubes (15 ml) | BD Falcon |
| Centrifuge tubes (50 ml) | BD Falcon |
| Cryotube (1,5 ml) | Nunc |
| Forceps | Ulrich Medizintechnik |
| Glass tubes | Brand |
| IPG-Strips (pH3-10NL) | GE-Healthcare |
| IPG-Strips (pH4-7NL) | GE-Healthcare |
| Needles 'Sterican' | Braun |
| Nitrocellulose membrane | GE Healthcare |
| Paper Wicks | GE Healthcare |
| Pipette filter tips (1 ml) | Biozym |
| Pipette filter tips (10 µl) | Biozym |
| Pipette filter tips (200 µl) | Biozym |
| Pipette tips (1 ml) | StarLab |
| Pipette tips (10 µl) | StarLab |
| Pipette tips (200 µl) | StarLab |
| QIAshredder | Qiagen |
| Scalpel | B/Braun |
| Scissors | Roth |
| Syringe (5 ml, 25G needle) | B/Braun |
| Tissue culture flask ($75cm^2$) | BD Falcon |
| Tubes (1,5 ml), safe-lock | Eppendorf |
| Whatman paper | Schleicher & Schuell |

## 2.1.2 Primers for Electrophoretic Mobility Shift Assay (EMSA)

**Table 2: Primers for Electrophoretic Mobility Shift Assay**

| Primer | Sequence |
|---|---|
| NF-κB sense | 5´- AGT TGA GGG GAC TTT CCC AGG C - 3´ |
| NF-κB anti-sense | 5´- GCC TGG GAA AGT CCC CTC AAC T - 3´ |
| NFAT sense | 5´- TCT AAG AGG AAA ATT TCA TG - 3´ |
| NFAT anti-sense | 5´- AGA TTA |
| AP-1 sense | 5´- CGC TTG ATG AGT CAG CCG GAA - 3´ |
| AP-1 anti-sense | 5´- GCG AAC TAC TCA GTC GGC CTT - 3´ |

## 2.1.3 Conjugated antibodies for flow cytometry

**Table 3: Conjugated antibodies for flow cytometry**

| Protein | Dye | Origin | Isotype | Company |
|---|---|---|---|---|
| **CD4** | FITC | mouse | IgG1 | BD |
| **CD8** | PE | mouse | IgG1 | BD |
| **CD95** | PE | mouse | IgG1 | BD |
| **mIgG1** | FITC | mouse | IgG1 | BD |
| **mIgG1** | PE | mouse | IgG1 | BD |

## 2.1.4 Kits

**Table 4: Kits**

| Kit | Company | Use |
|---|---|---|
| BCA Protein Assay | Thermo Scientific | Determination of protein concentration |
| Dynal CD8 Positive Isolation Kit | Dynal | Isolation of $CD8^+$ T cells |
| Dynal CD4 Positive Isolation Kit | Dynal | Isolation of $CD4^+$ T cells |
| RosetteSep | Stem Cell Technology | Isolation of pan T cells |

## 2.1.5 Antibodies for Western Blot analysis

**Table 5: Antibodies for Western Blot analysis**

| Protein | MW in kDa | Origin | Isotype | Company |
|---|---|---|---|---|
| **A20** | 70 | mouse | IgG1 | NatuTec |
| **Bcl-10 (H-197)** | 33 | rabbit | polyclonal | Santa Cruz |
| **Bcl-3 (C-14)** | 60 | rabbit | polyclonal | Santa Cruz |
| **CARD11 (N-20)** | 133 | goat | polyclonal | Santa Cruz |
| **CARD11=CARMA1** | 130 | rabbit | polyclonal | Cell Signaling |
| **caspase-10** | 58 | mouse | IgG1 | MBL |
| **caspase-3** | 35, 19, 17 | rabbit | polyclonal | Cell Signaling |
| **caspase-8** | 55, 54, 43, 41, 18 | mouse | IgG2b | Alexis |
| **CD59** | xxx | mouse | IgG2b | Exbio |
| **c-FLIP** | 55, 43, 41, 28 | mouse | xxx | xxx |
| **c-Rel** | 75 | rabbit | polyclonal | Santa Cruz |
| **FADD** | 27 | mouse | IgG1 | BD Pharmingen |
| **Fas=CD95** | 48 | rabbit | polyclonal | Santa Cruz |
| **FasL=CD95L** | 45 | mouse | IgG1 | Santa Cruz |
| **IκBα** | 40 | rabbit | polyclonal | Santa Cruz |
| **IκBβ** | 40 | rabbit | polyclonal | Santa Cruz |
| **IKKα** | 85 | rabbit | polyclonal | Santa Cruz |
| **IKKα, β** | 87,85 | rabbit | polyclonal | Santa Cruz |
| **IKKβ** | 87 | rabbit | polyclonal | Cell Signaling |
| **IKKγ** | 48 | rabbit | polyclonal | Santa Cruz |
| **LAT** | 38,36 | mouse | IgG | Upstate |
| **Lck** | 56 | mouse | IgG1 | Exbio |
| **MALT1 (C-16)** | 93 | goat | polyclonal | Santa Cruz |
| **p50** | 105, 50 | rabbit | polyclonal | Santa Cruz |
| **p52** | 100, 52 | rabbit | polyclonal | Santa Cruz |
| **p65** | 65 | rabbit | polyclonal | Santa Cruz |
| **PARP** | 116, 89 | rabbit | polyclonal | Cell Signaling |
| **PKCθ** | 79 | mouse | IgG2a | BD Biosciences |
| **PLCγ1** | 162 | rabbit | polyclonal | Cell Signaling |
| **RelB** | 68 | rabbit | polyclonal | Santa Cruz |
| **RIP1** | 74 | mouse | IgG1 | BD Pharmingen |
| **TRAF2** | 56 | rabbit | polyclonal | MBL |
| **TRAF6** | 55 | rabbit | polyclonal | ZYMED |
| **Zap70** | 70 | mouse | IgG2a | BD Biosciences |

## 2.1.6 Buffers and solutions

**Table 6: Buffers and solutions**

| Buffer | Ingredients |
|---|---|
| 10x SDS-PAGE running buffer | 30.3 g/ l Tris base, 144 g/ l glycin, 10 g/ l SDS |
| 6x loading buffer | 70% UGB, 0.38 g/ ml glycerol, 0.1 g/ ml SDS, 93 mg/ ml DTT, 0.12 mg/ ml bromphenol blue |
| Anode running buffer (2D-DIGE) | 0.025 M Tris; 0.192 M glycin; 0.1% SDS |
| Binding buffer | 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris/HCl, pH 7.5, 4% glycerol |
| Blocking buffer | 5 % (w/v) milk powder; 0.1% Tween-20 |
| Blotting buffer | 5.8 g/ l Tris base, 2.9 g/ l glycin, 0.037% SDS, 20% methanol |
| Buffer A (nuclear extraction) | 10 mM HEPES-OH pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl |
| Buffer B (nuclear extraction) | 20 mM HEPES-OH pH 7.9; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.5 mM EDTA; 25% glycerol |
| Cathode running buffer (2D-DIGE) | 0.05 M Tris; 0.384 M glycin; 0.2% SDS |
| Cell lysis buffer | 30 mM Tris/HCl pH 7.5; 150 mM NaCl; 1% Triton X-100; 10% glycerol; 1 mM PMSF; 1 µM DTT |
| CyDye labeling buffer | 7 M urea; 2 M thiourea, 4% (w/v) CHAPS; 30 mM Tris/HCl pH 8.1 |
| Displacing solution | 25% (v/v) 1.5 M Tris/HCl; 50% (v/v) glycerol; 0.0002% (w/v) bromephenol blue |
| Equilibration buffer 1 | 1 % DTT; 50 mM Tris/HCl pH 8.8; 3% SDS; 6 M urea; 30% (v/v) glycerin |
| Equilibration buffer 2 | 4% IAA; 50 mM Tris/HCl pH 8.8; 3% SDS; 6 M urea; 30% glycerin |
| FACS buffer | 1 x PBS, 10% FBS, 0.1% sodium azide |
| Glycolipid enriched membrane (GEM) lysis buffer | 50 mM HEPES pH 7,4; 100 mM NaCl; 3 % (v/v) Brij58; 1 mM PMSF; 5 mM EDTA; 1 mM sodium ortovanadate; 50 mM NaF; 10 mM sodium pyrophosphate |
| MNE-buffer | 25 mM MES; 5 mM EDTA; 150 mM NaCl; pH 6.5 |
| Red blood cell (RBC) lysis buffer | 8.29 g/ l ammonium chloride, 1.0 g/ l potassium hydrogen carbonate, 0.037 g/ l EDTA, pH 7.2 - 7.4 |
| Upper gel buffer (UGB) | 60 g/ l Tris base, 4 g/ l SDS, pH 6.8 |

### 2.1.7 Reagents and solutions

**Table 7: Reagents and solutions I (A-L)**

| **Chemical** | **Company** |
|---|---|
| 1x PBS | Biochrom AG |
| Acetic acid | Sigma |
| Aceton | Roth |
| Acrylamid | Sigma |
| Agarose | Sigma |
| $AgNO_3$ | Sigma |
| APO-1 (anti-CD95 antibody) | self-made |
| APS | Serva |
| Bay | Sigma |
| Biocoll | Biochrom AG |
| Brij58 | Pierce |
| Butanol | Sigma |
| Cape | Sigma |
| CHAPS | Merck |
| Deoxycholacid | Sigma |
| DeStreak rehydration solution | GE Healthcare |
| DMSO | Merck |
| Dry Strip Cover Fluid | GE Healthcare |
| DTT | Sigma |
| EDTA | Sigma |
| Ethanol | Roth |
| FBS | Lonza |
| FBS | Gibco |
| Gentamycin | Biochrom AG |
| Glycerin | Sigma |
| Glycin | Sigma |
| HEPES pH 7.4 | Biochrom AG |
| IL-2, human, recombinant | PeproTech |
| Iodacetamid | Sigma |
| IPG buffer (pH 4-7 NL) | GE Healthcare |
| IPG buffer (pH 4-7 NL) | GE Healthcare |
| L-Glutamine (200 mM) | Gibco |

**Table 8: Reagents and solutions II (M-Z)**

| Chemical | Company |
|---|---|
| MES | Sigma |
| Methanol | Sigma |
| MG-132 | Sigma |
| Milk powder | Roth |
| Mitomycin C | Sigma |
| $Na_2CO_3$ | Sigma |
| NaF | Roth |
| Nitrocellulose membrane | Amersham Bioscience |
| NP-40 | Merck |
| Penicillin/Streptomycin | Gibco |
| peqGOLD Protein Marker | Peqlab |
| PMA | Sigma |
| PMSF | Sigma |
| RPMI 1640 | Gibco |
| SDS | Sigma |
| Sodium ortovanadate | Sigma |
| Sodium pyrophosphate | Sigma |
| Sodium pyruvate (100 mM) | Biochrom AG |
| SPI | Sigma |
| Staurosporine | Sigma |
| TCA | Sigma |
| Temed | Sigma |
| Thiourea | Sigma |
| TRIS | Sigma |
| Urea | Sigma |
| Z-VRPR-FMK | Enzo |

## 2.1.8 Equipment

**Table 9: Equipment**

| Equipment | Company |
|---|---|
| Centrifuge | Heraeus Christ |
| Centrifuge | Heathrow scientific |
| Centrifuge '5417R' | Eppendorf |
| Centrifuge, Variofuge | Heraeus Christ |
| Clean bench | BDK |
| Developer Kodak M1000 | Kodak |
| ELISA ELx 800 | BioTek Instruments Inc. |
| FACS LSRII | Becton Dickinson |
| FACSScan | Becton Dickinson |
| Gel electrophoresis power supply EPS30 | Pharmacia Biotech |
| Gel electrophresisis system | Peqlab |
| Gel Imager Intas UV system | Intas |
| Homogenisator (douncer) | Fisher scientific |
| Incubator | Forma Scientific |
| Incubator BBD 6220 | Heraeus Instruments |
| L-70 ultracentrifuge | Beckmann |
| Microscope | Leica |
| N2 freezer Chromos | Messer Griesheim |
| Pipet 100-1000 µl | Finnpipette |
| Pipet 1-10 µl | Finnpipette |
| Pipet 20-200 µl | Finnpipette |
| Pipet 5-50 µl | Finnpipette |
| Spot-picker | GE-Healthcare |
| SW41 rotor | Beckmann |
| Ultra-clear centrifuge tubes | Beckmann |
| Fixed-angle rotor F-45-30-11 for Centrifuge 5417 C / 5417 R | eppendorf |

## 2.1.9 Cell Lines

**Table 10: Cell lines**

| Cell line | Description |
|---|---|
| .721 | HLA-A1$^{+}$; used for stimulation of HLA-A1$^{-}$ T cells |
| Je6.1 | CD95-sensitive Jurkat cell line |
| JM | CD95-sensitive Jurkat cell line |
| JM301 | CD95-resistant Jurkat cell line, created out of JM cells through constitutive incubation with APO-1 |
| JM319 | CD95-resistant Jurkat cell line, created out of JM cells through constitutive incubation with APO-1 |

## 2.1.10 Gels

**Table 11: Gels**

| Gel | Ingredients | Use |
|---|---|---|
| Stacking gel - polyacrylamide | 5% acrylamide, 0.1% SDS, 125 mM Tris/HCl pH 6.8, 0.1% APS, 0.1% TEMED | Western Blot analysis |
| Resolving gel - polyacrylamide | 10% or 12% acrylamide, 0.1% SDS, 250 mM Tris/HCl pH 8.8, 0.1% APS, 0.04% TEMED | 2D-DIGE<br>Western Blot analysis |
| 1.5% Agarose gel | 1.5% (w/v) Agarose in 1xTAE | RNA purity |

## 2.2 Methods

### 2.2.1 Cell culture

The B cell lymphoma cell line .721 and the Jurkat cell lines Je6.1, JM , JM301 and JM319 were cultured in RPMI 1640 with 10% FCS (Gibco), 1% glutamine and 1% sodium pyruvate. Human primary cells were cultured in the same medium including 0.1% β-mercaptoethanol. The culture conditions were 37°C and 7.5% $CO_2$. To remove dead cells, cell suspensions were adjusted to $2.5x10^7$ cells/ ml in PBS and 1 volume of FICOLL was overlaid with 2 volumes of the cell suspension and centrifuged at 789 g for 15 min at RT without brake to avoid mixing of the gradient. Living cells were collected from the interphase, washed three times in PBS and resuspended in their respective medium.

### 2.2.2 Generation of alloreactive long-term-activated, CD95-resistant T cells

#### 2.2.2.1 Isolation of human peripheral blood lymphocytes (PBLs) from Buffy Coats

1x phosphate buffered saline (PBS) was added to 20-30 ml human blood from the „DRK-Blutspendezentrale Ulm“ to a final volume of 50 ml. 10 ml Biocoll were overlayed with 25 ml diluted human blood and centrifuged for 15 min at 789 g without brake. The interphase was collected and 1x PBS was added to a final volume of 50 ml. Cells were washed three times with 1x PBS. To remove erythrocytes, cells were resuspended in 7 ml erythrocyte lysis buffer and incubated for 5 min at 37°C. After two more washing steps with 1x PBS, cells were resuspended in RPMI 1640 with 2% FBS, 1% glutamine and 0.1% β-mercaptoethanol to a final cell concentration of $2*10^6$ cells/ml with a maximum volume of 50 ml per 75 $cm^2$ culture flask.

### 2.2.2.2 Mitomycin treatment of HLA-A1$^+$ .721 cells

T cells were stimulated with human leukocyte antigen (HLA) –A1$^+$ .721 cells. For this purpose, .721 cells were treated with mitomycin to block cell division. Cells were distributed to 50 ml falcon tubes with 1-1.5*10$^7$ cells/ml and 200 µg/ml mitomycin were added to the cell suspension. After 40-60 min at 37°C, cells were pooled, washed three times with 1x PBS at 789 g and resuspended in primary cell medium for the stimulation of PBLs.

### 2.2.2.3 Stimulation of HLA-A1$^-$ human PBLs with .721 cells

Biocoll-separated peripheral blood mononuclear cells (PBMCs) from HLA-A1$^-$ donors were adjusted to 1*10$^6$ cells/ml and incubated with mitomycin C-treated HLA-A1$^+$ .721 stimulator cells at a ratio of 10 to 1 in the presence of 30 U/ml human recombinant IL-2. Dead cells were removed by Ficoll densitiy gradient centrifugation, if cultures contained more than 30% dead cells. Stimulation was performed weekly and 30 U/ml IL-2 were given twice a week. Short-term-activated T cells presented T cells between the 3$^{rd}$ and 7$^{th}$ stimulation and are characterized by high CD95 sensitivity, while long-term-activated T cells were stimulated at least 9 times and exhibit CD95 resistance.

### 2.2.2.4 Determination of CD95 sensitivity

2*10$^5$ cells per well were seeded in a 48-well plate and incubated with 10, 100 or 1000 ng APO-1 /ml at 37°C for 24 h. Cells were harvested, washed once with 1x PBS and transferred into a 96-well round bottom plate. To distinguish between CD4$^+$ and CD8$^+$ T cell populations, cells were stained for 30 min with CD8- phycoerythrin (PE) and CD4- fluorescein (FITC) antibodies at 4°C in darkness. The cells were washed twice with FACS medium and analyzed on an LSRII flow cytometer. CD4$^+$ and CD8$^+$ subpopulations were gated and cell death was determined by measuring FSC/SSC. CD95 sensitivity was routinely determined in triplicates.

Specific apoptosis was calculated according to the formula:

100 x [experimental cell death (%) – spontaneous cell death (%)]/[100 – spontaneous cell death (%)].

### 2.2.3 Western Blot

#### 2.2.3.1 Protein extraction of whole cell lysate for Western Blot

1-10*$10^7$ cells were washed twice with 1x PBS and centrifuged for 5 min at 789 g and 4°C. The supernatant was discarded and cells were resuspended in 100 µl of cell lysis buffer per 1*$10^7$ cells. The lysis reaction was transferred into a 1.5 ml eppendorf tube and incubated for 15 min on ice. The tubes were centrifuged for 10 min at 17.900 g and 4°C and the supernatant containing the proteins was transferred into a new 1.5 ml eppendorf tube. Protein lysates were stored at -80°C until further use. Determination of protein concentration was performed using the BCA protein assay kit (Thermo Scientific) and the BioTek EL800 with the analysis software Gen5 1.07.

#### 2.2.3.2 Sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE)

For SDS separation of protein lysates, aqua bidest was added to 20 µg of protein to a final volume of 50 µl. After addition of 10 µl 6x loading buffer, proteins were denatured for 5 min at 95°C and loaded onto a 12% SDS gel. SDS-PAGE was performed overnight at 15-20 V.

#### 2.2.3.3 Electro-blotting and antibody staining

The protein gels were electro-blotted onto Hybond ECL nitrocellulose membrane by using 1 mA/$cm^2$ of membrane. Membranes were washed for 10 min in 1x PBS with 0.1% Tween-20 and incubated for 1 h in blocking buffer. After washing with 1x PBS, the membranes were incubated overnight with the primary antibody. Membranes were washed three times in 1x PBS with 0.1% Tween-20 before adding the Horseraddish peroxidase- (HRP-) conjugated secondary antibody. After 1 h incubation, the membranes were washed again three times with 1x PBS with 0.1% Tween-20. Detection was performed by incubating membranes for 1 min in freshly

mixed enhanced chemoluminescene (ECL) solution according to the instructions of the manufacturer.

### 2.2.4 Lipid Rafts

Because of the high content of cholesterol within lipid rafts, these structures can be isolated by sucrose gradient ultracentrifugation.

#### 2.2.4.1 Isolation of lipid rafts

Cells were lysed for 10 min on ice with 1 ml GEM lysis buffer with up to $1.5*10^8$ cells. Lysates were mixed with 1 ml ice-cold 80% sucrose and transferred to a douncer. After 10 quick regular thrusts with the ice-cold douncer, the solution was transferred into an ultracentrifuge tube and carefully overlayed with 5 ml ice-cold 30% sucrose and 5 ml 5% sucrose. The tubes were centrifuged for 18-20 h at 40.000 rpm in the ultracentrifuge with a SW41 Ti rotor (Becton Dickinson) at 4°C without brake. Twelve 1 ml fractions were collected and immediately frozen at -80°C until protein precipitation was performed.

#### 2.2.4.2 Identification of lipid raft fractions

Figure 7A depicts the protocol for the isolation of lipid raft fractions. LAT is a palmitoylated membrane-associated protein, which is constitutively found in lipid rafts in T cells and, therefore, acts as a marker for the lipid raft fraction.[172] Western Blot analysis of each fraction revealed that LAT was exclusively found in fractions 5-6 of activated T cells, whereas the non-raft marker α-tubulin was found in fractions 10 and 11 (Figure 7B). Alternatively, lipid raft-containing fractions can be determined by staining dot-blots with an antibody against CD59, recognizing the native protein, as raft marker (Figure 7C). For this 2 µl of each 1 ml fraction were spotted on a nitrocellulose membrane to determine in which fractions the lipid rafts can be found. The following steps were performed after the western blot protocol using CD59 as

raft marker.

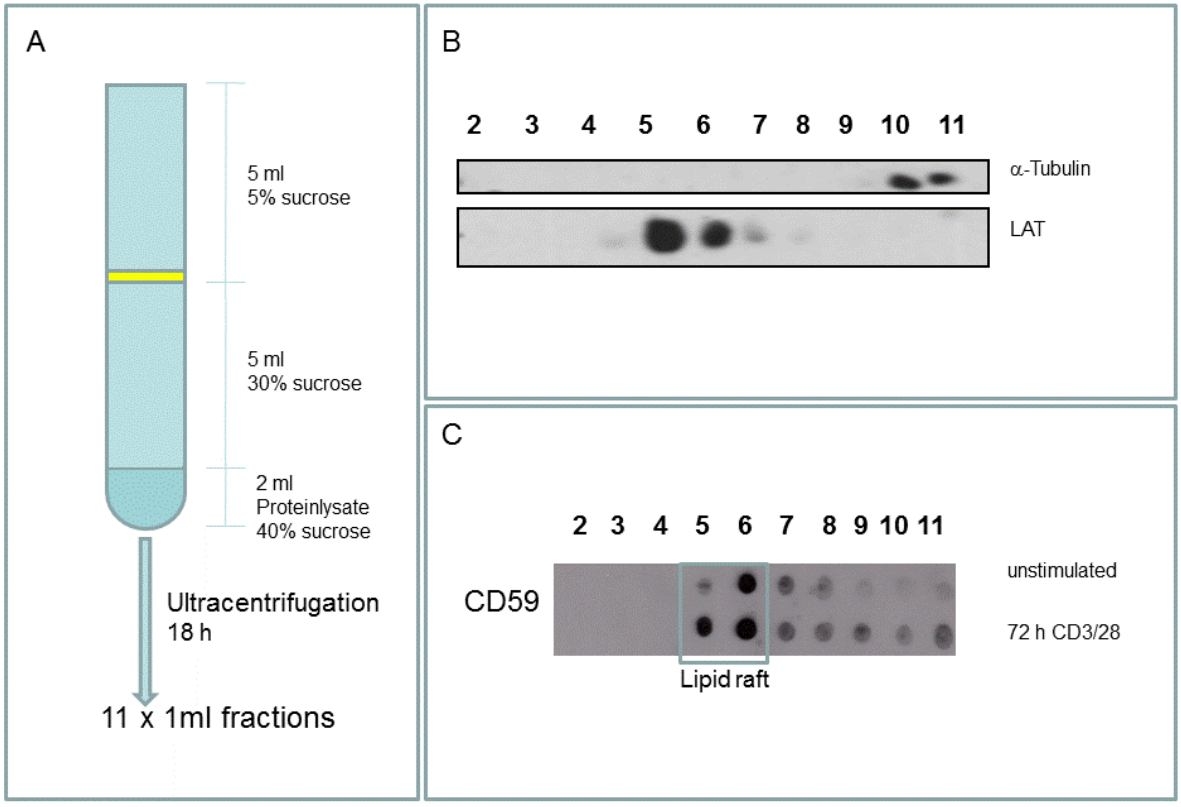



**Figure 7: Determination of lipid raft fractions**

Schemata of lipid raft isolation. 11 1 ml fractions were taken and the 12$^{th}$ fraction was discarded (B) Western Blot of the fractions 2-11 with the lipid raft marker LAT and non-raft marker α-Tubulin of 1x10$^{8}$ T cells activated by 25 µg CD3/CD28. 50 µl of each fraction were loaded (C) Dot-Blot of the fractions 2-11 with CD59 antibody of unstimulated and 72 h CD3/28 activated T cells. 2 µl of each fraction were used. The raft fractions 5-6 were pooled and are shown in Figure 15.

#### 2.2.4.3 Protein precipitation of lipid raft fractions for Western Blot analysis

Lipid raft fractions were precipitated to reduce the volume for SDS-PAGE. 2 µl 10% deoxychol acid were added to each 1 ml fraction and samples were incubated for 15 min at room temperature (RT). After addition of 300 µl 20% trichloroacetic acid (TCA), samples were further incubated for 1 h on ice, centrifuged for 10 min at 20.700 g and the supernatant was discarded. The precipitated proteins were washed

twice with ice-cold acetone by centrifugation at 14.000 rpm (16.000 g) and dried at RT. Dried proteins were resolved in 1x loading buffer for western blot analysis.

### 2.2.5 Electrophoretic Mobility Shift Assay (EMSA)

EMSA is used to study protein/DNA interactions and was used to analyze NF-κB, NFAT and AP-1 activity in long-term- compared to short-term-activated T cells. Supershifts were performed to determine which NF-κB subunits are active in short- and long-term-activated T cells.

#### 2.2.5.1 Extraction of nuclear fractions

Buffer A and B were supplemented with 1% 0.5 mM dithiothreitol (DTT), 1% phosphatase inhibitor cocktail and 1% sodium vanadate before use. Cells were collected in a 1.5 ml tube and washed twice with ice-cold 1x PBS. All following steps were performed on ice. After centrifugation, the pellets were resuspended in 100 µl buffer A per $1x10^7$ cells by gently flipping the tube. After swelling of the cells on ice for 10-15 min, 10% vol. of a 10% solution of NP-40 in buffer A were added. The tubes were vortexed vigorously for 30 sec for cell lysis. Afterwards, the samples were centrifuged for 5 min at 1.300 g and 4°C and the cytoplasmic fraction (supernatant) was stored at -80°C. The nuclear pellets were resuspended in 25 µl buffer B per $1x10^7$ cells and incubated for 15-20 min on ice while vortexing the tubes at regular intervals. Samples were centrifuged for 8 min at 17.900 g and 4°C and the nuclear extracts (supernatant) were frozen at -80°C.

#### 2.2.5.2 $^{32}$P-Labeling of oligonucleotides

1 µl of the sense oligonucleotide was mixed with 4 µl aqua bidest, 1 µl 10x buffer, 1 µl polynucleotide kinase (PNK) and 3 µl dATP ($^{32}$P). The mixture was incubated for 30 min at 37°C in a water bath for labeling. Samples were transferred on ice and 1 µl $MgCl_2$ (10 mM) and 2 µl of the anti-sense oligonucleotide were added. The mixture

was subsequently incubated for 5 min at 90°C in a heat block and then cooled down overnight. To purify the labeled oligonucleotides the column matrix was washed twice with 500 µl aqua bidest and dried by centrifugation for 2 min at 2.700 g. 60 µl aqua bidest were added to the labeling mixture which was then carefully pipetted onto the column matrix placed in a 1.5 ml eppendorf tube and centrifuged for 4 min at 2.700 g.

#### 2.2.5.3 Electrophoresis of nuclear extracts

For detection of transcription factor binding, 5 µg nuclear extract, 1 µg poly(dI:dC), 10.000 cpm labeled oligonucleotides and binding buffer were incubated for 30 min on ice. Samples were separated by electrophoresis on a non-denaturing 6% polyacrylamide gel, using 0.3 M TBE as running buffer. For supershift experiments, binding reactions containing 2.5 µg nuclear extract were incubated with 4 µg of either a p50-, p65-, Rel-B-, c-Rel- or p52-binding antibody for 30 min before adding the labeled oligonucleotides. The gel was dried and bands corresponding to the protein/DNA complexes were visualized by autoradiography.

### 2.2.6 Gene Chips

RNA was isolated from $1x10^7$ cells using the Qiagen RNA isolation kit according to the instructions of the manufacturer. RNA was separated in a size- dependent manner by agarose gel electrophoresis to check RNA quality. Non-degraded RNA shows specific bands of ribosomal RNA (rRNA). For this purpose, samples were mixed with 1/6 volume loading dye and loaded on 2 % TAE agarose gels. As running buffer 1x TAE was used. Ribosomal RNA was visualized by UV light and documented using the Intas gel imaging system. RNA of the Jurkat cell lines Je6.1, JM, JM301 and JM319 was sent to the Chip Facility Ulm for Affimatrix whole genome chip analysis.

### 2.2.7 2D-Difference-In-Gel-Electrophoresis (2D-DIGE)

2D-DIGE was used for a proteomic screen of the CD95-sensitive cell lines Je6.1 and JM compared to the CD95-resistant Jurkat cell lines JM301 and JM319.

Fluorescence labeling of the protein samples makes it possible to analyze the proteome of two different donors on the same gel. The use of a fluorescent-labeled internal standard provides the possibility to overlay different runs. (Figure 8) For 2D-DIGE it is important to isolate the proteins without using detergents and ions, because they interfere with CyDye-labeling and isoelectric focusation.

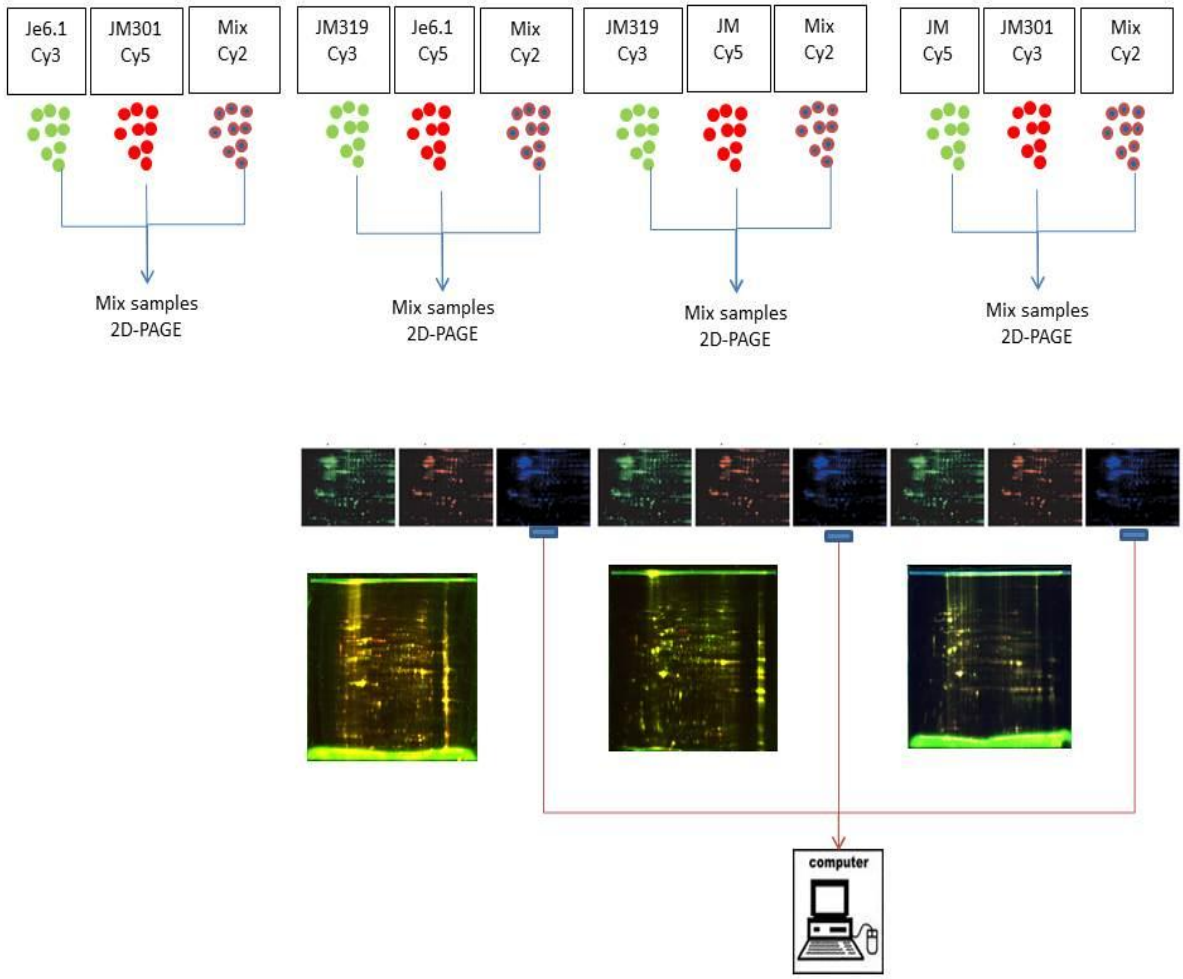



**Figure 8: Scheme of 2D-DIGE analysis**

On each 2D-DIGE gel, a protein sample pair derived from one CD95-sensitive and one -resistant cell line was compared. In total, four gels were ran to compare each possible combination of the 2 CD95-sensitive and the 2 -resistant Jurkat cell lines. One protein sample was crosslinked with Cy3, the second with Cy5. As internal standard, a mix of all protein lysates labeled with Cy2 was used to overlay all gels in computational analysis to compare the proteome of CD95-sensitive and -resistant Jurkat cell lines.

#### 2.2.7.1 2D-DIGE sample preparation

Cells were resuspended with $2*10^7$ cells per 100 µl 1x PBS and lysed by three 5 second sonication repeats and subsequent centrifugation for 10 min at 20.700 g and

4°C. The protein lysate was transferred to a new 1.5 ml tube and stored for further use at -80°C. Triplicates of 5 µl were used for the determination of protein concentration. 150 µg of protein lysate were pipetted into a new 1.5 ml tube and mixed with 1 ml of ice-cold aceton. After incubation for 30 min at -20°C, the tubes were centrifuged for 10 min at 20.700 g. The protein pellet was resuspended in 40 µl 2D-DIGE buffer, using a supersonic bath three times for one minute and shaking at RT overnight. Immobilized pH-gradient (IPG) -stripes were rehydrated with 400 µl Destreak rehydration solution and overlayed with Cover-Fluid overnight at RT.

#### 2.2.7.2 CyDye-labeling

Each sample, containing 130-150 µg protein, was labeled with 200 pmol of Cy3 or Cy5. The single samples were labeled either Cy3 or Cy5 for a dye-switched comparison to avoid potential dye-to-protein preferences. The internal standard consisting of an aliquot of each individual sample was labeled with Cy2 in the same dye-to-protein ratio. The samples were combined and diluted 1.33 x in a stock solution for subsequent isoelectric focusing.

#### 2.2.7.3 Isoelectric focusing of proteins

The IPG-Phor was used for isoelectric focusing overnight. For optimal transfer of the proteins from the first to the second dimension, proteins were equilibrated first in 25 ml equilibration buffer 1 with DTT, to reduce disulfide bonds, and SDS for electrophoretic mobility. In a second step, IPG stripes were equilibrated with equilibration buffer 2 containing iodacetamid, to prevent reoxidation of the thiol groups and SDS. The stripes were incubated for 20 min in 25 ml equilibration buffer 1 and 2 with a maximum of 4 stripes in a closable plastic tube under constant shaking.

#### 2.2.7.4 2D-DIGE: second dimension and spot analysis

For the second dimension, the Ettan DALT II System was used. 12.5% polyacrylamid gels were always prepared freshly one day before use. Electrophoresis was

performed at 3.5 W/gel overnight at RT. The fluorescence signals of the Cy-labeled protein samples were detected using the DIGE-Imager, recording emission wavelengths of 520 nm (Cy2), 580 nm (Cy3) and 670 nm (Cy5). Decyder software was used to determine spots of interest, which were manually picked and identified by mass spectrometry at the Max-Planck Institute.

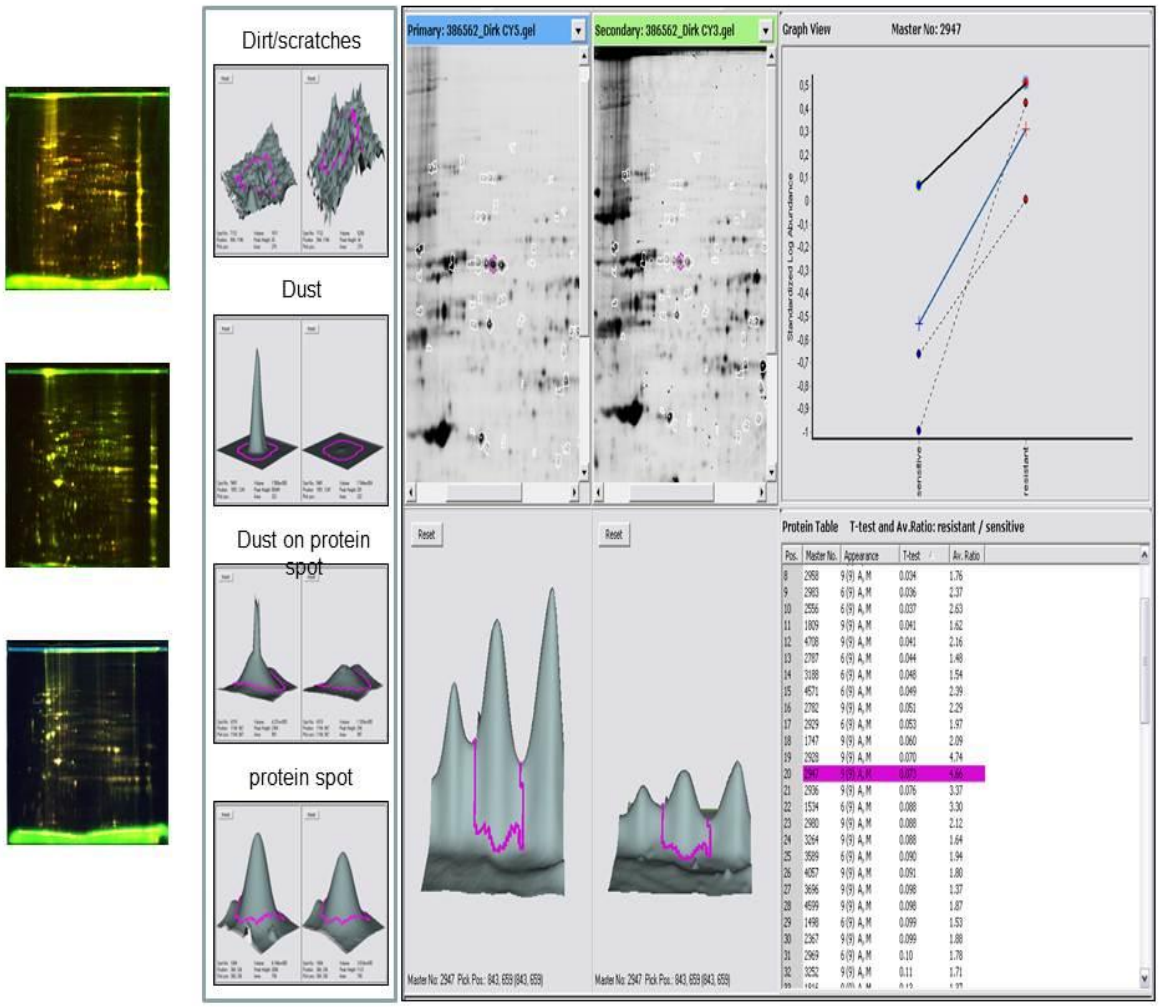



**Figure 9: Scheme of 2D-DIGE analysis**

Decyder software was used to overlaying more than one gel by using the Cy-2-labeled protein mix. Each spot was checked for dirt or scratches on each gel, which may have influenced a detected difference in protein expression.

# 3. Results

## 3.1 Part I: Relevance of NF-κB for CD95 resistance in long-term-activated T cells

The NF-κB signaling pathway was analyzed for differences in short- and long-term-activated T cells. Relevant components of TCR-, NF-κB- and CD95 signaling pathways were analyzed by Western Blot of whole cell lysates and lipid rafts. NF-κB activity in short- and long-term-activated T cells was compared by EMSA and the involved NF-κB subunits were determined by supershifts using specific antibodies.

### 3.1.1 Repeated TCR stimulation induces CD95 resistance in long-term-activated T cells

HLA-A1$^-$ T cells were stimulated weekly with the alloantigen HLA-A1$^+$ expressed on the lymphoblastoid cell line .721. CD95 sensitivity of CD4$^+$ and CD8$^+$ T cells was monitored weekly at day five after restimulation (Figure 10). While naïve T cells were resistant to CD95-induced apoptosis, activated T cells acquired sensitivity after 3-6 days of stimulation. Maximum apoptosis sensitivity was reached after the 7$^{th}$ stimulation. These cells were called short-term-activated T cells. However, after the 8$^{th}$ stimulation, apoptosis sensitivity decreased and long-term-activated T cells became CD95-resistant again. The population of CD95-resistant, long-term-activated T cells consisted exclusively of CD8$^+$ T cells, whereas short-term-activated, CD95-sensitive T cells still represented a mixture of CD4$^+$ and CD8$^+$ T cells. Approximately 1-10% of all HLA-A1$^-$ T cells are alloreactive against HLA-A1, resulting in their activation after stimulation with HLA-A1$^+$ APCs and in overgrowth of the initial T cell pool. Maximum growth was usually reached after the 5$^{th}$ round of stimulation.

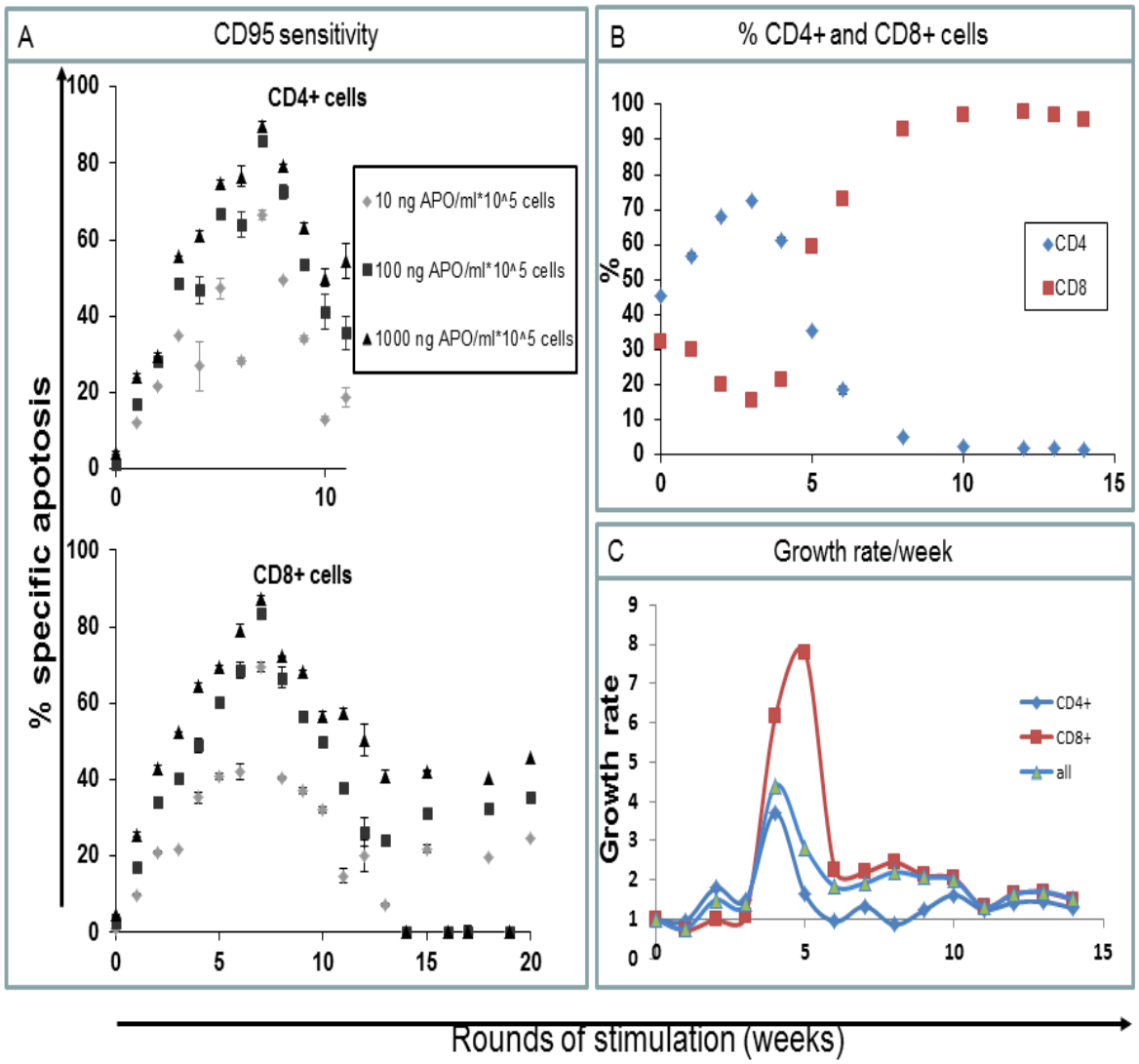



**Figure 10: Repetitive alloantigen stimulation induces CD95-resistant alloantigen-specific T cells.**

HLA-A1$^-$ T cells were stimulated weekly with HLA-A1$^+$ .721 cells at a ratio of 10:1. For analysis of CD95 sensitivity T cells were incubated for 24h in a 48-well plate with 2*10$^5$ cells/well and 10, 100 or 1000 ng/ml APO-1 on day 5 after stimulation. Apoptosis was determined by measuring FSC/SSC of CD4$^+$ and CD8$^+$ T cells. Data represent the mean of triplicates (A). The CD4$^+$/CD8$^+$ ratio of each stimulation is shown in (B) and the growth rate in (C).

### 3.1.2 Both, short- and long-term-activated T cells, display constitutive caspase activation

The expression of proteins involved in T cell activation and CD95 signaling was analyzed in HLA-A1-specific, short- and long-term-activated T cells. (Figure 11)

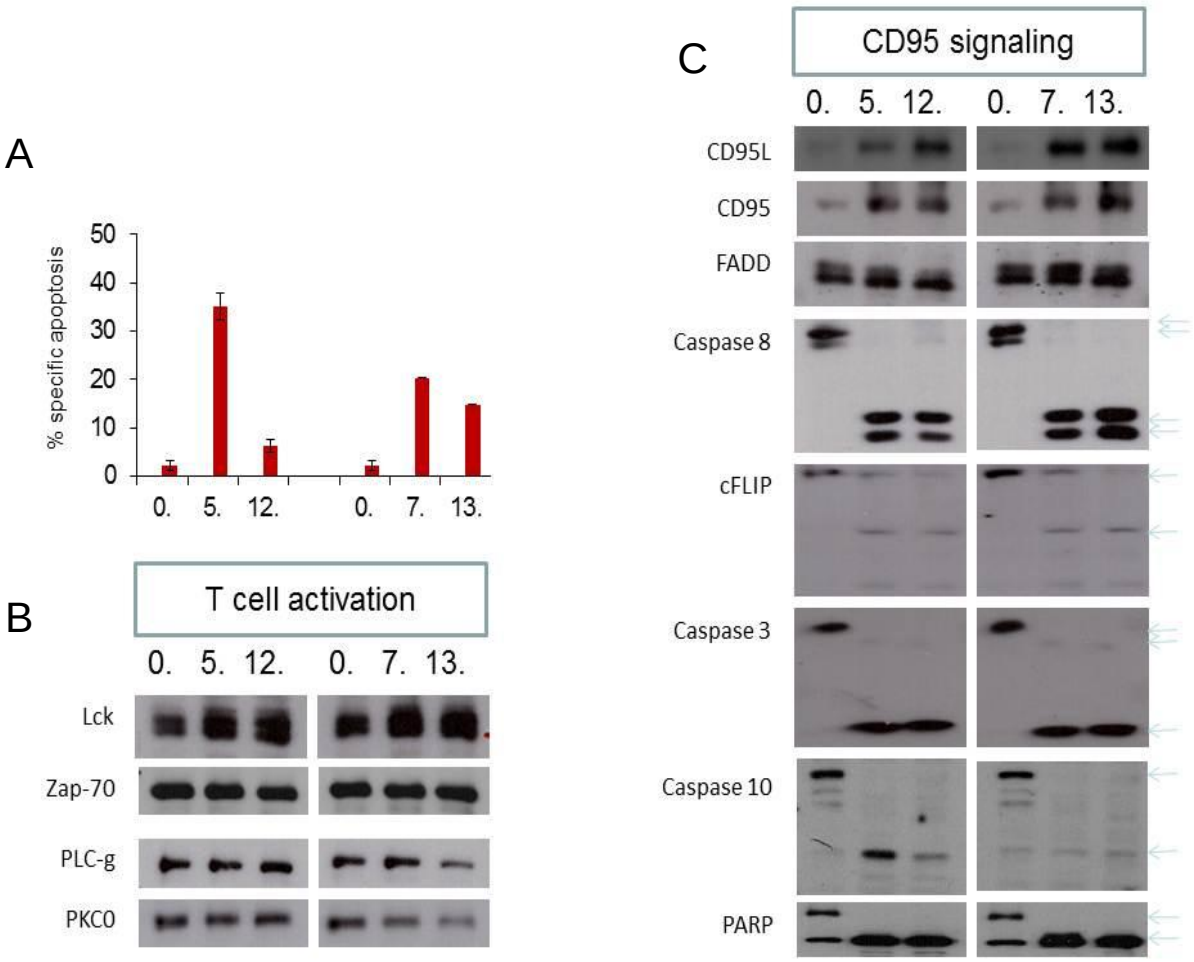



**Figure 11: Caspases are constitutively cleaved in short- and long-term-activated T cells.**

Unstimulated, short- and long-term-activated $CD8^+$ T cells were compared by CD95 sensitivity (A) and levels of proteins involved in T cell activation (B) and CD95 signaling (C). Lysates of isolated $CD8^+$ T cells were prepared at day 4 after restimulation. Results are shown for two different blood donors.

The expression profile of molecules induced in T cell signaling was comparable in both cell types, with the exception of PLC-γ and PKC-θ, These two were both downregulated in long-term- compared to short-term-activated T cells in one out of

two blood donors analyzed. Lck and Zap-70 levels were unaltered in short- and long-term-activated T cells. In line with published data, CD95L expression increased upon activation and was generally higher in long-term-activated than in short-term activated T cells.[148] Furthermore, recruitment of CD95L to lipid rafts was more efficient in long-term- compared to short-term-activated T cells (Figure 16). FADD expression was unaltered in short- and long-term-activated T cells. C-FLIP, the initiator caspases-8 and -10 and the effector caspase-3, as well as its target protein PARP were cleaved upon T cell activation, however, this was the case in both, short- and long-term-activated T cells.

### 3.1.3 Expression of NF-κB signaling mediators differs between short- and long-term-activated T cells

The expression of proteins involved in NF-κB signaling was analyzed by Western Blot in HLA-A1-specific, short- and long-term-activated T cells (Figure 12). CARMA 1, the main initiator of NF-κB activation in response to T cell stimulation, was found to be expressed in unstimulated, but not in activated T cells, independent of their stimulation round. Malt1 levels did not change upon activation and were also indifferent between short- and long-term-activated T cells. The signaling mediators TRAF2 and TRAF6 were upregulated, however, to the same extent in short- and long-term-activated T cells. When analyzing total Bcl-10 levels, a second band, indicating its potential phosphorylation, was observed in short-term-, but not in long-term-activated T cells. The phosphorylation status of Bcl-10 must be confirmed by the use of a specific antibody detecting phosphorylated Bcl-10. The expression of the NF-κB inhibitors A20 and IκBα was lower in long-term- compared to short-term-activated T cells, suggesting an increased activation of NF-κB in these cells. While unstimulated T cells did not express IκBβ, it was strongly upregulated upon activation. However, only slight differences were found between short- and long-term-activated T cells. IKK-α, -β and -γ were equally expressed independent of activation status or stimulation round. The same was true for the NF-κB subunits p50, p52, p65 and c-Rel. Interestingly, Bcl-3, which is known to interact with NF-κB, resulting in enhanced transcription of a variety of genes, was slightly upregulated in long-term-activated T cells.

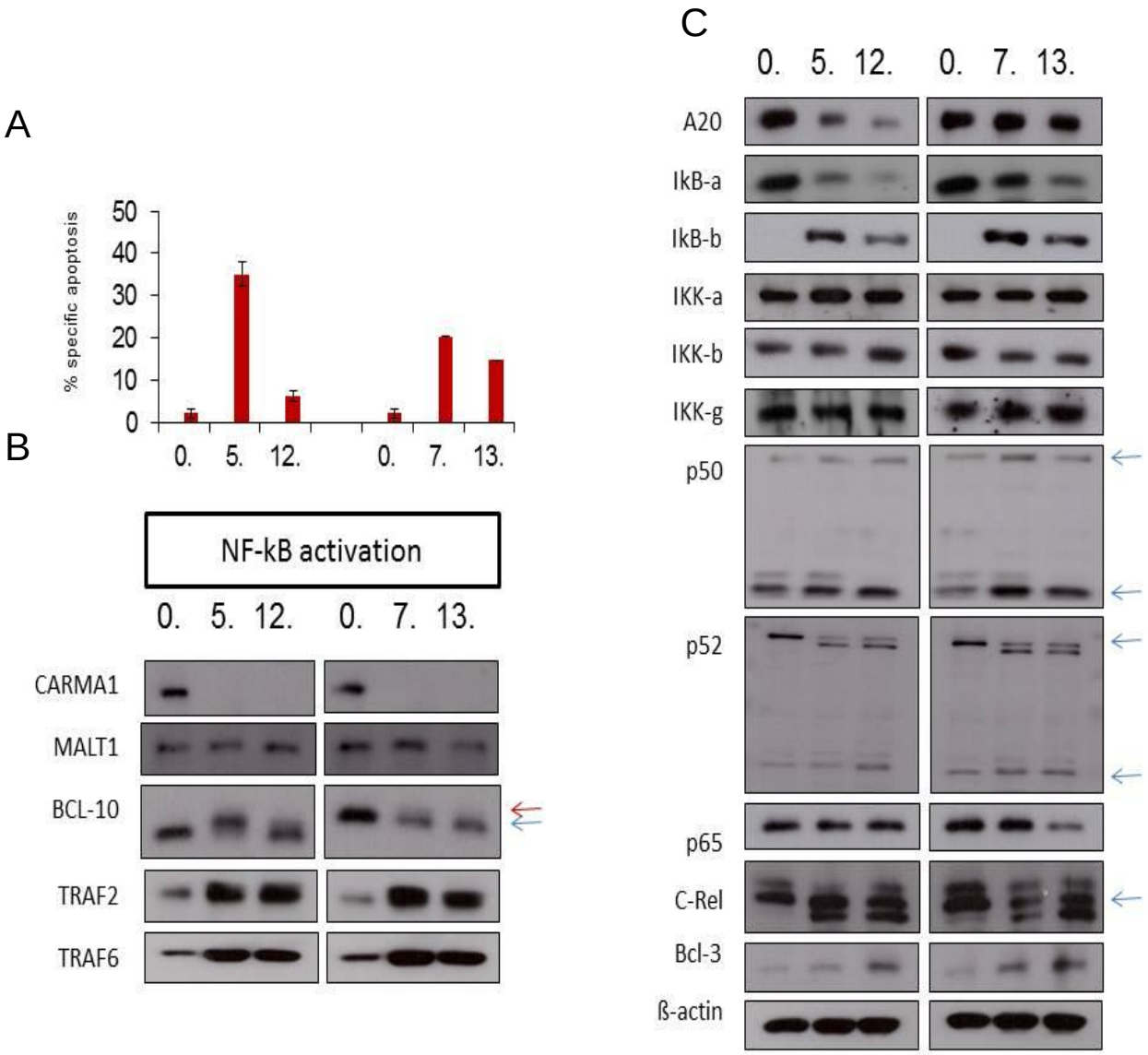



**Figure 12: Expression of NF-κB signaling molecules in short- and long-term activated T cells**

Unstimulated, short- and long-term-activated $CD8^+$ T cells were compared by CD95 sensitivity (A) and levels of proteins involved in NF-κB signaling (B) and (C). Lysates of isolated $CD8^+$ T cells were prepared at day 4 after restimulation. Results are shown for two different blood donors.

### 3.1.4 NF-κB is persistently active in short- and long-term-activated T cells

To determine the relevance of NF-κB activation for the development of CD95 resistance, NF-κB activity was assessed in unstimulated, short- and long-term-activated T cells by EMSA (Figure 13). Supershifts detected p65/p50 and p50/p50 as the only active NF-κB-dimers. p65/p50 and p50/p50 have been shown to be constitutively active in both, short- and long-term-activated T cells to the same extent. Immediately after activation with antibodies against CD3 and CD28 or with Phorbol 12-myristate 13-acetate (PMA) and Ionomycin, higher levels of p65/p50 and lower levels of p50/p50 were detected, but still without a difference between short- and long-term-activated T cells. Hence, NF-κB activation does not correlate with CD95 sensitivity or resistance of activated T cells. The main transcription factors of activated T cells, AP-1 and NFAT, also showed comparable activity, irrespective of the responsiveness to CD95 signaling. Thus, CD95 resistance in long-term- activated T cells probably develops independent of the activity of NF-κB, NFAT and AP-1.

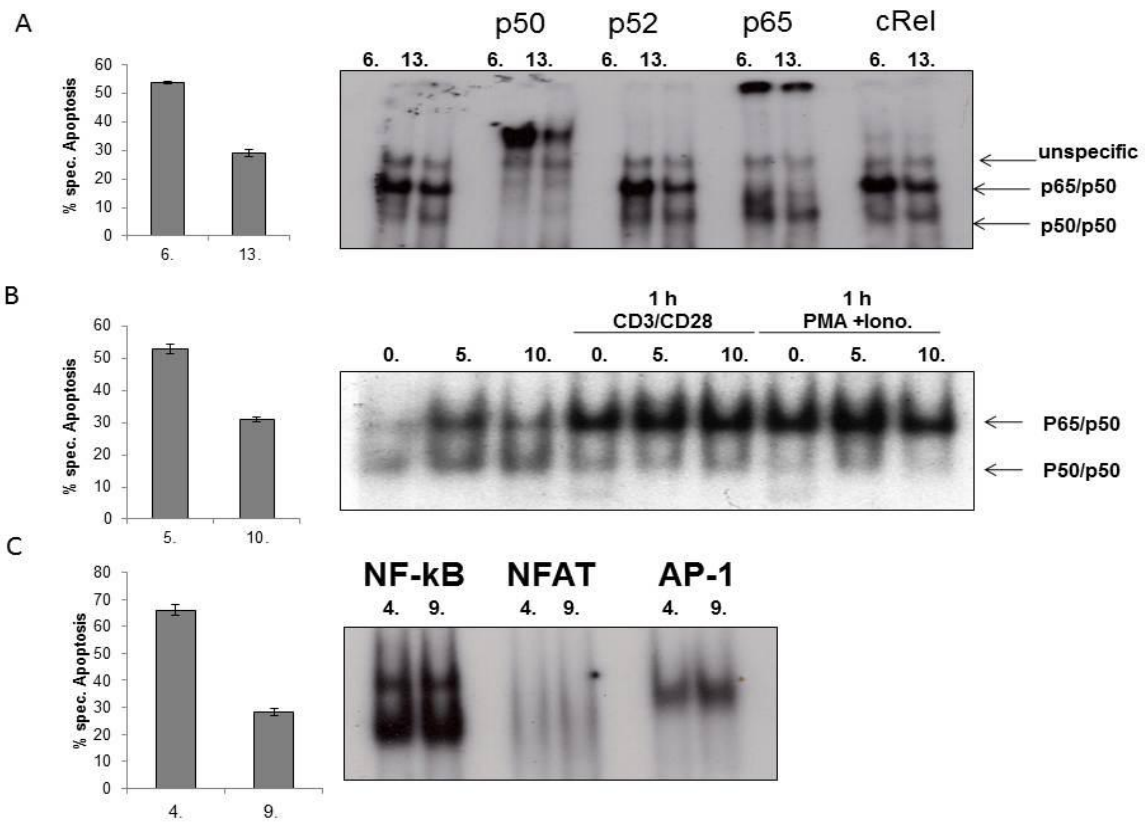



**Figure 13: Electro Mobility Shift Assay of short- and long-term-activated T cells**.

Nuclear extracts were prepared on day 5 after restimulation. CD95 sensitivity at this point in time is shown on the left side. (A) In order to determine which NF-κB subunits are responsible for the shifted bands observed, the EMSA signals were supershifted by adding NF-κB subunit-specific antibodies for p50, p52, p65 and c-Rel to the binding reactions of a 6$^{th}$ and a 13$^{th}$ Stimulation. (B) EMSA of unstimulated and a 5$^{th}$ and a 10$^{th}$ stimulation after 1h incubation with 10 µg CD3/28 antibody or 1h with 250 ng/ml PMA+Ionomycin (C) Analysis of NFAT, AP-1 and NF-kB activation by EMSA of a 4$^{th}$ and a 9$^{th}$ stimulation.

### 3.1.5 Inhibition of NF-κB at different sites did not revert CD95 resistance of long-term-activated T cells

To prove that NF-κB has no influence on CD95 resistance in long-term-activated T cells, CD95 sensitivity was analyzed in the presence of different NF-κB inhibitors. Caffeic acid phenetyl ester (CAPE) is a specific inhibitor of NF-κB and affects PKC activity.[116] Bay inhibits IκBα phosphorylation and therefore its proteasomal degradation.[112] Velcade and MG-132 inhibit NF-κB in a more unspecific manner by preventing proteasome function and thus degradation of IκBs, which in turn retain

NF-κB in the cytosol.[4,96] Z-VRPR-FMK specifically inhibits the proteolytic activity of Malt1, thereby preventing cleavage of the cellular NF-κB inhibitor A20.[123] Inhibition of NF-κB at different sites did not revert CD95 resistance of long-term-activated T cells from two independent blood donors (Figure 14), indicating that the development of resistance probably occurs independent of NF-κB activity.

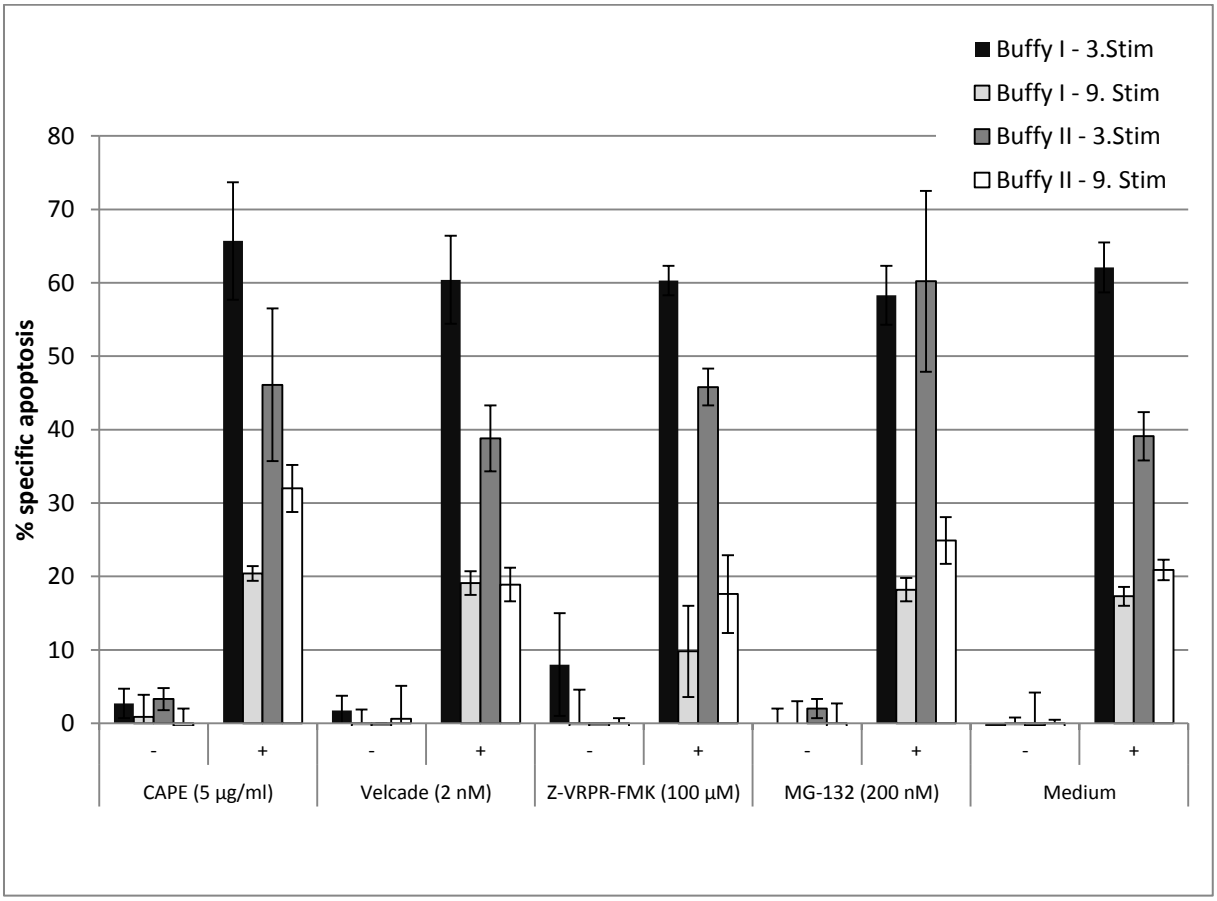



**Figure 14: Inhibition of NF-κB does not alter CD95 sensitivity of short- and long-term-activated T cells.**

CD95 sensitivity is shown as specific apoptosis after 24 h incubation with 100 ng/ ml APO (+) or without (-) and 2h preincubation with the NF-κB inhibitors CAPE, Velcade, Z-VRPR-FMK, MG-132 and NF-κB activator PMA two pairs of a 3$^{th}$ and a 9$^{th}$ stimulation were used. The highest used DMSO concentration to solve the inhibitors was used as negative control. The shown data are representative for two independent experiments, performed with even higher and lower concentrations which are not shown.

### 3.1.6 NF-κB signaling mediators are recruited into lipid rafts after TCR activation

Especially in T cells, lipid rafts act as signaling domains and a different protein composition within the rafts results in a different outcome of signaling. First, CD3/28-antibody-activated T cells were used to demonstrate lipid raft recruitment of NF-κB signaling mediators after T cell activation. As expected, the NF-κB mediators PLC-γ, A20, TRAF2 and TRAF6 moved into lipid rafts after T cell activation. Interestingly, again CARMA1 was only detected in the non-raft fraction of unstimulated, but not of activated T cells, although cells were only stimulated for 3 days in this experiment (Figure 15).

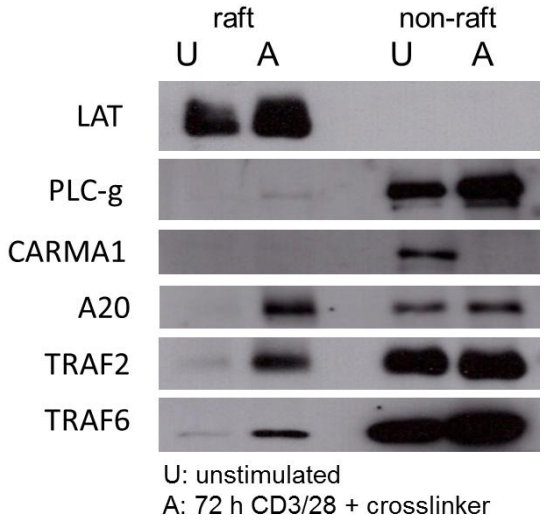



**Figure 15: NF-κB signaling mediators are recruited into lipid rafts upon anti-CD3/28-mediated T cell activation.**

Western Blot analysis of NF-kB mediators in isolated lipid rafts of unstimulated and 72h after stimulation with CD3/CD28 antibodies. $1x10^8$ cells were used. Determined lipid raft fractions were pooled and precipitated.1/5 of lipid raft pool and 1/100 of non-raft fraction were loaded.

### 3.1.7 Mediators of TCR-, NF-κB- and CD95 signaling are constitutively associated with lipid rafts in apoptosis-sensitive and -resistant T cells

The early events of T cell activation, NF-κB- and CD95 signaling take place in lipid rafts.[5,44,103,110] Constitutive alterations within the protein complex structures might represent a possible mechanism of CD95 resistance. Therefore, lipid rafts of

short- and long-term-activated T cells were analyzed for differences in the localization of CD95 and NF-κB signaling molecules (Figure 16).

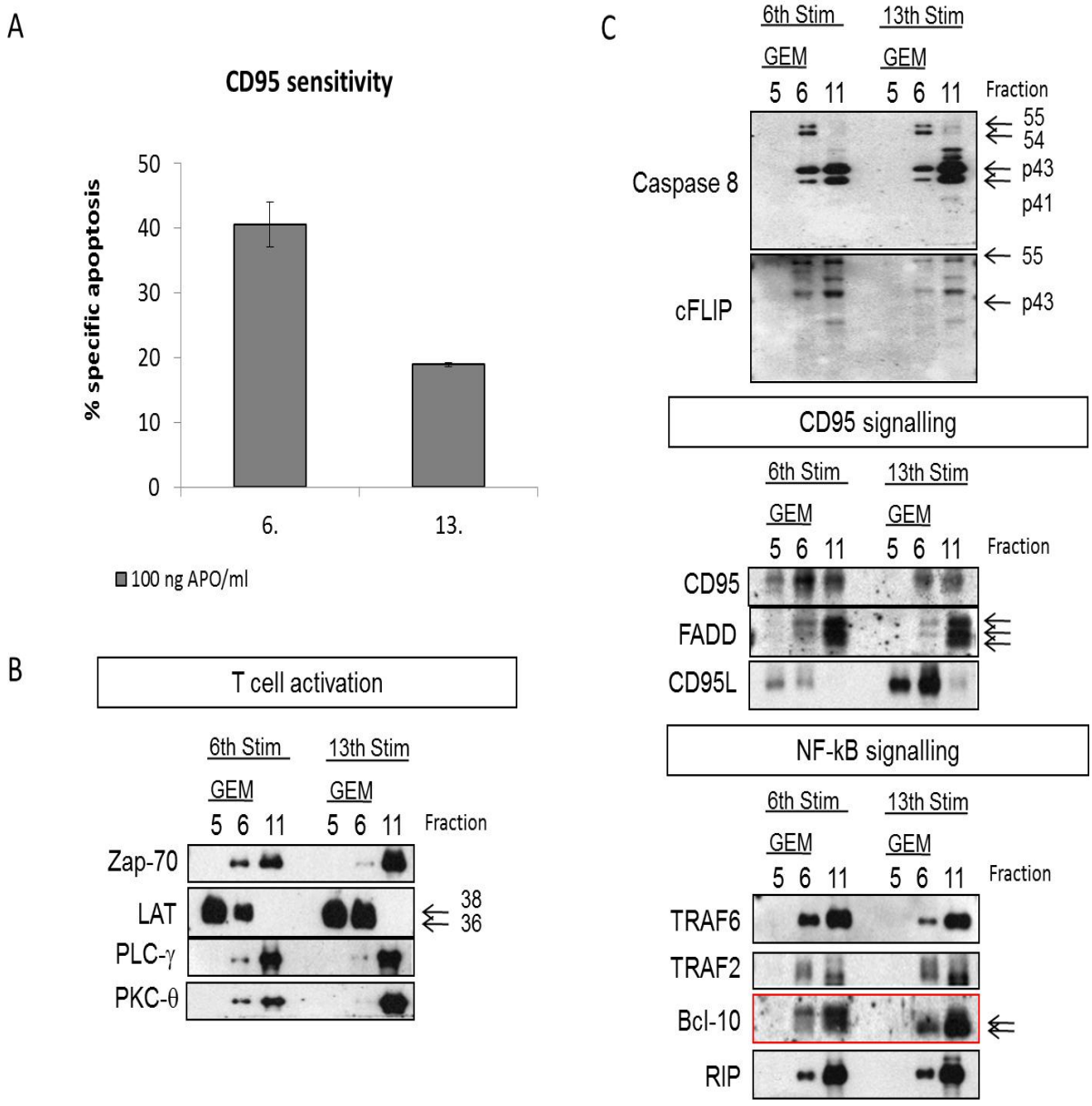



**Figure 16: Constitutive localization of signaling proteins in lipid rafts of short- and long-term-activated T cells**

**(A):** CD95 sensitivity of short- and long-term activated T cells used for lipid raft analysis of proteins important for T cell activation, CD95 signaling and NF-κB activation. CD95 sensitivity was monitored after 24h incubation with 100 ng APO/ml by measuring FSC/SSC of isolated CD8+-T cells. Data represent the mean of triplicates **(B+C):** $1.5*10^8$ T cells were used for the isolation of lipid rafts. Determined lipid raft fractions 5 and 6 were precipitated. 1/5 of the lipid raft fractions were loaded whereas only a 1/150 were loaded of the non-raft fraction 11. The shown results are representative for two independent experiments. (GEM: glycolipid-enriched membranes=lipid rafts)

RIP1 and caspase-8 showed no differences in lipid raft recruitment. However, a small portion of pro-caspase-8 could still be detected which had completely localized to lipid rafts. Thus, caspase-8 is not completely cleaved.

Zap-70 recruitment to lipid rafts is a sign for active TCR signaling. Zap-70 was found in lower amounts within lipid rafts of long-term- compared to short-term-activated T cells. This can be hypothesized as the reason for the reduced recruitment of PLC-γ and PKC-θ into lipid rafts. Zap-70 can usually be found within lipid rafts as long as the ITAMs of the TCR are phosphorylated. Subsequently, ITAM-bound Zap-70 phosporylates LAT and SLP-76, leading to the recruitment of PLC-γ.[160] PLC-γ cleaves $PIP_2$ to $IP_3$ and leaves DAG in the membrane.[78] PKC-θ binds to DAG and gets activated. Thus, the lower amounts of PKC-θ are probably a result of the reduced localization of PLC-γ to lipid rafts. Furthermore, the generation of $IP_3$ can lead to the opening of $Ca^{2+}$ channels and $Ca^{2+}$ signaling is important for the induction of CD95-mediated apoptosis in T cells.[70] Therefore, the reduced localization of PLC-γ to lipid rafts in long-term-activated T cells might also contribute to their CD95 resistance via the reduction of $Ca^{2+}$ signaling, indirectly shown by the lowered recruitment of PKC-θ.

One target of PKC-θ is Bcl-10. Bcl-10 localized in lipid rafts was found in a phosphorylated state in short- term-activated T cells, but not in long-term-activated T cells, where PKC-θ recruitment was also reduced. Bcl-10 has many phosphorylation sites with various functions, e.g. cytoskeletal rearrangement through stimulation of actin polymerization[129] and could be important for the induction of apoptosis.

Lipid raft localization of the NF-κB signaling mediator TRAF6 was reduced in long-term-activated T cells. Other studies have shown a direct implication of TRAF6 in inducing apoptosis by interacting with caspase-8.[62,97]

CD95 and CD95L are only recruited to lipid rafts if palmitoylated. CD95 was found in reduced levels in lipid rafts of long-term-activated T cells but comparable whole lysate expression (Figure 11). PI3K activity negatively regulates CD95 palmitoylation,[14] and increased PI3K activity was shown for long-term-activated T

cells.[148] Thus, reduced amounts of CD95 in lipid rafts in long-term-activated T cells might be a consequence of the inhibition of its palmitoylation by PI3K. CD95-recruitment to lipid rafts is important for inducing apoptosis, at least in type I cells.[14,45,90]

CD95L, which is also known to be expressed in higher amounts in long-term-activated T cells and which is relevant for the induction of apoptosis in target cells[12], was completely recruited into lipid rafts, which is a sign for complete palmitoylation of CD95L. A direct interaction of CD95 and CD95L on the same cell has to be shown in future experiments.

These findings suggest that long-term-activated T cells are resistant towards CD95L-induced apoptosis due to reduced recruitment of CD95 to lipid rafts and weaker TCR signaling, leading in turn to reduced $Ca^{2+}$ signaling, which is important for CD95-mediated apoptosis.

Further experiments need to be conducted to clarify the relevance of these findings in the given context.

### 3.1.8 Screening by 2D-DIGE revealed no proteins known to be directly involved in regulation of apoptosis

For further analysis of CD95 resistance a genechip analysis of short and long-term activated T cells was planned for screening on RNA-level and 2D-DIGE on protein level. 2D-DIGE analysis was performed with 4 pairs of short- and long-term-activated T cells. (Figure 17)

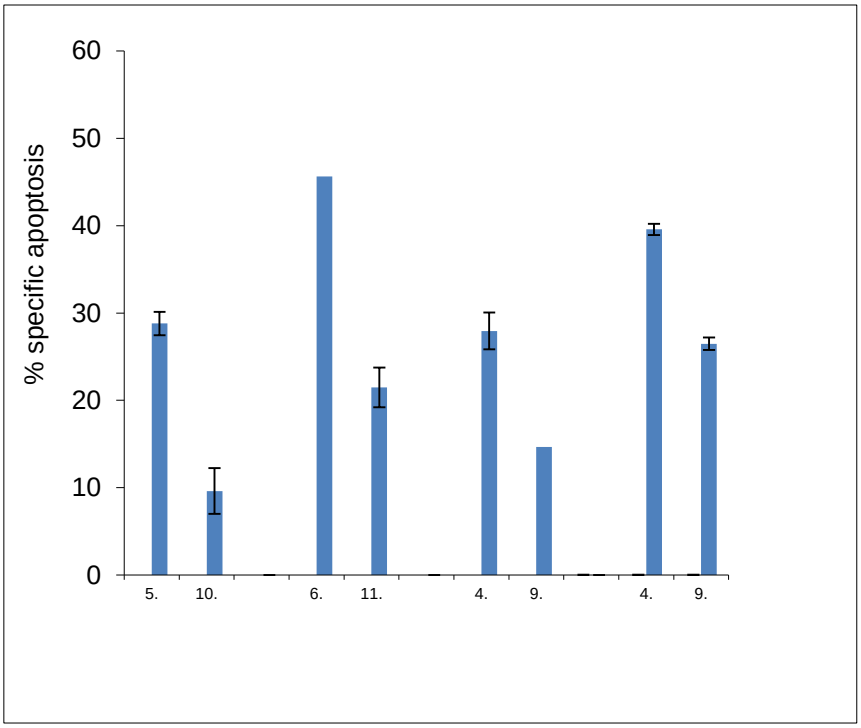



**Figure 17: CD95 sensitivity of short and long-term activated T cells used for 2D-DIGE**

CD95 sensitivity of 4 pairs of short and long-term activated T cells was monitored after 24h incubation with 100 ng APO/ml by measuring FSC/SSC of isolated CD8+-T cells. Data represent the mean of triplicates.

But the quality of the gels was not sufficient enough for an overlay for analysis. Therefore, these gels were analysed separately from each other and were not overlayed for analysis. The number of picked and identified protein spots are shown in Table 12.

**Table 12:** Numbers of picked and identified protein spots of each 2D-DIGE experiment with CTLs

| Gel | Cy5-labeling | Cy3-labeling | pH range | number of overlayed gels | picked **protein** spots | identified protein spots |
|---|---|---|---|---|---|---|
| 1 | 5.Stim | 10.Stim | 4-7 | 1 | 55 | 3 |
| 2 | 11.Stim | 6.Stim | 4-7 | 1 | 74 | 24 |
| 3 | 3.Stim | 9.Stim | 4-7 | 1 | 56 | 24 |
| 4 | 9.Stim | 3.Stim | 4-7 | 1 | 45 | 1 |

The number of identified protein was not sufficient. Only a low amount of protein spots were identified by the 2-DIGE analysis of short-and long-term activated T cells. Still a number of 52 proteins were identified but none of these are known to be

directly involved in regulation of apoptosis or cell survival. The complete list of identified proteins can be found in the appendix. 2D-DIGE of short-and long-term activated T cells has to be repeated in the future, as well as a gene chip analysis on RNA-level.

## 3.2 Part II: CD95 resistance in Jurkat cell lines

The generation of long-term-activated T cells resistant towards CD95 stimulation is a big hurdle and requires 10-14 weeks of primary cell culture. Jurkat cells have been used for a long time as a model system to study the behaviour of T cells. They are usually CD95-sensitive, however, two CD95-resistant Jurkat lines have been established by continuous CD95 stimulation.[69] This allowed us to study CD95 resistance mechanisms without generating long-term-activated T cells. Four Jurkat cell lines were chosen: Je6.1 and JM which are CD95-sensitive, and the two CD95-resistant cell lines JM301 and JM319, which are both derived from JM cells. These cell lines were used to screen on RNA level by gene chip analysis and on protein level by 2D-DIGE for differences between CD95-sensitive and -resistant representatives.

### 3.2.1 The Jurkat cell lines JM301 and JM319 are CD95-resistant despite receptor surface expression

First, it was analyzed by flow cytometry whether CD95 resistance in JM301 and JM319 was due to loss of CD95 surface expression. All four cell lines showed comparable levels of CD95 (Figure 18). Thus, it was excluded that absent CD95 surface expression rendered JM301 and JM319 cells resistant to CD95-mediated apoptosis.

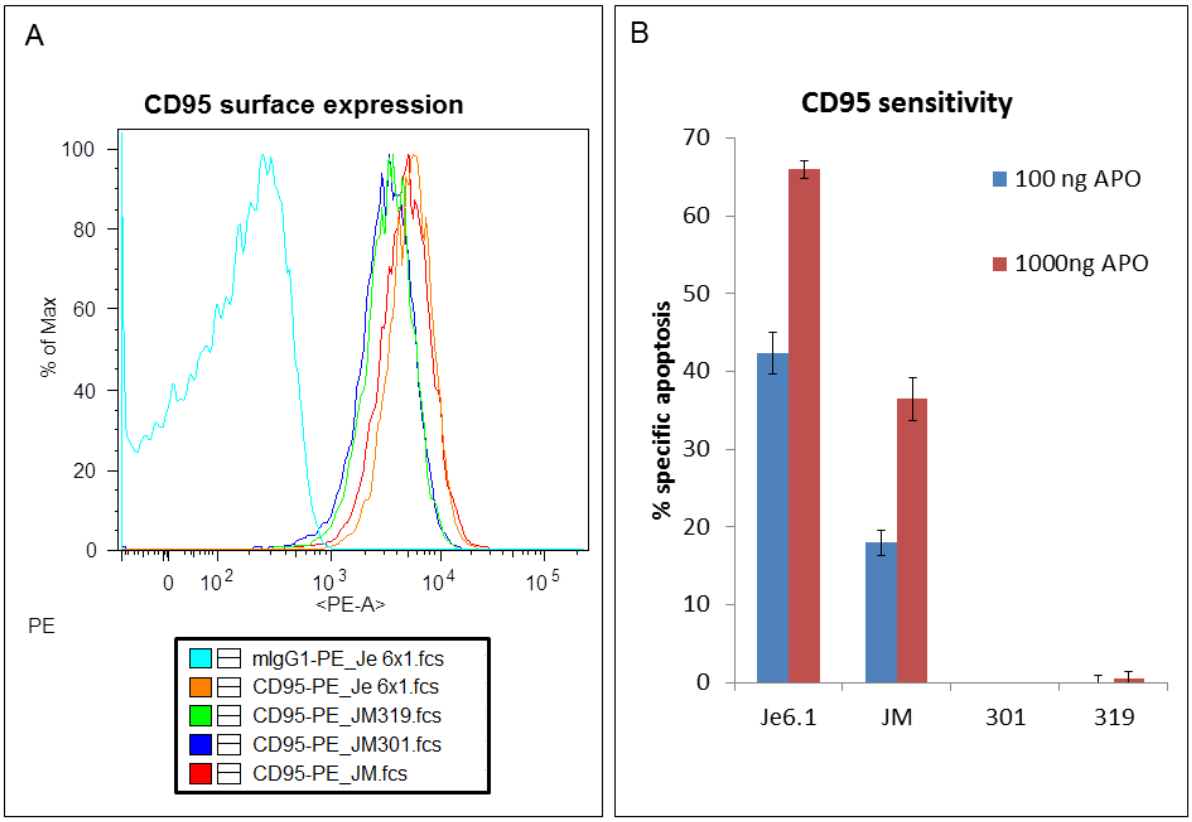



**Figure 18: CD95-sensitive and –resistant Jurkat cells show comparable CD95 surface expression.**

(A) CD95 surface expression of CD95-sensitive (Je6.1, JM) and -resistant (JM301 and JM319) Jurkat cell lines was compared by flow cytometry. (B) CD95 sensitivity of Jurkat cell lines was determined by incubating the cells for 24h with 100 or 1000 ng/ml APO-1. Cell death was measured by FSC/SSC. Specific apoptosis was calculated relative to the medium control.

### 3.2.2 The gene expression profile of Je6.1, JM, and JM301 and JM319 correlates with CD95 sensitivity

The CD95-resistant cell lines JM319 and JM301 were generated out of JM cells by continuous CD95 stimulation. Surprisingly, gene expression profiling revealed that less genes were differentially regulated between the two CD95-sensitive cell lines JM and Je6.1 than between JM and its daughter cell lines JM301 and JM319. This means that the gene expression profile of all four cell lines correlated with their CD95 sensitivity (Figure 19).

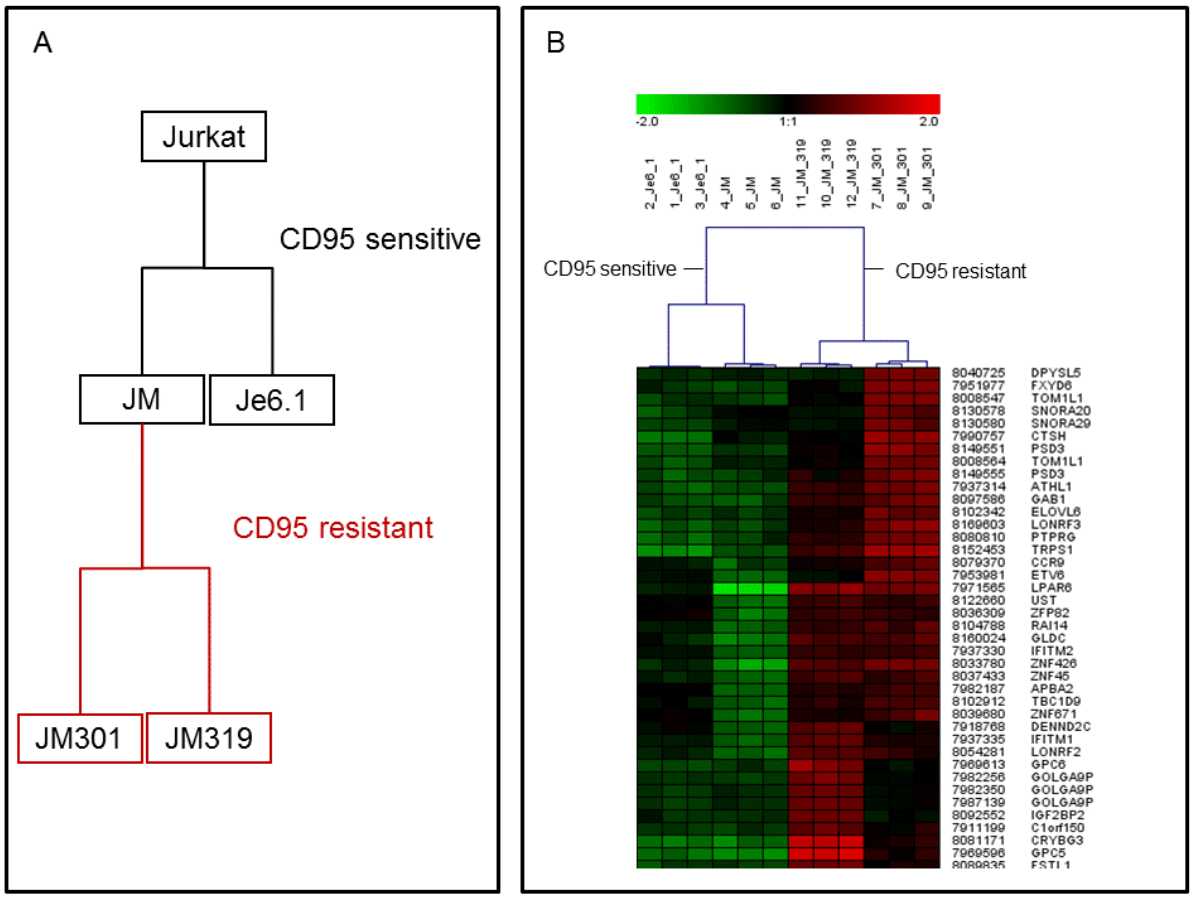



**Figure 19: The gene expression profile correlates with CD95 sensitivity**

(A) family tree of jurkat cell lines. (B) Triplicates were used for Gene expression profiling of the four Jurkat cell lines.The dendrogram was calculated taking all differences into account.

### 3.2.3 Gene chip analysis revealed 276 differentially regulated genes between CD95-sensitive and -resistant Jurkat cell lines

Gene chip analysis showed hundreds of differences between CD95-sensitive and –resistant Jurkat cell lines (Table 13). The gene expression profile of all CD95-sensitive Jurkat cells was compared to that of their CD95-resistant counterparts. Only changes which were repeatedly found in all comparisons were taken into account, resulting in a total number of 276 alterations in gene expression with 1.5 fold difference and 154 with a 2 fold change.

**Table 13:** Number of gene expression changes, which were higher than 1.5 or 2 fold in all 4 cell lines compared to each other. For further analysis, those changes were determined which were always the same between CD95-sensitive and -resistant cell lines in all comparisons.

| | cell line | number of changes 1.5 fold | number of changes 2 fold |
|---|---|---|---|
| resistant vs resistant | JM301 vs JM 319 | 272 | 42 |
| sensitive vs sensitive | JM vs Je6.1 | 1858 | 527 |
| | Je6.1 vs JM301 | 2185 | 644 |
| | Je6.1 vs JM319 | 2257 | 648 |
| | JM vs JM301 | 856 | 235 |
| | JM vs JM319 | 1168 | 285 |
| sensitive vs resistant | JM/Je6.1 vs JM301/JM319 | **276** | **154** |

Genes with a 3 fold change in expression between CD95-resistant and -sensitive Jurkat cell lines can be found in the appendix. However, Gominer pathway analysis (data not shown) could not link these changes to apoptosis or cell survival. None of the differently regulated gene products are directly involved in CD95 signaling, except the Rho/ROCK signaling pathway, which is required for CD95-induced apoptosis.[64,121]

### 3.2.4 Gene chip analysis revealed differences in the Rho/ROCK-signaling pathway, which is required for CD95 signaling

Gene chip analysis of the Rho/ROCK signaling pathway revealed that Rho-GEFs, which are required for activation of the pathway were downregulated, whereas Rho-GTPases leading to a shut-down were upregulated in the CD95-resistant cell lines JM301 and JM319. ROCK itself is only in JM301 cells downregulated, but not in JM319 cells (Table 14). These results suggest that the Rho/ROCK pathway might be inhibited in CD95-resistant Jurkat cells. Next, we sought to confirm the relevance of the Rho/ROCK signaling pathway for CD95-induced apoptosis using a specific ROCK inhibitor.

**Table 14:** Differential expression of genes involved in Rho signaling between CD95-sensitive and -resistant Jurkat cell lines (sen = CD95-sensitive, res = CD95-resistant)

| sen/res | res/sen | Gene | Cell lines |
|---|---|---|---|
| 0,5 | 1,9 | Rho GTPase activating protein 10 | JMvsJM319 |
| 0,6 | 1,7 | Rho GTPase activating protein 10 | JMvsJM301 |
| 0,6 | 1,6 | Rho GTPase activating protein 10 | Je6.1vsJM301 |
| 0,6 | 1,8 | Rho GTPase activating protein 10 | Je6.1vsJM319 |
| 0,6 | 1,7 | Rho GTPase activating protein 12 | JMvsJM319 |
| 0,6 | 1,6 | Rho GTPase activating protein 12 | Je6.1vsJM301 |
| 0,5 | 1,9 | Rho GTPase activating protein 12 | Je6.1vsJM319 |
| 0,7 | 1,5 | Rho GTPase activating protein 18 | Je6.1vsJM319 |
| 0,7 | 1,5 | Rho GTPase activating protein 5 | Je6.1vsJM319 |
| 0,6 | 1,6 | SLIT-ROBO Rho GTPase activating protein 1 | JMvsJM319 |
| 0,6 | 1,8 | SLIT-ROBO Rho GTPase activating protein 1 | JMvsJM301 |
| 0,5 | 2,0 | SLIT-ROBO Rho GTPase activating protein 1 | Je6.1vsJM301 |
| 0,6 | 1,7 | SLIT-ROBO Rho GTPase activating protein 1 | Je6.1vsJM319 |
| 1,6 | 0,6 | Rho guanine nucleotide exchange factor (GEF) 11 | Je6.1vsJM319 |
| 3,4 | 0,3 | Rho guanine nucleotide exchange factor (GEF) 12 | JMvsJM319 |
| 5,1 | 0,2 | Rho guanine nucleotide exchange factor (GEF) 12 | JMvsJM301 |
| 6,7 | 0,1 | Rho guanine nucleotide exchange factor (GEF) 12 | Je6.1vsJM301 |
| 4,5 | 0,2 | Rho guanine nucleotide exchange factor (GEF) 12 | Je6.1vsJM301 |
| 1,6 | 0,6 | RhoA/RAC/CDC42 exchange factor | Je6.1vsJM301 |
| 1,7 | 0,6 | RhoA/RAC/CDC42 exchange factor | Je6.1vsJM319 |
| 1,9 | 0,5 | Rho-associated, coiled-coil containing protein kinase 1 | JMvsJM301 |
| 1,8 | 0,5 | Rho-associated, coiled-coil containing protein kinase 1 | Je6.1vsJM301 |

### 3.2.5 Inhibition of ROCK1 renders CD95-sensitive Jurkat cells apoptosis-resistant

To clarify whether the ROCK pathway was indeed required for CD95 signaling in our system, we blocked this pathway using a well-described specific inhibitor of ROCK1, Y-72632. Inhibition of the Rho/ROCK pathway by Y-72632 should lead to CD95 resistance in CD95-sensitive Jurkats.[64]

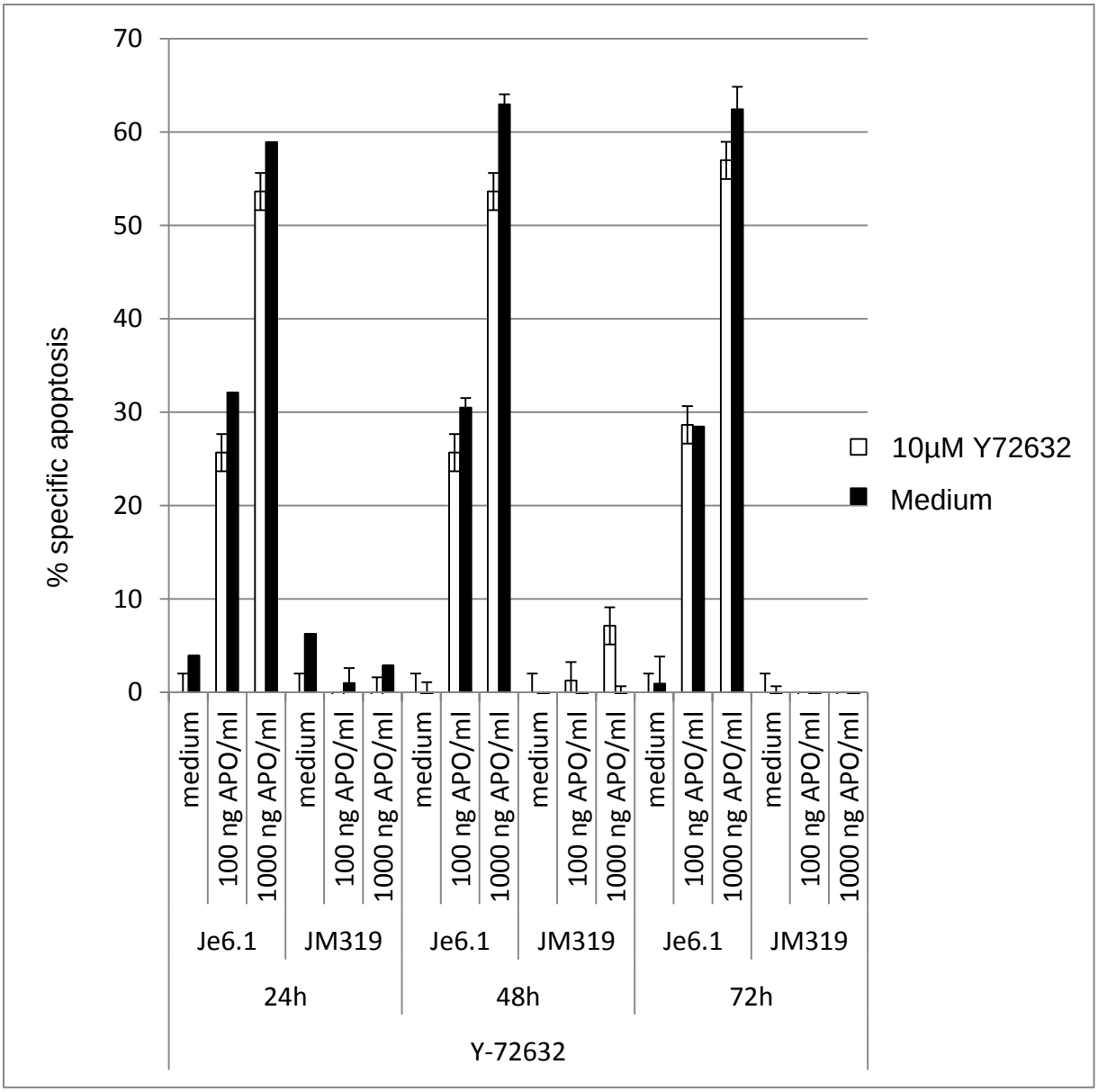



**Figure 20: Inhibition of ROCK1 by Y-72632 leads to CD95 resistance in Je6.1 cells.**

CD95 sensitivity of apoptosis-sensitive Je6.1 and -resistant JM319 cells was determined using 100 ng/ml APO-1 or 1000 ng/ml APO-1 in the presence or absence of 10 µM Y-72632. CD95 sensitivity was monitored after 24, 48 and 72h by measuring FSC/SSC. Data represent the mean of triplicates of two independent experiments.

As demonstrated in Figure 20, inhibition of the Rho/ROCK pathway indeed led to the development of CD95 resistance in originally apotosis-sensitive Je6.1 cells, indicating that ROCK activity is required for CD95-mediated apoptosis.

Inhibition of this pathway by upregulation of Rho-GAPs and downregulation of Rho-GEFs (Table 14) might therefore explain the development of apoptosis resistance of JM301 and JM316 cells at least in part. But the result is not very impressive.

### 3.2.6 A total of 95 differentially regulated proteins were identified by 2D-DIGE of Jurkats

2D-DIGE analysis was performed to screen the cell lines on protein level for differences responsible for CD95 resistance. Each possible combination of CD95-sensitive and -resistant Jurkat cell lines was analyzed by 2D-DIGE in two rounds. Decyder-softer was used to overlay the gels. Only proteins were picked with the same difference in expression on each single gel. In the second run with a different pH range one gel had to be discarded, so that only 3 gels were analysed instead of 4. Table 15shows the number of picked and identified protein spots of both runs.

**Table 15:** Numbers of picked and identified protein spots of each 2D-DIGE experiment with Jurkats

| Run | Gel | Cy5 labeling | Cy3 labeling | pH range | number of overlayed gels | picked protein spots | identified protein spots |
|---|---|---|---|---|---|---|---|
| | 1 | Je6.1 | JM301 | | | | |
| | 2 | JM319 | Je6.1 | | | | |
| | 3 | JM | JM319 | | | | |
| 1 | 4 | JM301 | JM | 3-10 | 4 | 65 | 44 |
| | 1 | Je6.1 | JM319 | | | | |
| | 2 | JM | JM319 | | | | |
| 2 | 3 | JM301 | JM | 4-7 | 3 | 83 | 51 |

A total of 147 differentially regulated proteins were picked of which 95 protein spots were identified by mass spectrometry. The complete list of picked proteins can be found in the appendices. None of these proteins are known to be directly involved in regulation of apoptosis or cell survival, except ezrin, which was shown to be 1.3 fold down-regulated in CD95 resistant Jurkat cells.

### 3.2.7 The intersection of 2D-DIGE analysis of Jurkats and CTLs lowered the amount of candidate proteins for further analysis

To minimize the amount of data given by 2D-DIGE of CTLs and Jurkats for further analysis the intersection of 2D-DIGE analysis of Jurkats and CTLs was created taken also the gene expression profile of the jurkats into account. Table 16 shows the intersection of proteins of all experiments.

**Table 16:** List of proteins, which could be picked in 2D-DIGE of CTLs and Jurkats and which have the same difference on RNA level of Jurkats found by gene chip analysis. Shown are fold changes of CD95 resistant cells compared to CD95 sensitive cells.

| Protein | CD95-resistant | |
|---|---|---|
| | 2D-DIGE JURKATS | 2D-DIGE CTLs |
| Annexin A1 | -1,69 | -1,73 |
| Proteasome activator complex subunit 2 | -1,46 | -1,65 |
| Coronin | -1,47 | -1,22 |
| proteasome subunit alpha type-6 | -1,51 | -1,37 |
| Heat shock protein HSP 90-alpha | 1,24 | 1,25 |
| Protein disulfide-isomerase | 1,33 | 1,6 |
| Eukaryotic initiation factor 4A-II | 1,58 | 1,67 |
| 78kDa glucose-regulated protein(GRP-78) | 1,7 | 1,79 |

If gene chip analysis and 2D-DIGE results of Jurkats and CTLs are combined, the following candidate proteins are ideal for further analysis: Annexin A1, Coronin, proteasome subunit alpha type-6, proteasome activator complex subunit 2, Hsp90α, protein disulfide isomerase (PDI), eucaryotic initiation factor 4A-II and 78kDa glucose-regulated protein (Grp78).

To clarify the relevance as the next step we inhibited the function of these candidate proteins. For Hsp90, a specific inhibitor was known. In the case of Grp78, substances which are known to result in an up- or downregulation of Grp78 were used. The relevance of the other proteins must be shown in future experiments.

### 3.2.8 Hsp90 is not involved in CD95 resistance

Hsp90 is a chaperone which is mostly located in the cytosol and functions as part of a multi-chaperone complex that folds, activates and assembles proteins, such as p53, Bcr-Abl, Her2, Akt, Raf-1 and B-Raf. Activation of T cells is accompanied by increased expression of Hsp90.[150] To analyze whether Hsp90 has a relevance for CD95 sensitivity, the CD95-resistant Jurkat cell line JM319 was treated with APO-1 in the presence of the Hsp90 inhibitor 17-allylamino-17-demethoxygeldamycin (17-AAG) (Figure 21).

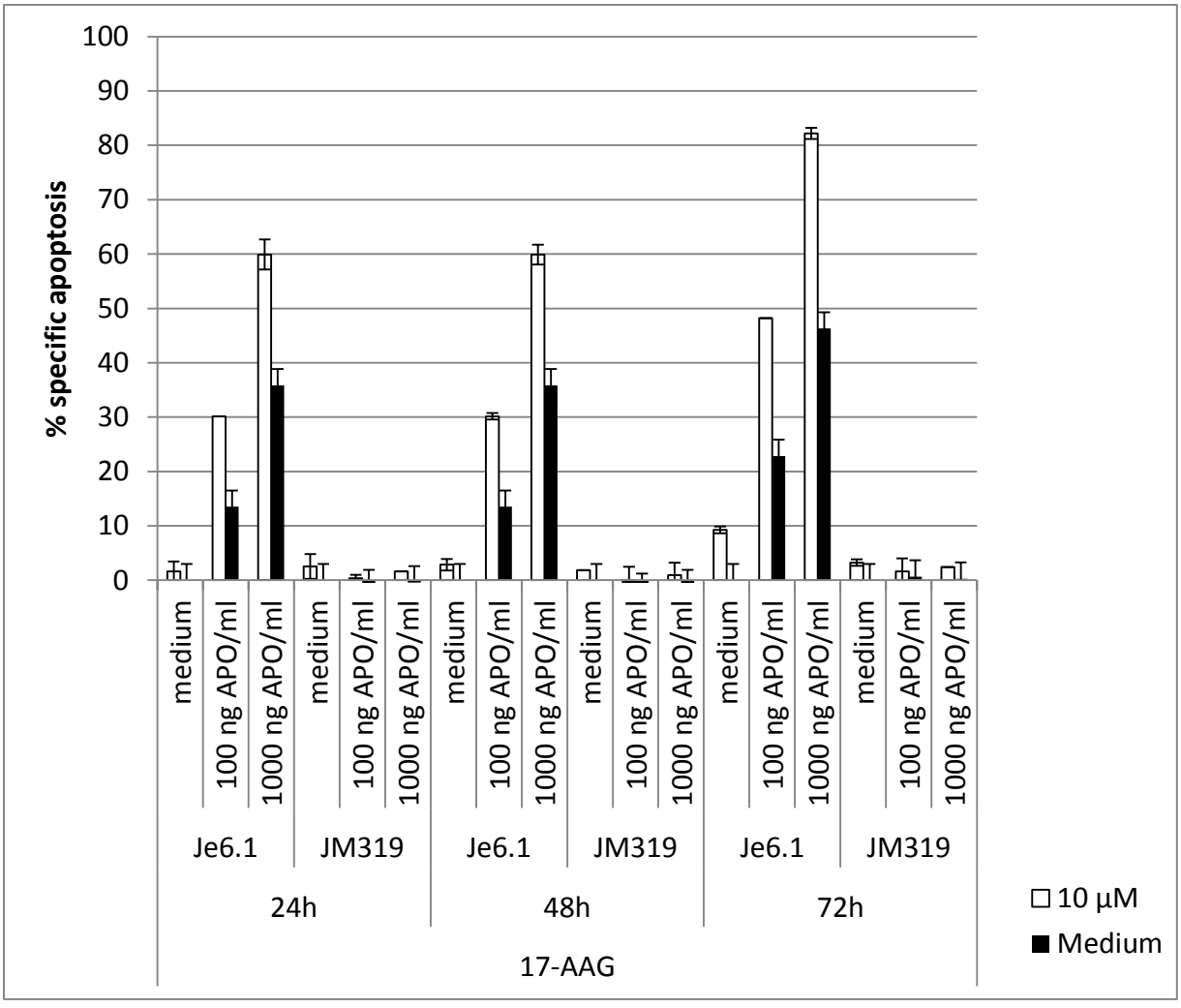



**Figure 21: Hsp90 is not involved in CD95 resitance**

CD95 sensitivity of the CD95-sensitive Jurkat cell line Je6.1 and the CD95-resistant Jurkat cell line JM319 with medium, 100 ng/ml APO and 1000 ng/ml APO with or without 10 µM 17-AAG, which was the highest non toxic determined concentration. CD95 sensitivity was monitored after 24h, 48h and 72h by measuring FSC/SSC. Data represent the mean of triplicates of two independent experiments.

17-AAG is an ansamycin antibiotic and specifically binds and inhibits Hsp90 by disrupting the Hsp90-client protein complexes, resulting in the degradation of the client proteins.[134] 17-AAG doubled specific apoptosis of the already sensitive cell line Je6.1 but did not sensitize CD95-resistant JM319 cells. Hsp90 was shown to be upregulated in CD95 resistant T cells. Therefore, the inhibition should also lead to CD95 sensitivity in the CD95 resistant Jurkat lines. So Hsp90 is not involved in CD95 resistance.

### 3.2.9 Downregulation of Grp78 by deoxynivalenol sensitizes to CD95-induced apoptosis

PDI and Grp78 are chaperones, which are mostly upregulated upon stress conditions, and which were overexpressed in CD95-resistant cells. PDI is a chaperone in the ER that catalyzes the formation and breakage of disulfide bonds to catalyze protein folding. Grp78, also known as binding immunoglobulin protein (BiP) or heat shock 70 kDa protein 5 (HSPA5), is also located in the lumen of the ER and binds unfolded proteins for subsequent folding by PDI and regulates protein transport across the membrane. Grp78 has also a strong $Ca2^{+}$-binding capacity. Upregulation of Grp-78 in many cancers has been shown to have anti-apoptotic properties.[87] 2D-DIGE analysis revealed an upregulation of Grp78 in CD95 resistant Jurkats and long-term activated T cells. Deoxynivalenol (DON, vomitoxin) is a naturally occurring mycotoxin produced by several species of Fusarium fungi and has been shown to downregulate Grp78.[175] Since deoxynivalenol acts in an unspecific manner, it also affects expression of a variety of other proteins. Grp78 was found at significantly higher expression levels in CD95-resistant Jurkat cell lines on RNA- and protein level. Downregulation of Grp78 by deoxynivalenol lead to sensitization of both, the CD95-sensitive Jurkat cell line Je6.1 and the CD95-resistant Jurkat cell line JM319. (Figure 22) Thus, Grp78 might be relevant for the development of CD95 resistance. Western blot analysis of Jurkat cells treated with deoxynivalenol must be performed to prove downregulation of Grp78.

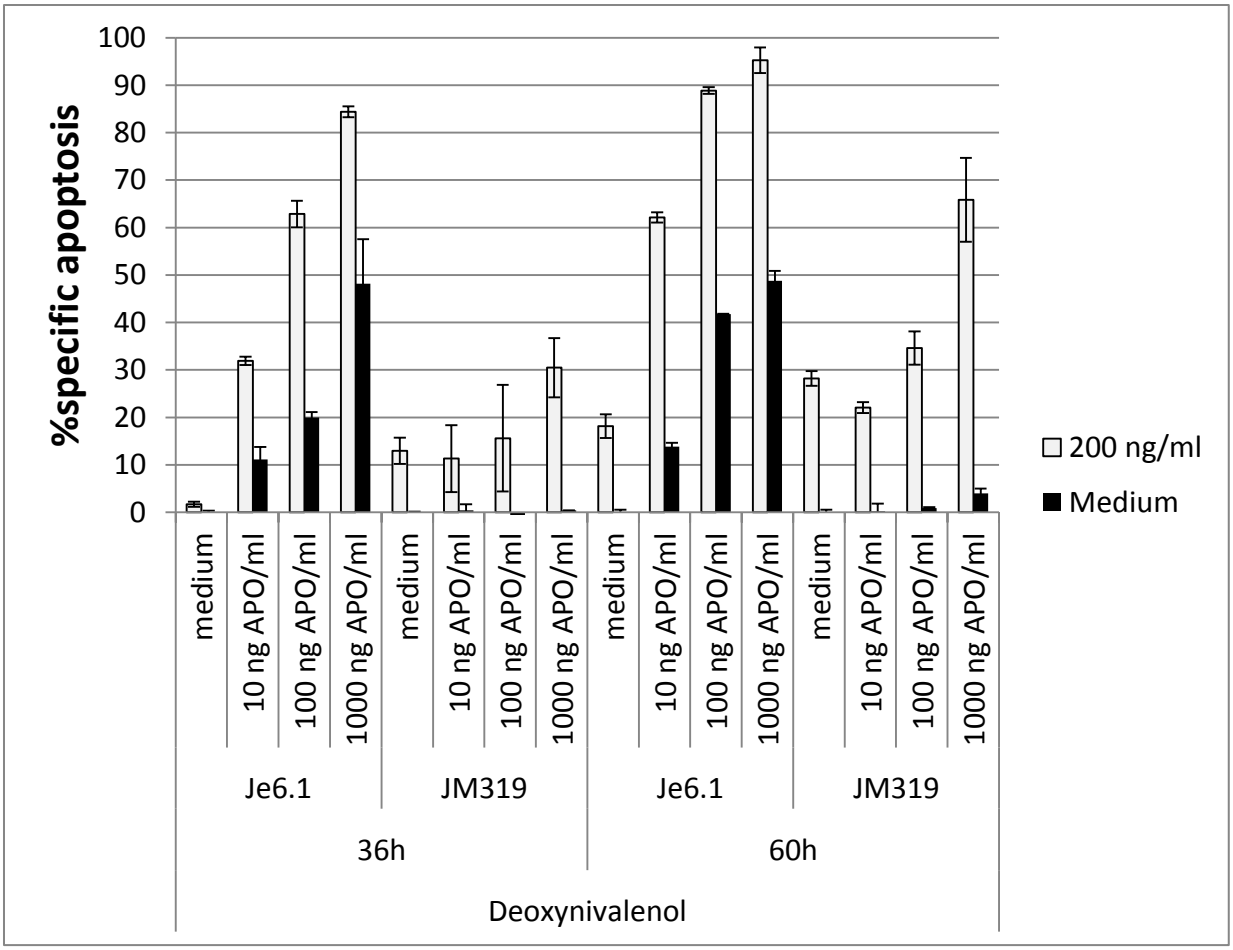



**Figure 22: Downregulation of Grp78 sensitizes to CD95-induced apoptosis**

CD95 sensitivity of the CD95 sensitive Jurkat cell line Je6.1 and the CD95 resitant jurkat cell line JM319 with medium, 100 ng/ml APO and 1000 ng/ml APO under the influence with or without 200 ng/ml Deoxynivalenol, which was the highest non toxic determined concentration. CD95 sensitivity was monitored after 36h and 60h by measuring FSC/SSC. Data represent the mean of triplicates of two independent experiments.

### 3.2.10 Upregulation of Grp78 by valproic acid increased apoptosis in CD95-sensitive Jurkat cells

Valproic acid enhances chaperone expression by a mechanism that involves histone hyperacetylation. Grp78 expression is enhanced by valproic acid.[138] Downregulation of Grp78 by deoxynivalenol had sensitized CD95-resistant Jurkat cell lines for apoptosis. Upregulation of Grp78 by valproic acid should, therefore, lead to CD95 resistance in CD95-sensitive Jurkat cell lines. Surprisingly, valproic acid

increased apoptosis sensitivity of the CD95-sensitive Jurkat cell line Je6.1 instead. Valproic acid had no effect on CD95 resistance in JM319 cells (Figure 23).

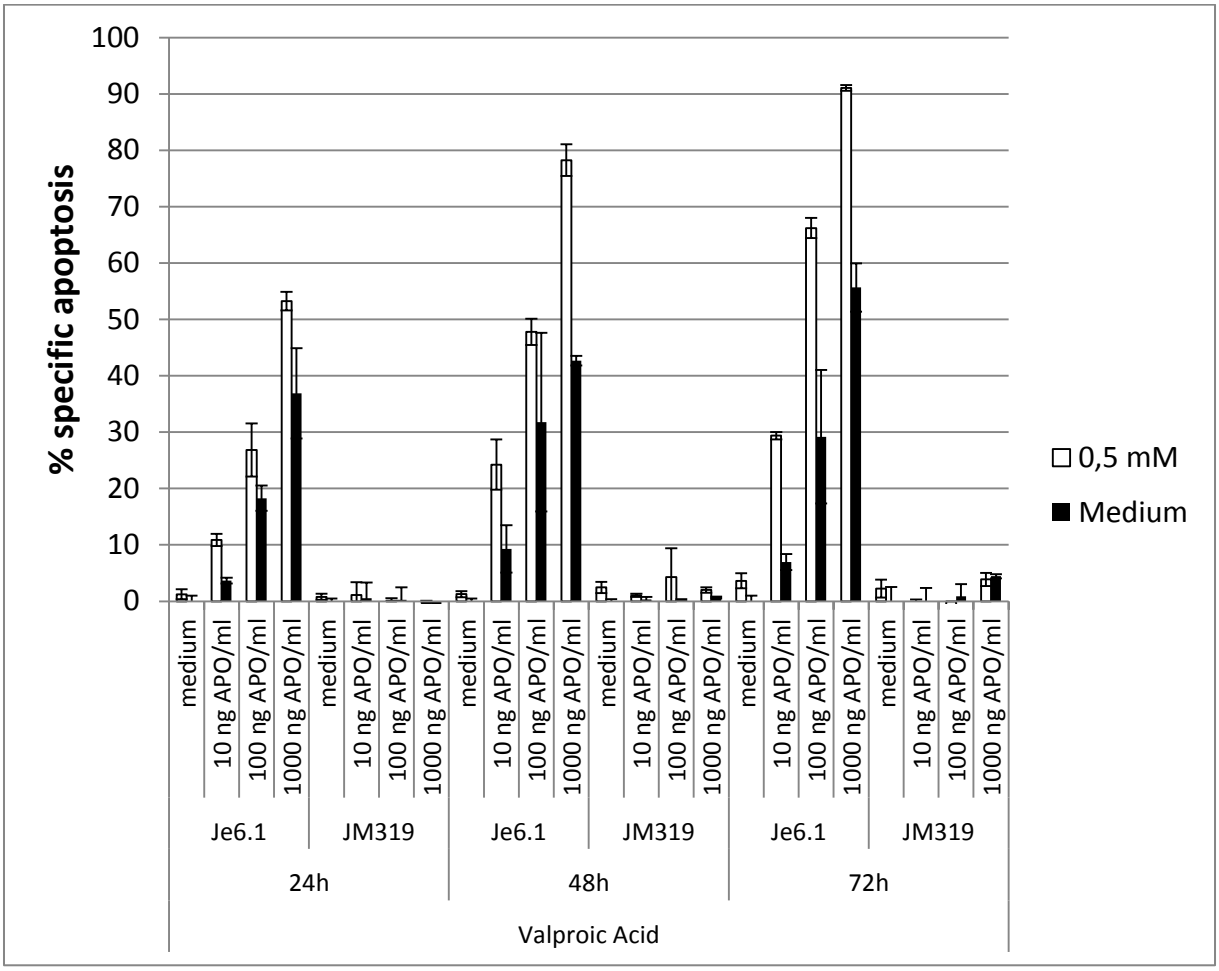



**Figure 23: Upregulation of Grp78 by Valproic acid sensitizes unexpected Je6.1 cells**

CD95 sensitivity of the CD95 sensitive Jurkat cell line Je6.1 and the CD95 resitant jurkat cell line JM319 with medium, 100 ng/ml APO and 1000 ng/ml APO under the influence with or without 0,5 mM Valproic acid, which was the highest non toxic determined concentration. CD95 sensitivity was monitored after 24h, 48h and 72h by measuring FSC/SSC.

Incubation with valproic acid affects the expression of many proteins. Therefore, si-RNA-mediated knock-down of Grp78 in CD95-resistant Jurkat cells would be more useful to clarify if Grp78 overexpression in CD95-resistant cells is relevant.

### 3.2.11 Detection of a hypothetical protein with a known CD95 binding site by 2D-DIGE

Mass spectrometric analysis showed a hypothetical protein, which displayed complete sequence homology to Ezrin, but had a longer N-terminus. The peptide analyzed was derived from a region which is homologous in both proteins. Therefore, the hypothetical protein could only be determined indirectly by its isoelectric point and size and not by the peptide itself. Figure 24 shows a short-cut of picked and identified proteins by mass spectrometry with their theoretical isoelectric point (pI) and molecular weight (Mw). Comparing surrounding sequenced protein spots and their theoritical pI and molecular weight, it was shown that one of the protein spots has to be the hypothetical protein DKFZp762H157.1, whereas the other one is Ezrin. Moesin must be constitutively phosphorylated, shown by a shift to lower pI.

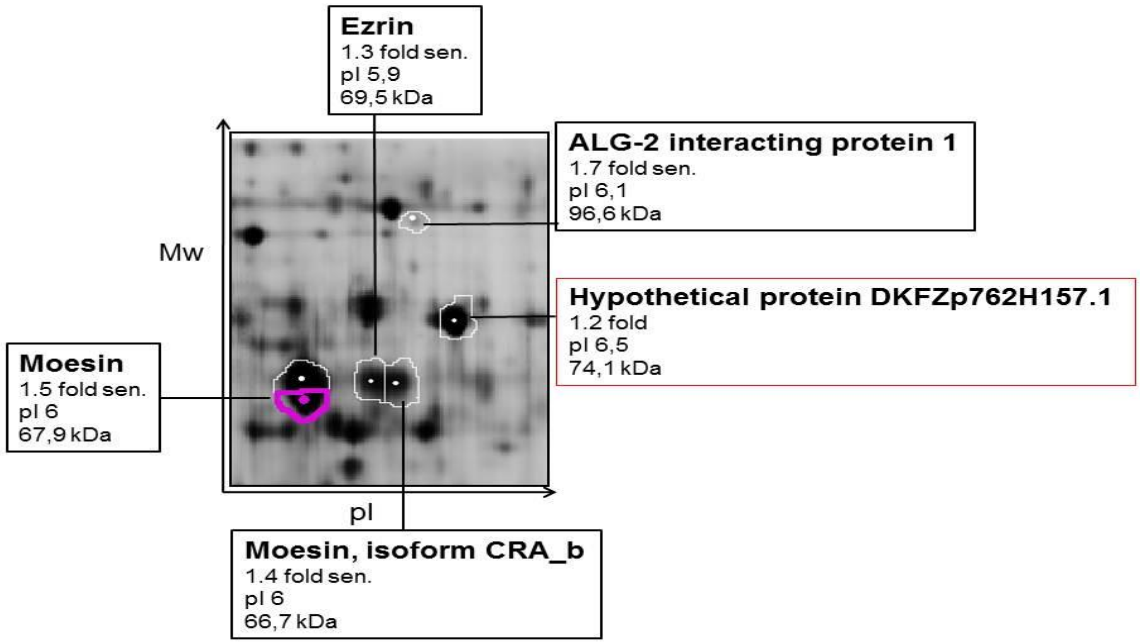



**Figure 24: Detection of the hypothetical protein DKFZp762H157.1 by 2D-DIGE**

Short-cut of picked and identified proteins of 2D-DIGE of Jurkats

Ezrin binds to CD95, thereby linking the receptor-complex to the cytoskeleton.[99] The hypothetical protein and Ezrin shared the same known CD95 binding site, however, the N-terminus of the hypothetical protein was 44 amino acids longer (Figure 25). The hypothetical protein was only 1.2 fold and Ezrin was found to be 1.3

fold downregulated in CD95 resistant Jurkats, which could explain CD95 resistance in these cells at that level at least in part.

```
001  DKFZp762H157.1 grvggrvgaa gstrstagag lgqrlrccwi vvfsgiedth qkpkmpkpin vrvttmdael
001  Ezrin                                                          mpkpin vrvttmdael

061 DKFZp762H157.1  efaiqpnttg kqlfdqvvkt iglrevwyfg lhyvdnkgfp twlkldkkvs aqevrkenpl
017 Ezrin           efaiqpnttg kqlfdqvvkt iglrevwyfg lhyvdnkgfp twlkldkkvs aqevrkenpl

121 DKFZp762H157.1  qfkfrakfyp edvaeeliqd itqklfflqv kegilsdeiy cppetavllg syavqakfgd
077 Ezrin           qfkfrakfyp edvaeeliqd itqklfflqv kegilsdeiy cppetavllg syavqakfgd
                                CD95-binding site
181 DKFZp762H157.1  ynkevhksgy ls serlipqr vmdqhkltrd q wedriqvwh aehrgmlkdn amleylkiaq
137 Ezrin           ynkevhksgy ls serlipqr vmdqhkltrd q wedriqvwh aehrgmlkdn amleylkiaq

241 DKFZp762H157.1  dlemyginyf eiknkkgtdl wlgvdalgln iyekddkltp kigfpwseir nisfndkkfv
197 Ezrin           dlemyginyf eiknkkgtdl wlgvdalgln iyekddkltp kigfpwseir nisfndkkfv

301 DKFZp762H157.1  ikpidkkapd fvfyaprlri nkrilqlcmg nhelymrrrk pdtievqqmk aqareekhqk
257 Ezrin           ikpidkkapd fvfyaprlri nkrilqlcmg nhelymrrrk pdtievqqmk aqareekhqk

361 DKFZp762H157.1  qlerqqlete kkrretvere keqmmrekee lmlrlqdyee ktkkaerels eqiqralqle
317 Ezrin           qlerqqlete kkrretvere keqmmrekee lmlrlqdyee ktkkaerels eqiqralqle

421 DKFZp762H157.1  eerkraqeea erleadrmaa lrakeelerq avdqiksqeq laaelaeyta kialleearr
377 Ezrin           eerkraqeea erleadrmaa lrakeelerq avdqiksqeq laaelaeyta kialleearr

481 DKFZp762H157.1  rkedeveewq hrakeaqddl vktkeelhlv mtappppppp vyepvsyhvq eslqdegaep
437 Ezrin           rkedeveewq hrakeaqddl vktkeelhlv mtappppppp vyepvsyhvq eslqdegaep

541 DKFZp762H157.1  tgysaelsse girddrneek riteaekner vqrqlltlss elsqardenk rthndiihne
497 Ezrin           tgysaelsse girddrneek riteaekner vqrqlltlss elsqardenk rthndiihne

601 DKFZp762H157.1  nmrqgrdkyk tlrqirqgnt kqridefeal
557 Ezrin           nmrqgrdkyk tlrqirqgnt kqridefeal
```

**Figure 25: Sequence alignment of ezrin and the hypothetical protein DKFZp762H157.1**

The hypothetical protein shows complete sequence homology to ezrin with a known CD95 binding site, but has has a 44 aa longer N-terminus.

# 4. Discussion

The aim of this work was to investigate the mechanism leading to CD95 resistance in long-term-activated T cells. CD95-resistant T cells have been shown to play an important role in the development of several autoimmune diseases, such as rheumatoid arthritis[179], multiple sclerosis[28] and type 1 diabetes[115]. This T cell subpopulation is thought to develop in response to permanent stimulation by autoantigen. Similarly, CD95-resistant T cells develop in response to constitutive alloantigen stimulation.[148] As a model system for this situation, HLA-A1$^-$ T cells were weekly restimulated with HLA-A1-expressing .721 cells to create long-term-activated, CD95-resistant T cells. These *in vitro*-generated, apoptosis-resistant CD8$^+$ T cells were used in the first part of this study to analyze the relevance of NF-κB for CD95 signaling. The second part uses CD95-sensitive and -resistant Jurkat cell lines for genome-wide and proteome-wide screening to find new molecular targets involved in the development of apoptosis resistance. It has been shown that *in vitro*-created, CD95-resistant T cells display an effector/memory phenotype, characterized by the loss of CD62L and CCR7 and increased IL-5, IL-10 and IFN-γ production.[148] Memory cells protect from reinfection with the same or related pathogens, such as influenza and herpes viruses which infect humans repeatedly. Memory T cells also play a major role in preventing systemic dissemination caused by persistent virus infections. Analyzing the mechanisms of CD95 resistance of long-term-activated T cells can help to better understand the function of memory T cells and to identify new molecular targets that help to control these cells in infectious, oncological and autoimmune diseases.

## 4.1 Constitutive alloantigen stimulation of human T cells induces CD95 resistance

T cells only acquire sensitivity to CD95-mediated apoptosis some days after antigen activation and its sensitivity increases upon restimulation.[148] CD95 sensitivity increased with each stimulation up to the 5$^{th}$-7$^{th}$ stimulation and subsequently decreased again until the 9$^{th}$-15$^{th}$ stimulation. It is still not clear whether changes in CD95 sensitivity in repeatedly stimulated T cells could be due to altered subset ratios

of a CD95-sensitive and a CD95-resistant subpopulation without major changes within individual cells, or whether repeated antigen stimulation alters CD95 sensitivity within each cell. A genome wide microarray analysis revealed that each stimulation results in the differential regulation of several hundreds of genes. T cells in different rounds of restimulation differ substantially in their molecular signature while also sharing a small group of identically expressed genes.[173] Usually 1-10% of T cells are alloreactive.[50] The HLA-A1$^{-}$ CD8$^{+}$ T cells alloreactive against HLA-A1 were expanded by weekly stimulation so that these cells usually overgrow the culture.

## 4.2 Short-and long-term-activated T cells show no difference in NF-κB activity

NF-κB can protect and sensitize against CD95-mediated death.[41] In this study, both, short- and long-term-activated T cells showed persistent activation of NF-κB p65/p50. Thus, NF-κB has no influence on CD95 sensitivity in long-term-activated T cells. In some cell types CD95 can also directly mediate activation of NF-κB, protecting them from apoptosis, which has not been analyzed in electrophoretic mobility shift assays. However, inhibition of NF-κB did not lead to apoptosis sensitization in long-term-activated T cells, which would have been expected if NF-κB activation after CD95 stimulation had protected long-term-activated T cells from apoptosis.

## 4.3 Differences in the phosphorylation status of Bcl-10 could be relevant in the regulation of cytoskeletal rearrangements, which are important for the induction of apoptosis

The first steps of CD95 signaling and NF-κB signaling take place in lipid rafts, which act as signaling domains within the plasma membrane.[14,44,110] Interestingly, lipid raft isolation of short- and long-term-activated T cells showed some differences among components of the NF-κB signaling pathway, some of which might also play a

role in other signaling pathways. Bcl-10 acts as an adaptor protein between CARMA-1 and MALT1 in the CARMA-1 signalosome within the NF-κB pathway in T cells. IKKβ-mediated phosphorylation of Bcl-10 interferes with the Bcl-10/MALT1 interaction and therefore negatively regulates NF-κB.[178] In Western Blots comparing short- and long-term-activated T cells, we found an additional band only in short-term-activated, apoptosis-sensitive T cells, probably corresponding to phosphorylated Bcl-10. Bcl-10 has many Ser/Thr phosphorylation sites and a novel function of Bcl-10 has been shown in the control of TCR-induced actin polymerization, where Bcl-10 is phosphorylated upon TCR signal transduction at $Ser^{138}$ which does not affect NF-κB signaling at all.[129] Cytoskeletal rearrangements are required for the induction of CD95-mediated apoptosis at least during the recruitment of neighboring lipid rafts and the process of CD95 signaling complex internalization, but also in the process of membrane blebbing during the execution phase of apoptosis.[3,44,121] Higher amounts of potentially phosphorylated Bcl-10 were found in lipid rafts of short-term-activated, CD95-sensitive T cells. Simultaneously, PKC-θ recruitment into lipid rafts was more efficient in short-term- than in long-term-activated T cells. So Bcl-10 is probably phosphorylated by PKC-θ. Since Bcl-10 has various phosphorylation sites associated with different functions it still has to be determined which of them are phosphorylated in short-term-activated T cells by the use of specific anti-phospho-Bcl-10 antibodies.

## 4.4 The NF-κB signaling mediators TRAF2 and TRAF6 can also be associated to apoptosis signaling and were both differentially recruited into lipid rafts of short- and long-term-activated T cells

TRAF6 and TRAF2 are important signal transducers of NF-κB signaling, but are also associated with other functions, such as regulation of apoptosis. TRAF2 was recruited at slightly higher levels to lipid rafts of long-term-activated T cells, whereas lipid raft localization of TRAF6 was significantly reduced in these cells. Overexpression of TRAF2 has been shown to be responsible for CD95 resistance in pancreatic tumor cells[156], whereas TRAF6 has also the capacity to interact with

and activate caspase-8 leading to apoptosis. This was confirmed by TRAF6 knockdown which resulted in resistance to CD95 induced apoptosis.[97] Therefore, differential recruitment of these signaling mediators might contribute to the differences in CD95 sensitivity of short- and long-term-activated T cells. A potential direct interaction of TRAF6 with caspase-8 must be clarified by analyzing the composition of the DISC in these cells.

## 4.5 Reduced constitutive lipid raft recruitment of Zap-70, PLC-γ and PKC-θ suggests reduced $Ca^{2+}$ influx in long-term-activated T cells protecting them from apoptosis

The main signaling events that induce an increase in intracellular $Ca^{2+}$ in T cells involve TCR signaling which leads, via activation of a cascade of protein kinases beginning with Zap-70 bound to phosphorylated ITAMs on the TCR, to the activation of PLCγ1.[160] PLCγ1 hydrolyses $PIP_2$ to DAG and $IP_3$ and leads to the binding of $IP_3$ to IP[153]$_3$R leading to the release of $Ca^{2+}$ from intracellular endoplasmic reticulum (ER) $Ca^{2+}$ stores.[92] In this study, Zap-70 was found in lower amounts in lipid rafts of long-term-activated T cells which, in consequence, might result in the reduced found PLC-γ1 recruitment. PKC-θ is only associated with the membrane if bound to DAG which is a product of membrane-associated PLC-γ1.[20] PKC-θ was also recruited in reduced amounts in lipid rafts. Interstingly PKC-$\theta^{-/-}$ T cells in knock-out mice displayed resistance to CD95-mediated apoptosis as well as AICD[106], which would fit with these findings. PKC-theta-deficient mice are also protected from Th1-dependent antigen-induced arthritis.[63] The lowered PKC-θ recruitment in lipid rafts indicates indirectly that Calcium influx might be lowered in long-term-activated T cells by the lack of sufficient TCR-induced signaling. Similar results have been obtained in an *in vivo* model in which mutation of Zap-70 results in strongly reduced TCR signals including $Ca^{2+}$ signaling.[158] Disregulated $Ca^{2+}$ responses of T cells have been found to be associated with rheumatoid arthritis, and apoptosis-resistant T cells have been identified in patients suffering from this disease.[21,179] T cells that are deficient in type 1 $IP_3$ receptors are resistant to apoptosis induced by CD95 and several other stimuli, and it has been shown that this resistance could be reversed by

raising cytoplasmic $Ca^{2+}$ levels.[70] Therefore, $Ca^{2+}$ is an important trigger of apoptosis in lymphocytes. Interestingly, Bcl-$x_L$ alters calcium signaling by downregulation of IP3R[93] and could be found in increased levels in long-term-activated T cells[148]. Increased $Ca^{2+}$-signaling also leads to activation of the calcium/calmodulin-dependent phosphatase calcineurin, which controls the translocation of NFAT from the cytoplasm to the nucleus of activated T cells.[153] Differences in $Ca^{2+}$-signaling should therefore also lead to differences in NFAT activity. However, EMSA from nuclear extracts of short- and long-term- activated T cells showed no differences in NFAT activity.

## 4.6 Reduced CD95 recruitment to lipid rafts might be due to constitutive PI3K activity in long-term-activated T cells

Localization of the death receptor CD95 to lipid rafts is crucial for CD95-mediated cell death signaling.[14,44] Cholesterol depletion and the resulting loss of lipid rafts lead to CD95 resistance. Palmitoylation is required for the constitutive localization of CD95 to rafts which is necessary for inducing cell death.[49] In this study, CD95 was found in decreased levels in lipid rafts of long-term-activated T cells, which might explain CD95 resistance at least in part. It has been shown that long-term-activated T cells show increased PI3K activity[148] and that PI3K activity leads to reduced lipid raft recruitment of CD95 by its depalmitoylation.[14] The role of lipid rafts in CD95 signaling is still differentially discussed and dependent on the cell type analyzed. Disturbance of lipid rafts by depletion of cholesterol might provide further insight into the role of lipid raft signaling for CD95 sensitivity of short- and long-term-activated T cells.

Palmitoylation of CD95 is also required for its binding to ezrin, thereby linking the CD95 receptor signaling complex to the cytoskeleton.[99] This step is necessary for lipid raft aggregation to form large CD95 clusters and efficient CD95 receptor complex internalization by clathrin-dependent endocytosis.[44,90] Blocking CD95 internalization result in the inhibition of DISC recruitment and apoptosis.[90] Membrane linkage of ezrin and clathrin by AP2 is dependent on $PIP_2$.[90,121] Altering $PIP_2$ levels by constitutive expression of a $PIP_2$ phosphatase leads to CD95

resistance.[90] Long-term-activated T cells show constitutive PI3K activity and loss of the phosphatase PTEN, a negative regulator of PI3K signaling.[148] This may alter the $PIP_2/PIP_3$ balance in favor of $PIP_3$ and may therefore excert an inhibitory effect on CD95 receptor complex internalization and CD95 clustering.

## 4.7 Overexpression of CD95L and complete localization into lipid rafts may inhibit CD95-induced apoptosis by blocking the binding site of CD95

The increased levels of CD95L in long-term-activated T cells are completely recruited into lipid rafts. Lipid raft localization of CD95L is also dependent on palmitoylation and is required for the induction of apoptosis in other cells.[58] Higher cytoxicity of long-term activated T cells was already shown.[148] High CD95L expression and lipid raft localization may also have a yet unknown inhibiting effect. Otherwise, signaling complex formation of CD95L and CD95 and induction of apoptosis could occur on the same cell without any extracellular stimulus. CD95L could be directly bound to CD95 on the same cell, thus, blocking external stimulation of CD95. Such an interaction of CD95 and CD95L must be shown in future experiments.

## 4.8 CD95-sensitive and -resistant Jurkat cells as model system for the study of long-term-activated T cells

Jurkat cells are often used as model system for the study of T cells. But as an immortal cell line there are only partially comparable to CTLs. While Jurkat cells are classified as CD95 type II cells[131], short-term-activated T cells are classified as CD95 type I cells[127,169]. For this study, two CD95-sensitive and two CD95-resistant Jurkat cell lines were used. Using these four cell lines, it was possible to study CD95 resistance mechanisms without depending on long-term primary cell cultures.

## 4.9 The Rho/ROCK signaling pathway is important for the induction of apoptosis in response to CD95 signaling

The four Jurkat cell lines were cross-analyzed by gene chip analysis. Only changes were taken into account which were present in all four possible cell line combinations. Appliying these rules, gene chip analysis revealed 276 genes to be differentially regulated between CD95-sensitive and -resistant Jurkat cells. These alterations could be correlated to CD95 resistance, since the CD95-sensitive Jurkat cell line JM was genetically more closely related to the second CD95-sensitive cell line Je6.1 than to its CD95-resistant daughter cell lines JM301 and JM319. But none of these changes were directly related to apoptosis or cell survival except the Rho/ROCK signaling pathway. Phosphorylation of ezrin by ROCK1 has been found to be critical for maintaining the active, open conformation of ezrin, competent for membrane localization and binding of CD95 and actin.[49,64] ROCK is an effector of the small GTPase Rho which acts as a molecular switch, cycling between an active GTP-bound and an inactive GDP-bound state. In the GTP-bound form, catalyzed by Rho-GEFs, Rho is able to interact with ROCK leading to phosphorylation of ezrin and PTEN. An intrinsic GTPase activated by Rho-GAPs returns the protein to the GDP-bound state and terminates signal transduction.[64] CD95-sensitive Jurkat cells showed increased GEF gene expression and decreased RNA levels of the inhibiting Rho-GTPases compared to CD95-resistant cells, suggesting reduced ROCK activity in CD95-resistant compared to -sensitive Jurkat cells. Furthermore, ROCK inhibition reduced CD95-mediated apoptosis to some extent in CD95-sensitive Jurkat cells, indicating that dysfunctional Rho/ROCK signaling might contribute to the development of apoptosis resistance.

## 4.10 2D-DIGE identified a hypothetical protein of the ezrin-radixin-moesin (ERM) family with a known CD95 binding site

The 2D-DIGE approach for protein profiling is an accessible and economical method that possesses high resolving power and enables the detection of hundreds of proteins in a single experiment by analyzing two protein mixtures at the same time.

The use of imagers and computers allows spot detection, normalization and background correction which makes it a powerful tool for proteomics comparing two different samples, in this case short- and long-term-activated T cells or Jurkat cell lines differing in CD95 sensitivity. Interestingly, a hypothetical protein was identified. The hypothetical protein is a member of the Ezrin-Radixin-Moesin (ERM) family and displays high sequence homology to Ezrin, but has a 44 amino acid longer N-terminus. The identified peptide can be found in both, Ezrin and the hypothetical protein, but they can be distinguished through their different size and isoelectric point (IEP) in 2D electrophoresis. A CD95 binding site of Ezrin is known[99] and can also be found in the hypothetical protein. Ezrin links the CD95 signaling complex to the cytoskeleton and was also found in decreased levels in CD95-resistant Jurkat cells together with the hypothetical protein, which may affect CD95 sensitivity. The longer N-terminus of the hypothetical protein may recruit yet unknown proteins to the CD95 signaling complex. .

## 4.11 Grp78 protects from CD95-induced apoptosis

In line with the previously suggested lowered Calcium signaling in long-term-activated T cells, $Ca^{2+}$ overload in the ER $Ca^{2+}$ pool can affect proper protein folding and result in ER stress. As a consequence, the unfolded protein response (UPR) pathway is activated leading to the induction of ER chaperones, such as Grp78 and PDI.[74] Both chaperones were found by 2D-DIGE to be overexpressed in CD95-resistant long-term-activated T cells and Jurkat cells. During UPR a significant proportion of Grp78 is also localized to the inter-membrane space, inner membrane and matrix of mitochondria.[151] Grp78 is a $Ca^{2+}$-binding protein and prevents apoptosis that arises from a disturbance of intracellular $Ca^{2+}$.[87] The relevance of Grp78 in inhibiting apoptosis was demonstrated by downregulation of Grp78 in CD95-resistant Jurkat cells which lead to their sensitization to CD95L-induced apoptosis. That upregulation of Grp78 by valproic acid led to further sensitization of CD95-sensitive Jurkat cells, is probably due to the fact that valproic acid changes the expression of several other proteins involved in apoptosis.

## 4.12 The proteasome and Annexin A1 play a role in regulation of apoptosis

Proteasomes are large complexes that are located in the nucleus and cytoplasm. The main function of the proteasome is to degrade proteins by proteolysis. The targeting of proteins can be specific affecting only ubiquitin-tagged proteins or more unspecific in immunoproteasomes.[126] The proteasome is a critical regulator of many cellular processes, such as cell cycle, regulation of gene expression, responses to oxidative stress, antigen presentation, proliferation, differentiation and apoptosis. Apoptosis can be induced by inhibition of the proteasome[139], but proteasome activation was also shown to be involved in apoptosis of thymocytes.[65] The fact that one protein complex can fulfill this variety of functions is due to the composition of its subunits.[57] The proteasome subunit alpha type-6 and proteasome activator complex subunit 2 were both downregulated in the CD95-resistant Jurkats and long-term activated T cells. The relevance of the differentially regulated proteasome subunits for CD95 resistance must be shown in further experiments.

Annexin A1 belongs to the annexin family of $Ca^{2+}$-dependent phospholipid-binding proteins and is a soluble cytoplasmic protein, moving to membranes when calcium levels are elevated. Annexin A1 has also been shown to move to the nucleus or outside the cells. The function of Annexin A1 is dependent on its localization within the cell. Annexin A1 is involved in cytoskeletal organization and cell differentiation. It has a phospholipase A2 inhibitory activity and also blocks activation of NF-κB by binding to the p65 subunit.[164,180] Annexin A-I has also been implicated in apoptosis. It promotes pro-apoptotic processes, if expressed on the surface of neutrophils where it is also involved in the removal of cells that have undergone apoptosis.[7] Annexin A1 is also a new functional linker between actin filaments.[122] The involvement of Annexin A1 in cytoskeletal organization could also be relevant in the process of apoptosis and was downregulated in CD95-resistant Jurkat and long-term activated T cells. Coronin 1 was another actin binding protein found to be downregulated in CD95 resistant Jurkats and long term-activated T cells. Future siRNA experiments are needed to show the relevance of Annexin A1 and Coronin 1, as well as the localization of Annexin A1 within the cell.

# 5. Summary

Long-term-activated T cells can be found in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis and are resistant to CD95-induced apoptosis. The major aim of this work was to investigate the mechanisms of CD95 resistance in order to find new molecular targets for the therapy of these diseases. Long-term-activated T cells show constitutive caspase activation, usually a sign for apoptosis, but are resistant towards CD95-induced cell death. Caspase-8 has been shown to interact with components of the NF-κB signaling pathway within lipid rafts during the first steps of NF-κB signaling, which can be pro- or antiapoptotic. Therefore, the goal of this work was to show, whether NF-κB plays a role in the development of CD95 resistance in long-term-activated T cells or not. For this purpose, *in vitro*-generated, short- and long-term-activated T cells were compared by EMSA and analysis of lipid raft composition to get a better understanding of constitutive lipid raft localization of signaling mediators important for CD95-induced apoptosis, TCR- and NF-κB signaling. Investigation of NF-κB activity by EMSA and inhibition of the pathway demonstrated that the activation status of NF-κB cannot be related to CD95 resistance in long-term-activated T cells. However, analysis of lipid raft composition in both cell types revealed differences in constitutive raft localization of TCR signaling proteins, such as reduced recruitment of Zap-70 and, subsequently, lower amounts of PLC-γ and PKC-θ within lipid rafts of long-term-activated T cells. Reduced raft localization of PLC-γ and PKC-θ may indicate decreased $Ca^{2+}$ signaling in these cells, which is required for T cell apoptosis. Besides its role in NF-κB activation, TRAF6 is also relevant for apoptosis induction and was also found in lower amounts in lipid rafts of long-term-activated T cells. The transmembrane proteins CD95 and CD95L are only associated to lipid rafts if palmitoylated. CD95 localization to lipid rafts is required for apoptosis induction and was reduced in long-term-activated T cells, whereas CD95L was present in high amounts, in line with its increased overall expression. Although raft localization of CD95L is a prerequisite for apoptosis induction in a variety of cell types, its overexpression and increased raft recruitment may also have a yet unknown inhibitory effect on CD95 signaling to prevent T cell suicide. Whether direct interaction of membrane-bound CD95L and CD95 within lipid rafts might block external CD95 stimuli and thus confer apoptosis

resistance to long-term-activated T cells must be shown in future experiments and may help to further delineate the role of lipid rafts in CD95-induced apoptosis.

Secondly, CD95-sensitive and CD95-resistant Jurkat cell lines were used as model system for the analysis of the mechanism of CD95 resistance. Genomic and proteomic comparison of CD95-sensitive and -resistant Jurkat cell lines revealed the relevance of chaperones, such as Grp78, in protecting from CD95-induced apoptosis, and of the Rho/ROCK pathway in inducing apoptosis. This work is also the first to identify the hypothetical protein DKFZp762H157.1 to be expressed in Jurkat cells. The hypothetical protein displayed high sequence homology to ezrin, including a common CD95 binding site, but had a 45 amino acids longer N-terminus, which might suggest its interaction with yet unknown binding partners, which could be relevant in apoptosis regulation. Further experiments are required to prove the significance of the presented results and to gain a better understanding of the mechanisms leading to CD95 resistance in long-term-activated T cells.

# 6. References


[1] O. Acuto and F. Michel, CD28-mediated co-stimulation: a quantitative support for TCR signalling, Nat. Rev. Immunol. 3 (2003) pp. 939 -951.

[2] K. Akashi, D. Traver, M. Kondo, I.L. Weissman, Lymphoid development from hematopoietic stem cells, Int. J. Hematol. 69 (1999) pp. 217 -226.

[3] A. Algeciras-Schimnich and M.E. Peter, Actin dependent CD95 internalization is specific for Type I cells, FEBS Lett. 546 (2003) pp. 185 -188.

[4] J. An, M. Fisher, M.B. Rettig, VHL expression in renal cell carcinoma sensitizes to bortezomib (PS -341) through an NF -kappaB-dependent mechanism, Oncogene 24 (2005) pp. 1563 -1570.

[5] S.M. Aouad, L.Y. Cohen, E. Sharif-Askari, E.K. Haddad, A. Alam, R.P. Sekaly, Caspase-3 is a component of Fas death-inducing signaling complex in lipid rafts and its activity is required for complete caspase-8 activation during Fas-mediated cell death, J. Immunol. 172 (2004) pp. 2316 -2323.

[6] A. Arcaro, C. Gregoire, N. Boucheron, S. Stotz, E. Palmer, B. Malissen, I.F. Luescher, Essential role of CD8 palmitoylation in CD8 coreceptor function, J. Immunol 165 (2000) pp. 2068 -2076.

[7] S. Arur, U.E. Uche, K. Rezaul, M. Fong, V. Scranton, A.E. Cowan, W. Mohler, D.K. Han, Annexin I is an endogenous ligand that mediates apoptotic cell engulfment, Dev. Cell 4 (2003) pp. 587 -598.

[8] Y. Baba, R. Pelayo, P.W. Kincade, Relationships between hematopoietic stem cells and lymphocyte progenitors, Trends Immunol. 25 (2004) pp. 645-649.

[9] A.S. Baldwin, Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB, J. Clin. Invest 107 (2001) pp. 241 -246.

[10] A.S. Baldwin, Jr., Series introduction: the transcription factor NF-kappaB and human disease, J. Clin. Invest 107 (2001) pp. 3 -6.

[11] R.C. Bargou, C. Leng, D. Krappmann, F. Emmerich, M.Y. Mapara, K. Bommert, H.D. Royer, C. Scheidereit, B. Dorken, High-level nuclear NF-kappa B and Oct-2 is a common feature of cultured Hodgkin/Reed-Sternberg cells, Blood 87 (1996) pp. 4340 -4347.

[12] M. Barry and R.C. Bleackley, Cytotoxic T lymphocytes: all roads lead to death, Nat. Rev. Immunol. 2 (2002) pp. 401 -409.

[13] V. Baud, Z.G. Liu, B. Bennett, N. Suzuki, Y. Xia, M. Karin, Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain, Genes Dev. 13 (1999) pp. 1297 -1308.

[14] M. Beneteau, M. Pizon, B. Chaigne-Delalande, S. Daburon, P. Moreau, G.F. De, F. Ichas, A. Rebillard, M.T. Dimanche-Boitrel, J.L. Taupin, J.F. Moreau, P. Legembre, Localization of Fas/CD95 into the lipid rafts on down-modulation of the phosphatidylinositol 3-kinase signaling pathway, Mol. Cancer Res. 6 (2008) pp. 604-613.

[15] J.R. Berenson, H.M. Ma, R. Vescio, The role of nuclear factor-kappaB in the biology and treatment of multiple myeloma, Semin. Oncol. 28 (2001) pp. 626-633.

[16] P. Bernardi and A. Rasola, Calcium and cell death: the mitochondrial connection, Subcell. Biochem. 45 (2007) pp. 481 -506.

[17] J. Bertin, CARD11 and CARD14 are novel caspase recruitment domain (CARD)/membrane -associated guanylate kinase (MAGUK) family

members that interact with BCL10 and activate NF-[kappa]B, J. Biol. Chem. 276 (2001) pp. 11877 -11882.

[18] P. Boya, K. Andreau, D. Poncet, N. Zamzami, J.L. Perfettini, D. Metivier, D.M. Ojcius, M. Jaattela, G. Kroemer, Lysosomal membrane permeabilization induces cell death in a mitochondrion-dependent fashion, J. Exp. Med. 197 (20 03) pp. 1323 -1334.

[19] S.K. Bromley, W.R. Burack, K.G. Johnson, K. Somersalo, T.N. Sims, C. Sumen, M.M. Davis, A.S. Shaw, P.M. Allen, M.L. Dustin, The immunological synapse, Annu. Rev. Immunol. 19 (2001) pp. 375 -396.

[20] S. Carrasco and I. Merida, Diacylglycerol-dependent binding recruits PKCtheta and RasGRP1 C1 domains to specific subcellular localizations in living T lymphocytes, Mol. Biol. Cell 15 (2004) pp. 2932-2942.

[21] D.M. Carruthers, W.G. Naylor, M.E. Allen, G.D. Kitas, P.A. Bacon, S.P. Young, Characterization of altered calcium signalling in T lymphocytes from patients with rheumatoid arthritis (RA), Clin. Exp. Immunol 105 (1996) pp. 291 -296.

[22] L. Chen, J.N. Glover, P.G. Hogan, A. Rao, S.C. Harrison, Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA, Nature 392 (1998) pp. 42 -48.

[23] X. Chen, K. Kandasamy, R.K. Srivastava, Differential roles of RelA (p65) and c-Rel subunits of nuclear factor kappa B in tumor necrosis factor-related apoptosis-inducing ligand signaling, Cancer Res. 63 (2003) pp. 1059-1066.

[24] Z.J. Chen, L. Parent, T. Maniatis, Site-specific phosphorylation of I[kappa]B[alpha] by a novel ubiquitination-dependent protein kinase activity, Cell 84 (1996) pp. 853 -862.

[25] M. Ciofani and J.C. Zuniga-Pflucker, Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism, Nat. Immunol. 6 (2005) pp. 881 -888.

[26] P. Cockwell, J.W. Calderwood, C.J. Brooks, S.J. Chakravorty, C.O. Savage, Chemoattraction of T cells expressing CCR5, CXCR3 and CX3CR1 by proximal tubular epithelial cell chemokines, Nephrol. Dial. Transplant. 17 (2002) pp. 734 -744.

[27] M.L. Coleman, E.A. Sahai, M. Yeo, M. Bosch, A. Dewar, M.F. Olson, Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I, Nat Cell Biol. 3 (2001) pp. 339 -345.

[28] C. Comi, M. Leone, S. Bonissoni, S. DeFranco, F. Bottarel, C. Mezzatesta, A. Chiocchetti, F. Perla, F. Monaco, U. Dianzani, Defective T cell fas function in patients with multiple sclerosis, Neurology 55 (2000) pp. 921-927.

[29] B. Coornaert, M. Baens, K. Heyninck, T. Bekaert, M. Haegman, J. Staal, L. Sun, Z.J. Chen, P. Marynen, R. Beyaert, T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20, Nat Immunol 9 (2008) pp. 263 -271.

[30] I.N. Crispe, Fatal interactions: Fas-induced apoptosis of mature T cells, Immunity. 1 (1994) pp. 347 -349.

[31] V. Dardalhon, A. Awasthi, H. Kwon, G. Galileos, W. Gao, R.A. Sobel, M. Mitsdoerffer, T.B. Strom, W. Elyaman, I.C. Ho, S. Khoury, M. Oukka, V.K. Kuchroo, IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3( -) effector T cells, Nat. Immunol. 9 (2008) pp. 1347 -1355.

[32] M.M. Davis, J.J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, Y. Chien, Ligand recognition by alpha beta T cell receptors, Annu. Rev. Immunol. 16 (1998) pp. 523 -544.

[33] H.A. de la, A. Acevedo, W. Marston, F. Sanchez-Madrid, Function of CD44(Pgp -1) homing receptor in human T cell precursors, Int. Immunol. 1 (1989) pp. 598 -604.

[34] D. Decaudin, I. Marzo, C. Brenner, G. Kroemer, Mitochondria in chemotherapy-induced apoptosis: a prospective novel target of cancer therapy (review), Int. J . Oncol. 12 (1998) pp. 141 -152.

[35] E.M. Degli, J. Tour, S. Ouasti, S. Ivanova, P. Matarrese, W. Malorni, R. Khosravi-Far, Fas death receptor enhances endocytic membrane traffic converging into the Golgi region, Mol. Biol. Cell 20 (2009) pp. 600-615.

[36] A. Degterev, M. Boyce, J. Yuan, A decade of caspases, Oncogene 22 (2003) pp. 8543 -8567.

[37] E. Dejardin, The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development, Biochem. Pharmacol. 72 (2006 ) pp. 1161 -1179.

[38] F. Denizot, A. Wilson, F. Battye, G. Berke, K. Shortman, Clonal expansion of T cells: a cytotoxic T-cell response in vivo that involves precursor cell proliferation, Proc. Natl. Acad. Sci. U. S. A 83 (1986) pp. 6089 -6092.

[39] R.L. Doughman, A.J. Firestone, R.A. Anderson, Phosphatidylinositol phosphate kinases put PI4,5P(2) in its place, J. Membr. Biol. 194 (2003) pp. 77 -89.

[40] C. Du, M. Fang, Y. Li, L. Li, X. Wang, Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition, Cell 102 (2000) pp. 33 -42.

[41] E. Dudley, F. Hornung, L. Zheng, D. Scherer, D. Ballard, M. Lenardo, NF-kappaB regulates Fas/APO-1/, Eur. J. Immunol 29 (1999) pp. 878 -886.

[42] C.K. Ea, L. Deng, Z.P. Xia, G. Pineda, Z.J. Chen, Activation of IKK by TNF[alpha] requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO, Mol. Cell 22 (2006) pp. 245 -257.

[43] M.J. Eck, S.K. Atwell, S.E. Shoelson, S.C. Harrison, Structure of the regulatory domains of the Src-family tyrosine kinase Lck, Nature 368 (1994) pp. 764 -769.

[44] A. Eramo, M. Sargiacomo, L. Ricci-Vitiani, M. Todaro, G. Stassi, C.G. Messina, I. Parolini, F. Lotti, G. Sette, C. Peschle, M.R. De, CD95 death-inducing signaling complex formation and internalization occur in lipid rafts of type I and type II cells, Eur. J. Immunol. 34 (2004) pp. 1930-1940.

[45] A. Eramo, M. Sargiacomo, L. Ricci-Vitiani, M. Todaro, G. Stassi, C.G. Messina, I. Parolini, F. Lotti, G. Sette, C. Peschle, M.R. De, CD95 death-inducing signaling complex formation and internalization occur in lipid rafts of type I and type II cells, Eur. J. Immunol. 34 (2004) pp. 1930-1940.

[46] A. Estelles, C.A. Charlton, H.M. Blau, The phosphoprotein protein PEA-15 inhibits Fas- but increases TNF-R1-mediated caspase-8 activity and apoptosis, Dev. Biol. 216 (1999) pp. 16 -28.

[47] V.A. Fadok, J.S. Savill, C. Haslett, D.L. Bratton, D.E. Doherty, P.A. Campbell, P.M. Henson, Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells, J. Immunol. 149 (1992) pp. 4029 -4035.

[48] M. Falk, S. Ussat, N. Reiling, D. Wesch, D. Kabelitz, S. Adam-Klages, Caspase inhibition blocks human T cell proliferation by suppressing appropriate regulation of IL-2, CD25, and cell cycle-associated proteins, J. Immunol 173 (2004) pp. 5077 -5085.

[49] C. Feig, V. Tchikov, S. Schutze, M.E. Peter, Palmitoylation of CD95 facilitates formation of SDS-stable receptor aggregates that initiate apoptosis signaling, EMBO J. 26 (2007) pp. 221 -231.

[50] N.J. Felix and P.M. Allen, Specificity of T-cell alloreactivity, Nat Rev Immunol 7 (2007) pp. 942 -953.

[51] J.D. Fontenot, M.A. Gavin, A.Y. Rudensky, Foxp3 programs the development and function of CD4+CD25+ regulatory T cells, Nat. Immunol. 4 (2003) pp. 330 -336.

[52] R. Fragoso, D. Ren, X. Zhang, M.W. Su, S.J. Burakoff, Y.J. Jin, Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling, J. Immunol. 170 (2003) pp. 913 -921.

[53] L. Galluzzi and G. Kroemer, Necroptosis: a specialized pathway of programmed necrosis, Cell 135 (2008) pp. 11 61-1163.

[54] S. Ghosh, M.J. May, E.B. Kopp, NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses, Annu. Rev Immunol 16 (1998) pp. 225 -260.

[55] D.I. Godfrey, J. Kennedy, T. Suda, A. Zlotnik, A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression, J. Immunol. 150 (1993) pp. 4244 -4252.

[56] D.R. Green and G. Kroemer, The pathophysiology of mitochondrial cell death, Science 305 (2004) pp. 626 -629.

[57] L.M. Grimm and B.A. Osborne, Apoptosis and the proteasome, Results Probl. Cell Differ. 23 (1999) pp. 209 -228.

[58] F. Guardiola-Serrano, A. Rossin, N. Cahuzac, K. Luckerath, I. Melzer, S. Mailfert, D. Marguet, M. Zornig, A.O. Hueber, Palmitoylation of human

FasL modulates its cell death-inducing function, Cell Death. Dis. 1 (2010) p.e88.

[59] M. Gyrd-Hansen and P. Meier, IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer, Nat. Rev. Cancer 10 (2010) pp. 561-574.

[60] C. Haanen and I. Vermes, Apoptosis and inflammation, Mediators. Inflamm. 4 (1995) pp. 5 -15.

[61] M.S. Hayden and S. Ghosh, Signaling to NF-kappaB, Genes Dev. 18 (2004) pp. 2195 -2224.

[62] L. He, X. Wu, R. Siegel, P.E. Lipsky, TRAF6 regulates cell fate decisions by inducing caspase 8-dependent apoptosis and the activation of NF-kappaB, J. Biol. Chem. 281 (2006) pp. 11235 -11249.

[63] A.M. Healy, E. Izmailova, M. Fitzgerald, R. Walker, M. Hattersley, M. Silva, E. Siebert, J. Terkelsen, D. Picarella, M.D. Pickard, B. LeClair, S. Chandra, B. Jaffee, PKC-theta-deficient mice are protected from Th1-dependent antigen-induced arthritis, J. Immunol. 177 (2006) pp. 1886-1893.

[64] M. Hebert, S. Potin, M. Sebbagh, J. Bertoglio, J. Breard, J. Hamelin, Rho-ROCK-dependent ezrin-radixin-moesin phosphorylation regulates Fas-mediated apoptosis in Jurkat cells, J. Immunol. 181 (2008) pp. 5963 -5973.

[65] T. Hirsch, B. Dallaporta, N. Zamzami, S.A. Susin, L. Ravagnan, I. Marzo, C. Brenner, G. Kroemer, Proteasome activation occurs at an early, premitochondrial step of thymocyte apoptosis, J. Immunol. 161 (1998) pp. 35 -40.

[66] M. Irmler, M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J.L. Bodmer, M. Schroter, K. Burns, C. Mattmann, D. Rimoldi, L.E. French,

J. Tschopp, Inhibition of death receptor signals by cellular FLIP, Nature 388 (1997) pp. 190 -195.

[67] N. Isakov and A. Altman, Protein kinase C(theta) in T cell activation, Annu. Rev. Immunol. 20 (2002) pp . 761-794.

[68] J. Jain, P.G. McCaffrey, Z. Miner, T.K. Kerppola, J.N. Lambert, G.L. Verdine, T. Curran, A. Rao, The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun, Nature 365 (1993) pp. 352-355.

[69] O. Janssen, A. Stocker, R. Sanzenbacher, H.H. Oberg, M.A. Siddiqi, D. Kabelitz, Differential regulation of activation-induced cell death in individual human T cell clones, Int. Arch. Allergy Immunol. 121 (2000) pp. 183 -193.

[70] T. Jayaraman and A.R. Marks, T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis, Mol. Cell Biol. 17 (1997) pp. 3005-3012.

[71] M. Karin, NF-kappaB as a critical link between inflammation and cancer, Cold Spring Harb. Perspect. Biol. 1 (2009) p.a0 00141.

[72] M. Karin and Y. Ben-Neriah, Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity, Annu. Rev Immunol 18 (2000) pp. 621 -663.

[73] T. Kataoka, K. Takaku, J. Magae, N. Shinohara, H. Takayama, S. Kondo, K. Nagai, Acidification is essential for maintaining the structure and function of lytic granules of CTL. Effect of concanamycin A, an inhibitor of vacuolar type H(+) -ATPase, on CTL-mediated cytotoxicity, J. Immunol. 153 (1994) pp. 3938 -3947.

[74] R.J. Kaufman, Orchestrating the unfolded protein response in health and disease, J. Clin. Invest. 110 (2002) pp. 1389 -1398.

[75] N.J. Kennedy, T. Kataoka, J. Tschopp, R.C. Budd, Caspase activation is required for T cell proliferation, J. Exp. Med. 190 (1999) pp. 1891 -1896.

[76] J.F. Kerr, A.H. Wyllie, A.R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, Br. J. Cancer 26 (1972) pp. 239 -257.

[77] J.Y. Kim, M. Morgan, D.G. Kim, J.Y. Lee, L. Bai, Y. Lin, Z.G. Liu, Y.S. Kim, TNFalpha induced noncanonical NF-kappaB activation is attenuated by RIP1 through stabilization of TRAF2, J. Cell Sci. 124 (2011) pp. 647 -656.

[78] M.J. Kim, E. Kim, S.H. Ryu, P.G. Suh, The mechanism of phospholipase C-gamma1 regulation, Exp. Mol. Med. 32 (2000 ) pp. 101 -109.

[79] U. Kordes, D. Krappmann, V. Heissmeyer, W.D. Ludwig, C. Scheidereit, Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells, Leukemia 14 (2000) pp. 399 -402.

[80] S.J. Korsmeyer, A. Gross, H. Harada, J. Zha, K. Wang, X.M. Yin, M. Wei, S. Zinkel, Death and survival signals determine active/inactive conformations of pro-apoptotic BAX, BAD, and BID molecules, Cold Spring Harb. Symp. Quant. Biol. 64 (1999) pp. 343 -350.

[81] S. Kothakota, T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T.J. McGarry, M.W. Kirschner, K. Koths, D.J. Kwiatkowski, L.T. Williams, Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis, Science 278 (1997) pp. 294 -298.

[82] G. Kroemer, Early work on the role of mitochondria in apoptosis, an interview with Guido Kroemer, Cell Death. Differ. 11 Suppl 1 (2004) p.S33 -S36.

[83] S. Krugmann, R. Williams, L. Stephens, P.T. Hawkins, ARAP3 is a PI3K and rap-regulated GAP for RhoA, Curr. Biol. 14 (2004) pp. 1380 -1384.

[84] A.M. Kruisbeek, Development of alpha beta T cells, Curr. Opin. Immunol 5 (1993) pp. 227 -234.

[85] J. Kucharczak, M.J. Simmons, Y. Fan, C. Gelinas, To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis, Oncogene 22 (2003) pp. 8961 -8982.

[86] M. Kundu and C.B. Thompson, Autophagy: basic principles and relevance to disease, Annu. Rev. Pathol. 3 (2008) pp. 427 -455.

[87] H.K. Lamb, C. Mee, W. Xu, L. Liu, S. Blond, A. Cooper, I.G. Charles, A.R. Hawkins, The affinity of a major Ca2+ binding site on GRP78 is differentially enhanced by ADP and ATP, J. Biol. Chem. 281 (2006) pp. 8796-8805.

[88] I. Lavrik, A. Golks, P.H. Krammer, Death receptor signaling, J. Cell Sci. 118 (2005) pp. 2 65-267.

[89] D.J. Leahy, R. Axel, W.A. Hendrickson, Crystal structure of a soluble form of the human T cell coreceptor CD8 at 2.6 A resolution, Cell 68 (1992) pp. 1145-1162.

[90] K.H. Lee, C. Feig, V. Tchikov, R. Schickel, C. Hallas, S. Schutze, M.E. Peter, A.C. Chan, The role of receptor internalization in CD95 signaling, EMBO J. 25 (2006) pp. 1009 -1023.

[91] C.M. Lewis, C. Broussard, M.J. Czar, P.L. Schwartzberg, Tec kinases: modulators of lymphocyte signaling and development, Curr. Opin. Immunol. 13 (2001) pp. 317 -325.

[92] R.S. Lewis, Calcium signaling mechanisms in T lymphocytes, Annu. Rev. Immunol. 19 (2001) pp. 497 -521.

[93] C. Li, C.J. Fox, S.R. Master, V.P. Bindokas, L.A. Chodosh, C.B. Thompson, Bcl-X(L) affects Ca(2+) homeostasis by alter ing expression of inositol 1,4,5-trisphosphate receptors, Proc. Natl Acad. Sci. U. S. A 99 (2002) pp. 9830 -9835.

[94] H. Li, H. Zhu, C.J. Xu, J. Yuan, Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis, Cell 94 (1998) pp. 491 -501.

[95] L.Y. Li, X. Luo, X. Wang, Endonuclease G is an apoptotic DNase when released from mitochondria, Nature 412 (2001) pp. 95 -99.

[96] Z.P. Lin, Y.C. Boller, S.M. Amer, R.L. Russell, K.A. Pacelli, S.R. Patierno, K.A. Kennedy, Prevention of brefeldin A-induced resistance to teniposide by the proteasome inhibitor MG-132: involvement of NF-kappaB activation in drug resistance, Cancer Res. 58 (1998) pp. 3059 -3065.

[97] S. Liu, J. Lutz, J. Chang, D. Liu, U. Heemann, M. Baumann, TRAF6 knockdown promotes survival and inhibits inflammatory response to lipopolysaccharides in rat primary renal proximal tubule cells, Acta Physiol (Oxf) 199 (2010) pp. 339 -346.

[98] S.K. Liu, D.M. Berry, C.J. McGlade, The role of Gads in hematopoietic cell signalling, Oncogene 20 (2001) pp. 6284 -6290.

[99] F. Lozupone, L. Lugini, P. Matarrese, F. Luciani, C. Federici, E. Iessi, P. Margutti, G. Stassi, W. Malorni, S. Fais, Identification and relevance of the CD95-binding domain in the N-terminal region of ezrin, J. Biol. Chem. 279 (2004) pp. 9199 -9207.

[100] P.C. Lucas, Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-[kappa]B signaling pathway, J. Biol. Chem. 276 (2001) pp. 19012 -19019.

[101] X. Luo, I. Budihardjo, H. Zou, C. Slaughter, X. Wang, Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors, Cell 94 (1998) pp. 481 -490.

[102] F. Macian, C. Lopez-Rodriguez, A. Rao, Partners in transcription: NFAT and AP-1, Oncogene 20 (2001) pp. 2476 -2489.

[103] T. Magee, N. Pirinen, J. Adler, S.N. Pagakis, I. Parmryd, Lipid rafts: cell surface platforms for T cell signaling, Biol. Res. 35 (2002) pp. 12 7-131.

[104] I. Maillard, T. Fang, W.S. Pear, Regulation of lymphoid development, differentiation, and function by the Notch pathway, Annu. Rev. Immunol. 23 (2005) pp. 945 -974.

[105] M. Malewicz, N. Zeller, Z.B. Yilmaz, F. Weih, NF kappa B controls the balance between Fas and tumor necrosis factor cell death pathways during T cell receptor-induced apoptosis via the expression of its target gene A20, J. Biol. Chem. 278 (2003) pp. 32825 -32833.

[106] S. Manicassamy, S. Gupta, Z. Sun, Selective function of PKC-theta in T cells, Cell Mol. Immunol. 3 (2006) pp. 263 -270.

[107] M. Matloubian, C.G. Lo, G. Cinamon, M.J. Lesneski, Y. Xu, V. Brinkmann, M.L. Allende, R.L. Proia, J.G. Cyster, Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1, Nature 427 (2004) pp. 355 -360.

[108] R. Matsumoto, Phosphorylation of CARMA1 plays a critical role in T-cell receptor-mediated NF-[kappa]B activation, Immunity 23 (2005) pp. 575-585.

[109] J.F. MILLER, Immunological function of the thymus, Lancet 2 (1961) pp. 748-749.

[110] R.S. Misra, J.Q. Russell, A. Koenig, J.A. Hinshaw-Makepeace, R. Wen, D. Wang, H. Huo, D.R. Littman, U. Ferch, J. Ruland, M. Thome, R.C. Budd, Caspase-8 and c-FLIPL associate in lipid rafts with NF-kappaB adaptors during T cell activation, J. Biol. Chem. 282 (2007) pp. 19365-19374.

[111] M. Miyasaka and T. Tanaka, Lymphocyte trafficking across high endothelial venules: dogmas and enigmas, Nat. Rev. Immunol. 4 (2004) pp. 360-370.

[112] N. Mori, Y. Yamada, S. Ikeda, Y. Yamasaki, K. Tsukasaki, Y. Tanaka, M. Tomonaga, N. Yamamoto, M. Fujii, Bay 11-7082 inhibits transcription factor NF-kappaB and induces apoptosis of HTLV-I-infected T-cell lines and primary adult T-cell leukemia cells, Blood 100 (2002) pp. 1828 -1834.

[113] K.M. Murphy and S.L. Reiner, The lineage decisions of helper T cells, Nat. Rev. Immunol. 2 (2002) pp. 933 -944.

[114] S. Nagata, Apoptotic DNA fragmentation, Exp. Cell Res. 256 (2000) pp. 12-18.

[115] M. Nakayama, M. Nagata, H. Yasuda, K. Arisawa, R. Kotani, K. Yamada, S.A. Chowdhury, S. Chakrabarty, Z.Z. Jin, H. Yagita, K. Yokono, M. Kasuga, Fas/Fas ligand interactions play an essential role in the initiation of murine autoimmune diabetes, Diabetes 51 (2002) pp. 1391 -1397.

[116] K. Natarajan, S. Singh, T.R. Burke, Jr., D. Grunberger, B.B. Aggarwal, Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B, Proc. Natl Acad. Sci. U. S. A 93 (1996) pp. 9090 -9095.

[117] L.A. O' Reilly, L. Tai, L. Lee, E.A. Kruse, S. Grabow, W.D. Fairlie, N.M. Haynes, D.M. Tarlinton, J.G. Zhang, G.T. Belz, M.J. Smyth, P. Bouillet, L. Robb, A. Strasser, Membrane-bound Fas ligand only is essential for Fas-induced apoptosis, Nature 461 (2009) pp. 659 -663.

[118] H. Park, Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, C. Dong, A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17, Nat Immunol 6 (2005) pp. 1133 -1141.

[119] Y.C. Park, V. Burkitt, A.R. Villa, L. Tong, H. Wu, Structural basis for self-association and receptor recognition of human TRAF2, Nature 398 (1999) pp. 533 -538.

[120] D.C. Parker, T cell-dependent B cell activation, Annu. Rev. Immunol. 11 (1993) pp. 331 -360.

[121] S. Parlato, A.M. Giammarioli, M. Logozzi, F. Lozupone, P. Matarrese, F. Luciani, M. Falchi, W. Malorni, S. Fais, CD95 (APO -1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: a novel regulatory mechanism of the CD95 apoptotic pathway, EMBO J. 19 (2000) pp. 5123 -5134.

[122] D.M. Patel, S.F. Ahmad, D.G. Weiss, V. Gerke, S.A. Kuznetsov, Annexin A1 is a new functional linker between actin filaments and phagosomes during phagocytosis, J. Cell Sci. 124 (2011) pp. 578 -588.

[123] F. Rebeaud, S. Hailfinger, A. Posevitz-Fejfar, M. Tapernoux, R. Moser, D. Rueda, O. Gaide, M. Guzzardi, E.M. Iancu, N. Rufer, N. Fasel, M. Thome, The proteolytic activity of the paracaspase MALT1 is key in T cell activation, Nat Immunol 9 (2008) pp. 272-281.

[124] H. Renganathan, H. Vaidyanathan, A. Knapinska, J.W. Ramos, Phosphorylation of PEA-15 switches its binding specificity from ERK/MAPK to FADD, Biochem. J. 390 (2005) pp. 729 -735.

[125] M.D. Resh, Palmitoylation of ligands, receptors, and intracellular signaling molecules, Sci. STKE. 2006 (2006) p.re14.

[126] K.L. Rock and A.L. Goldberg, Degradation of cell proteins and the generation of MHC class I-presented peptides, Annu. Rev. Immunol. 17 (1999) pp. 739-779.

[127] I. Rodriguez, K. Matsuura, K. Khatib, J.C. Reed, S. Nagata, P. Vassalli, A bcl-2 transgene expressed in hepatocytes protects mice from fulminant

liver destruction but not from rapid death induced by anti-Fas antibody injection, J. Exp. Med. 83 (1996) pp. 1031 -1036.

[128] S.S. Roy, M. Madesh, E. Davies, B. Antonsson, N. Danial, G. Hajnoczky, Bad targets the permeability transition pore independent of Bax or Bak to switch between Ca2+-dependent cell survival and death, Mol. Cell 33 (2009) pp. 377 -388.

[129] D. Rueda, O. Gaide, L. Ho, E. Lewkowicz, F. Niedergang, S. Hailfinger, F. Rebeaud, M. Guzzardi, B. Conne, M. Thelen, J. Delon, U. Ferch, T.W. Mak, J. Ruland, J. Schwaller, M. Thome, Bcl10 controls TCR- and FcgammaR-induced actin polymerization, J. Immunol 178 (2007) pp. 4373-4384.

[130] S.E. Ryu, A. Truneh, R.W. Sweet, W.A. Hendrickson, Structures of an HIV and MHC binding fragment from human CD4 as refined in two crystal lattices, Structure. 2 (1994) pp. 59 -74.

[131] C. Scaffidi, S. Fulda, A. Srinivasan, C. Friesen, F. Li, K.J. Tomaselli, K.M. Debatin, P.H. Krammer, M.E. Peter, Two CD95 (APO -1//Fas) signaling pathways, EMBO J. 17 (1998) pp. 1675 -1687.

[132] C. Scaffidi, I. Schmitz, P.H. Krammer, M.E. Peter, The role of c-FLIP in modulation of CD95-induced apoptosis, J. Biol. Chem. 274 (1999) pp. 1541-1548.

[133] M. Schirmer, A.N. Vallejo, C.M. Weyand, J.J. Goronzy, Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4+, J. Immunol 161 (1998) pp. 1018 -1025.

[134] T.W. Schulte and L.M. Neckers, The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin, Cancer Chemother. Pharmacol. 42 (1998) pp. 273 -279.

[135] J. Searle, J.F. Kerr, C.J. Bishop, Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance, Pathol. Annu. 17 Pt 2 (1982) pp. 229 -259.

[136] Y.K. Semra, O.A. Seidi, M.K. Sharief, Overexpression of the apoptosis inhibitor FLIP in T cells correlates with disease activity in multiple sclerosis, J. Neuroimmunol. 113 (2001) pp. 268 -274.

[137] M.K. Sharief and Y.K. Semra, Heightened expression of survivin in activated T lymphocytes from patients with multiple sclerosis, J. Neuroimmunol. 119 (2001) pp. 358 -364.

[138] Y. Shi, D. Gerritsma, A.J. Bowes, A. Capretta, G.H. Werstuck, Induction of GRP78 by valproic acid is dependent upon histone deacetylase inhibition, Bioorg. Med. Chem. Lett. 17 (2007) pp. 4491 -4494.

[139] K. Shinohara, M. Tomioka, H. Nakano, S. Tone, H. Ito, S. Kawashima, Apoptosis induction resulting from proteasome inhibition, Biochem. J. 317 ( Pt 2) (1996) pp. 385 -388.

[140] R.M. Siegel, Caspases at the crossroads of immune-cell life and death, Nat. Rev. Immunol. 6 (2006) pp. 308 -317.

[141] K. Simons and D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) pp. 31 -39.

[142] K. Sommer, Phosphorylation of the CARMA1 linker controls NF-[kappa]B activation, Immunity 23 (2005) pp. 561 -574.

[143] H.Y. Song, M. Rothe, D.V. Goeddel, The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappaB activation, Proc. Natl Acad. Sci. U. S. A 93 (1996) pp. 6721 -6725.

[144] J. Sprent and C.D. Surh, T cell memory, Annu. Rev Immunol 20 (2 002) pp. 551-579.

[145] I. Stancovski and D. Baltimore, NF-[kappa]B activation: the I[kappa]B kinase revealed?, Cell 91 (1997) pp. 299 -302.

[146] T.K. Starr, S.C. Jameson, K.A. Hogquist, Positive and negative selection of T cells, Annu. Rev. Immunol. 21 (2003) pp. 139 -176.

[147] A. Strasser, P.J. Jost, S. Nagata, The many roles of FAS receptor signaling in the immune system, Immunity. 30 (2009) pp. 180 -192.

[148] G. Strauss, I. Knape, I. Melzner, K.M. Debatin, Constitutive caspase activation and impaired death-inducing signaling complex formation in CD95-resistant, long-term activated, antigen-specific T cells, J. Immunol. 171 (2003) pp. 1172 -1182.

[149] G. Strauss, J.A. Lindquist, N. Arhel, E. Felder, S. Karl, T.L. Haas, S. Fulda, H. Walczak, F. Kirchhoff, K.M. Debatin, CD95 co-stimulation blocks activation of naive T cells by inhibiting T cell receptor signaling, J. Exp. Med. 206 (2009) pp. 1379 -1393.

[150] C. Stuehler, S. Mielke, M. Chatterjee, J. Duell, S. Lurati, F. Rueckert, H. Einsele, R.C. Bargou, M.S. Topp, Selective depletion of alloreactive T cells by targeted therapy of heat shock protein 90: a novel strategy for control of graft-versus-host disease, Blood 114 (2009) pp. 2829 -2836.

[151] F.C. Sun, S. Wei, C.W. Li, Y.S. Chang, C.C. Chao, Y.K. Lai, Localization of GRP78 to mitochondria under the unfolded protein response, Biochem. J. 396 (2006) pp. 31 -39.

[152] M. Svensson, B. Stockinger, M.J. Wick, Bone marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells, J. Immunol. 158 (1997) pp. 4229 -4236.

[153] C. Tabata, K. Ogita, K. Sato, H. Nakamura, Z. Qing, H. Negoro, K. Kumasawa, K. Temma-Asano, T. Tsutsui, K. Nishimori, T. Kimura,

Calcineurin/NFAT pathway: a novel regulator of parturition, Am. J. Reprod. Immunol. 62 (2009) pp. 44 -50.

[154] K. Takahashi, T. Sasaki, A. Mammoto, K. Takaishi, T. Kameyama, S. Tsukita, Y. Takai, Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein, J. Biol. Chem. 272 (1997) pp. 23371 -23375.

[155] N. Tanimura, S. Saitoh, S. Kawano, A. Kosugi, K. Miyake, Palmitoylation of LAT contributes to its subcellular localization and stability, Biochem. Biophys. Res. Commun. 341 (2006) pp. 1177 -1183.

[156] A. Trauzold, C. Roder, B. Sipos, K. Karsten, A. Arlt, P. Jiang, J.I. Martin-Subero, D. Siegmund, S. Muerkoster, L. Pagerols-Raluy, R. Siebert, H. Wajant, H. Kalthoff, CD95 and TRAF2 promote invasiveness of pancreatic cancer cells, FASEB J. 19 (2 005) pp. 620 -622.

[157] I.S. Trowbridge and M.L. Thomas, CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development, Annu. Rev. Immunol. 12 (1994) pp. 85 -116.

[158] H. Ueno, S. Matsuda, K. Katamura, M. Mayumi, S. Koyasu, ZAP-70 is required for calcium mobilization but is dispensable for mitogen-activated protein kinase (MAPK) superfamily activation induced via CD2 in human T cells, Eur. J. Immunol 30 (2000) pp. 78 -86.

[159] A.G. Uren, Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma, Mol. Cell 6 (2000) pp. 961 -967.

[160] J.E. van Leeuwen and L.E. Samelson, T cell antigen-receptor signal transduction, Curr. Opin. Immunol. 11 (1999) pp. 242 -248.

[161] M. Veldhoen, C. Uyttenhove, S.J. van, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, B. Stockinger, Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an

interleukin 9-producing subset, Nat. Immunol. 9 (2008) pp. 1341 -1346.

[162] I.M. Verma, J.K. Stevenson, E.M. Schwarz, A.D. Van, S. Miyamoto, Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation, Genes Dev. 9 (1995) pp. 2723 -2735.

[163] B.H. von and H.J. Fehling, Structure and function of the pre-T cell receptor, Annu. Rev. Immunol. 15 (1997) pp. 433 -452.

[164] B.P. Wallner, R.J. Mattaliano, C. Hession, R.L. Cate, R. Tizard, L.K. Sinclair, C. Foeller, E.P. Chow, J.L. Browing, K.L. Ramachandran, ., Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity, Nature 320 (1986) pp. 77 -81.

[165] C.Y. Wang, M.W. Mayo, R.G. Korneluk, D.V. Goeddel, A.S. Baldwin, Jr., NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation, Science 281 (1998) pp. 1680-1683.

[166] D. Wang, N. Muller, K.G. McPherson, H.M. Reichardt, Glucocorticoids engage different signal transduction pathways to induce apoptosis in thymocytes and mature T cells, J. Immunol. 176 (2006) pp. 1695 -1702.

[167] H.G. Wang, Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD, Science 284 (1999) pp. 339 -343.

[168] K. Wang, X.M. Yin, D.T. Chao, C.L. Milliman, S.J. Korsmeyer, BID: a novel BH3 domain-only death agonist, Genes Dev. 10 (1996) pp. 2859 -2869.

[169] M.C. Wei, W.X. Zong, E.H. Cheng, T. Lindsten, V. Panoutsakopoulou, A.J. Ross, K.A. Roth, G.R. MacGregor, C.B. Thompson, S.J. Korsmeyer, Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death, Science 292 (2001) pp. 727 -730.

[170] H.L. Weiner, Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells, Immunol. Rev. 182 (2001) pp. 207-214.

[171] A. Wilson and A. Trumpp, Bone-marrow haematopoietic-stem-cell niches, Nat. Rev. Immunol. 6 (2006) pp. 93 -106.

[172] B.S. Wilson, S.L. Steinberg, K. Liederman, J.R. Pfeiffer, Z. Surviladze, J. Zhang, L.E. Samelson, L.H. Yang, P.G. Kotula, J.M. Oliver, Markers for detergent-resistant lipid rafts occupy distinct and dynamic domains in native membranes, Mol. Biol. Cell 15 (2004) pp. 2580 -2592.

[173] T.C. Wirth, H.H. Xue, D. Rai, J.T. Sabel, T. Bair, J.T. Harty, V.P. Badovinac, Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation, Immunity. 33 (2010) pp. 128 -140.

[174] A.H. Wyllie, Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation, Nature 284 (1980) pp. 555 -556.

[175] G.H. Yang, S. Li, J.J. Pestka, Down-regulation of the endoplasmic reticulum chaperone GRP78/BiP by vomitoxin (Deoxynivalenol), Toxicol. Appl. Pharmacol. 162 (2000) pp. 207 -217.

[176] Y.F. Yang, M. Tomura, M. Iwasaki, T. Mukai, P. Gao, S. Ono, J.P. Zou, G.M. Shearer, H. Fujiwara, T. Hamaoka, IL-12 as well as IL-2 upregulates CCR5 expression on T cell receptor-triggered human CD4+ and CD8+ T cells, J. Clin. Immunol. 21 (2001) pp. 1 16-125.

[177] K.Y. Yu, B. Kwon, J. Ni, Y. Zhai, R. Ebner, B.S. Kwon, A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis, J. Biol. Chem. 274 (1999) pp. 13733-13736.

[178] H. Zeng, L. Di, G. Fu, Y. Chen, X. Gao, L. Xu, X. Lin, R. Wen, Phosphorylation of Bcl10 negatively regulates T-cell receptor-mediated NF-kappaB activation, Mol. Cell Biol. 27 (2007) pp. 5235 -5245.

[179] J. Zhang, T. Bardos, K. Mikecz, A. Finnegan, T.T. Glant, Impaired Fas signaling pathway is involved in defective T cell apoptosis in autoimmune murine arthritis, J. Immunol 166 (2001) pp. 4981 -4986.

[180] Z. Zhang, L. Huang, W. Zhao, B. Rigas, Annexin 1 induced by anti-inflammatory drugs binds to NF-kappaB and inhibits its activation: anticancer effects in vitro and in vivo, Cancer Res. 70 (2010) pp. 2379-2388.

# 7. Appendix

## 7.1 Gene chip analysis of Jurkats

**Table 17:** Genes with at least 3 fold expression levels in the CD95-resistant compared to -sensitive Jurkat cell lines

| Fold-change sensitive vs resistant | Fold-change resistant vs sensitive | Gene | |
|---|---|---|---|
| 0,1 | 15,9 | chordin-like 1 | CHRDL1 |
| 0,1 | 8,4 | BMP and activin membrane-bound inhibitor homolog | BAMBI |
| 0,1 | 7,7 | protein kinase, AMP-act., alpha 2 catalytic subunit | PRKAA2 |
| 0,1 | 7,6 | myosin VI | MYO6 |
| 0,1 | 7,0 | glycine amidinotransferase | GATM |
| 0,2 | 5,5 | actin, gamma 2, smooth muscle, enteric | ACTG2 |
| 0,2 | 4,7 | ephrin-B2 | EFNB2 |
| 0,2 | 4,2 | glypican 5 | GPC5 |
| 0,2 | 4,2 | laminin, beta 1 | LAMB1 |
| 0,2 | 4,1 | chromosome 6 open reading frame 105 | C6orf105 |
| 0,2 | 4,1 | ChaC, cation transport regulator homolog 1 (E. coli) | CHAC1 |
| 0,3 | 4,0 | nuclear receptor interacting protein 1 | NRIP1 |
| 0,3 | 3,9 | mannosidase, alpha, class 1A, member 1 | MAN1A1 |
| 0,3 | 3,9 | plastin 3 | PLS3 |
| 0,3 | 3,8 | beta-gamma crystallin domain containing 3 | CRYBG3 |
| 0,3 | 3,7 | family with sequence similarity 65, member B | FAM65B |
| 0,3 | 3,6 | low density lipoprotein receptor-related protein 2 | LRP2 |
| 0,3 | 3,5 | doublecortin-like kinase 2 | DCLK2 |
| 0,3 | 3,4 | trichorhinophalangeal syndrome I | TRPS1 |
| 0,3 | 3,4 | lysophosphatidic acid receptor 6 | LPAR6 |
| 0,3 | 3,3 | thioredoxin interacting protein | TXNIP |
| 0,3 | 3,3 | v-kit | KIT |
| 0,3 | 3,2 | G protein-coupled receptor 146 | GPR146 |
| 0,3 | 3,2 | glypican 4 | GPC4 |
| 0,3 | 3,1 | NK2 homeobox 1 | NKX2-1 |
| 0,3 | 3,0 | fibroblast growth factor 9 (glia-activating factor) | FGF9 |

**Table 18:** Genes with at least 3 fold higher expression levels in the CD95 sensitive Jurkat cell lines

| Fold-change sensitive vs resistant | Fold-change resistant vs sensitive | Gene | |
|---|---|---|---|
| 22,5 | 0,0 | deoxynucleotidyltransferase, terminal | DNTT |
| 8,1 | 0,1 | recombination activating gene 2 | RAG2 |
| 7,0 | 0,1 | selectin L | SELL |
| 7,0 | 0,1 | CD1e molecule | CD1E |
| 6,1 | 0,2 | CD2 molecule | CD2 |
| 5,4 | 0,2 | serpin peptidase inhibitor, clade B (ovalbumin), 8 | SERPINB8 |
| 5,4 | 0,2 | annexin A1 | ANXA1 |
| 4,9 | 0,2 | protein tyrosine phosphatase-like A domain cont. 2 | PTPLAD2 |
| 4,7 | 0,2 | Rho guanine nucleotide exchange factor (GEF) 12 | ARHGEF12 |
| 4,5 | 0,2 | sodium channel, voltage-gated, type III, alpha subunit | SCN3A |
| 4,3 | 0,2 | WW domain binding protein 5 | WBP5 |
| 3,8 | 0,3 | histone cluster 1, H2bh | HIST1H2BH |
| 3,6 | 0,3 | Osteoglycin | OGN |
| 3,4 | 0,3 | Src-like-adaptor 2 | SLA2 |
| 3,4 | 0,3 | FYN binding protein (FYB-120/130) | FYB |
| 3,3 | 0,3 | cholinergic receptor, nicotinic, alpha 3 | CHRNA3 |
| 3,3 | 0,3 | CD1a molecule | CD1A |
| 3,2 | 0,3 | B-cell CLL/lymphoma 11A (zinc finger protein) | BCL11A |
| 3,2 | 0,3 | CD5 molecule | CD5 |
| 3,2 | 0,3 | src kinase associated phospho-protein 1 | SKAP1 |
| 3,1 | 0,3 | histone cluster 1, H3f | HIST1H3F |

## 7.2 Identified protein spots in 2D-DIGE analysis of Jurkats

**Table 19**: list of identified proteins in 2D-DIGE analysis by mass spectrometry of the first run of CD95 sensitive and resistant Jurkats using a pH range of 3-10.

| Fold-change sen vs res | Fold-change res vs sen | Identified protein |
|---|---|---|
| 1,22 | | Cell division protein kinase 6 |
| 1,22 | | heterogeneous nuclear ribonucleoprotein H |
| 1,22 | | hypothetical protein DKFZp762H157.1 |
| 1,22 | | rho GDP-dissociation inhibitor 2 |
| 1,23 | | Eukaryotic initiation factor 4A-III |
| 1,24 | | thioredoxin-like protein |
| 1,25 | | proteasome activator complex subunit 1 isoform 1 |
| 1,25 | | T-complex protein 1 subunit theta |
| 1,26 | | proteasome subunit alpha type-1 isoform 2 |
| 1,28 | | Ezrin |
| 1,29 | | Tumor susceptibility gene 101 protein |
| 1,29 | | vacuolar protein sorting-associated protein 4B |
| 1,31 | | T-complex protein 1 subunit gamma |
| 1,33 | | Bifunctional purine biosynthesis protein PURH |
| 1,33 | | copine-3 |
| 1,33 | | thimet oligopeptidase |
| 1,33 | | Tubulin alpha-1B |
| 1,33 | | Unknown (protein for IMAGE:3543711) |
| 1,37 | | Proteasome subunit alpha type-6 |
| 1,38 | | T-complex protein 1 subunit beta |
| 1,38 | | T-complex protein 1 subunit zeta isoform a |
| 1,38 | | transformation upregulated nuclear protein |

**Table 20:** Follow-up of Table 19

| Fold-change sen vs res | Fold-change res vs sen | Identified protein |
|---|---|---|
| 1,39 | | T-complex protein 1 subunit gamma |
| 1,39 | | Tubulin alpha-1B |
| 1,39 | | tubulin beta-2C chain |
| 1,4 | | pre-mRNA-processing factor 19 [Homo sapiens] |
| 1,41 | | Coronin-1A |
| 1,42 | | Moesin |
| 1,43 | | moesin, isoform CRA_b |
| 1,46 | | Proteasome activator complex subunit 2 |
| 1,47 | | Coronin-1A |
| 1,49 | | Proliferation-associated protein 2 |
| 1,51 | | proteasome subunit alpha type-1 isoform 2 |
| 1,51 | | Tubulin beta-2C chain |
| 1,53 | | T-complex protein 1 subunit alpha |
| 1,56 | | Moesin |
| 1,69 | | Annexin A1 |
| 1,7 | | ALG-2 interacting protein 1 [Homo sapiens] |
| 2,2 | | Seryl-tRNA synthetase, cytoplasmic |
| | 1,35 | cytokeratin 9 [Homo sapiens] |
| | 1,99 | DNA replication licensing factor MCM7 |
| | 1,7 | GRP78 precursor |
| | 1,33 | Heat shock protein 105 kDa OS=Homo sapiens |
| | 1,24 | Heat shock protein HSP 90-alpha |

**Table 21:** List of identified proteins in 2D-DIGE analysis by mass spectrometry of the first run of CD95 sensitive and resistant Jurkats using a pH range of 4-7.

| Fold-change sen vs res | Fold-change res vs sen | Identified protein |
|---|---|---|
| 1,24 | | L-plastin variant |
| | 2,09 | 100 kDa coactivator [Homo sapiens] |
| | 1,48 | 3-phosphoglycerate dehydrogenase [Homo sapiens] |
| | 2,29 | 78 kDa glucose-regulated protein |
| | 1,47 | actin-related protein 2 isoform b [Homo sapiens] |
| | 2,1 | alpha-enolase [Homo sapiens] |
| | 1,63 | alpha-enolase [Homo sapiens] |
| | 1,83 | aminopeptidase B [Homo sapiens] |
| | 1,76 | Annexin A6 OS=Homo sapiens GN=ANXA6 PE=1 SV=3 |
| | 1,79 | cytokeratin 9 [Homo sapiens] |
| | 1,87 | deaminase a,adenosine |
| | 1,84 | dihydrolipoamide dehydrogenase precursor [Homo sapiens] |
| | 1,82 | Elongation factor 2 OS=Homo sapiens GN=EEF2 PE=1 SV=4 |
| | 1,58 | Eukaryotic initiation factor 4A-II |
| | 2,56 | fructose bisphosphate aldolase [Homo sapiens] |
| | 2,56 | fructose bisphosphate aldolase [Homo sapiens] |
| | 1,21 | galactokinase [Homo sapiens] |
| | 1,89 | GMP synthase [glutamine-hydrolyzing] [Homo sapiens] |
| | 1,21 | Heat shock cognate 71 kDa protein |
| | 1,31 | heat shock protein 70 |
| | 1,62 | heat shock protein 70 |
| | 1,54 | Heterogeneous nuclear ribonucleoprotein L |
| | 1,94 | inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205) |
| | 1,78 | inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205) |
| | 2,65 | Keratin, type II cytoskeletal 1 |

**Table 22:** Follow-up of Table 21

| Fold-change sen vs res | Fold-change res vs sen | Identified protein |
|---|---|---|
| | 1,31 | Keratin, type II cytoskeletal 1 |
| | 1,92 | Keratin, type II cytoskeletal 1 |
| | 1,48 | KIAA0070 [Homo sapiens] |
| | 1,74 | KIAA0088 [Homo sapiens] |
| | 1,64 | KIAA0128 [Homo sapiens] |
| | 2,05 | macrophage-capping protein |
| | 1,88 | mitochondrial aconitase |
| | 2,06 | mitochondrial aconitase |
| | 1,68 | p37 AUF1 [Homo sapiens] |
| | 2,3 | phospholipase C-alpha |
| | 2,51 | prolyl 4-hydroxylase alpha subunit (EC 1.14.11.2) |
| | 2,06 | Protein disulfide-isomerase |
| | 1,54 | protein phosphatase 5 |
| | 1,61 | Rab GDP dissociation inhibitor alpha |
| | 1,85 | S-adenosylhomocysteine hydrolase |
| | 1,76 | Septin-9 OS=Homo sapiens |
| | 1,64 | Serine/threonine-protein kinase PAK 2 |
| | 1,71 | Serine/threonine-protein kinase PAK 2 |
| | 2,16 | THO complex subunit 3 |
| | 2,37 | Transketolase |
| | 1,78 | Transketolase |
| | 1,37 | Tubulin alpha-1B chain |
| | 1,53 | ubiquitin activating enzyme E1 |
| | 1,55 | X-ray repair cross-complementing protein |
| | 1,52 | X-ray repair cross-complementing protein 6 |

## 7.3 Identified protein spots in 2D-DIGE analysis of short- and long-term activated T cells

**Table 23:** List of picked proteins from 2D-DIGE of CD95 sensitive and resitant CTLs

| Run | Fold-change sen vs res | Fold-change res vs sen | Identified protein |
|---|---|---|---|
| 1 | | 1,2 | Keratin, type I cytoskeletal 10 |
| | | 1,25 | Heat shock cognate 71 kDa protein |
| | | 1,28 | leucine rich repeat containing 50 |
| 2 | | 1,28 | alpha-enolase |
| | | 1,3 | Keratin, type II cytoskeletal 1 |
| | | 1,3 | alpha enolase |
| | | 1,3 | Keratin, type I cytoskeletal 10 |
| | | 1,34 | alpha enolase |
| | | 1,4 | Annexin A6 |
| | | 1,5 | Keratin, type I cytoskeletal 10 |
| | | 1,54 | nuclear chloride channel |
| | | 1,55 | Rab GDP dissociation inhibitor beta |
| | | 1,56 | pyruvate kinase [Homo sapiens] |
| | | 1,57 | Elongation factor 1-alpha 1 |
| | | 1,59 | Putative beta-actin-like protein 3 |
| | | 1,6 | Protein disulfide-isomerase |
| | | 1,6 | phosphoglycerate kinase 1 |
| | | 1,7 | malate dehydrogenase precursor |
| | | 1,75 | Cap-G |
| | | 1,78 | Malate dehydrogenase |
| | | 1,79 | 78 kDa glucose-regulated protein |
| | | 1,8 | Tubulin alpha-1B chain |
| | | 1,8 | malate dehydrogenase precursor |
| | 1,5 | | Moesin |
| | 1,71 | | annexin A1 |
| | 1,63 | | annexin A1 |
| | 1,65 | | Proteasome activator complex subunit 2 |

**Table 24:** Follow-up of Table 23

| Run | Fold-change sen vs res | Fold-change res vs sen | Identified protein |
|---|---|---|---|
| | 1,6 | | L-plastin variant |
| | 1,2 | | tyrosyl-tRNA synthetase |
| | 1,22 | | L-plastin variant |
| | 1,3 | | L-plastin variant |
| | 1,3 | | L-plastin variant |
| | 1,36 | | 60 kDa HSP mitochondria |
| | 1,44 | | Tubulin alpha 1B |
| | 1,57 | | L-plastin variant |
| | 3,09 | | Keratin, type I cytoskeletal 10 |
| | | 1,33 | thyroid hormone binding protein precursor |
| | | 1,33 | Apical endosomal glycoprotein |
| | | 1,9 | heat shock cognate 71 kDa protein isoform 1 |
| | | 1,24 | Annexin A6 |
| | | 1,24 | Heat shock 70 kDa protein 1A/1B |
| | | 2,21 | CD276 antigen |
| | | 1,38 | Tyrosine-protein kinase ZAP-70 |
| | | 1,23 | alpha-enolase |
| | | 1,5 | Glyceraldehyd-3-Phosphatase |
| | | 1,2 | Rho GTPase Activating Protein 10 |
| | | 2,91 | ATP-synthase subunit beta |
| | | 1,25 | Heat shock protein HSP 90-beta |
| | | 1,67 | Eucaryotic Initiation Factor 4AII |
| | | 1,49 | Rho GDP-dissociation inhibitor 2 |
| 3 | | 1,7 | heat shock cognate 71 kDa protein isoform 1 |
| 4 | 1,43 | | Sialic acid-binding like lectin 10 |

# Statutory Declaration

I hereby declare that I wrote the present dissertation with the topic:

**CD95 resistance in long-term activated cytotoxic T lymphocytes**

independently and used no other aids that those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current „Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis“ [Rules of the University of Ulm for Assuring Good Scientific Practice].

Ulm, 2011

# CURRICULUM VITAE

**"Aus Gründen des Datenschutzes wurde in der Online-Version der Lebenslauf entfernt."**