

Regulation of T cell apoptosis and T cell activation  
through costimulatory signals

Dissertation zur Erlangung des akademischen Grades

-Dr. rer. nat.-

vorgelegt von

Dipl. Biol. Lilian Stärck  
aus Harmanli (Bulgarien)

eingereicht beim

Fachbereich Biologie, Pharmazie und Chemie

Freie Universität zu Berlin

2005

1. Gutachter: Prof. Dr. Thomas Blankenstein
2. Gutachter: Prof. Dr. Peter T. Daniel

Tag der Disputation: 30. September 2005

## Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel angefertigt habe.

Die praktischen Arbeiten wurden in der Abteilung für Klinische und Molekulare Onkologie (Leiter Prof. Dr. P. Daniel) an der Robert Rössle-Klinik (Klinik mit Schwerpunkt Hämatologie, Onkologie und Tumorimmunologie, Charité Campus Berlin-Buch; Ärztl. Direktor: Prof. Dr. B. Dörken) durchgeführt.

Berlin, den 19. April 2005

Lilian Stärck

## Acknowledgements

Ich möchte Herrn Prof. Dr. Bernd Dörken danken für die Möglichkeit an der Robert-Rössle-Klinik unter optimalen Bedingungen promovieren zu können.

Herrn Prof. Dr. Thomas Blankenstein danke ich für den wissenschaftlichen Austausch, die Begutachtung meiner Arbeit und deren Vertretung vor dem Fachbereich Biologie, Chemie und Pharmazie der Freien Universität Berlin.

Mein Dank gilt Herrn Prof. Dr. Peter T. Daniel für die Bereitstellung des Themas, die Begutachtung und Betreuung meiner Arbeit, sowie die kontinuierliche Unterstützung und die ergebnisreichen Diskussionen.

Dem Graduiertenkolleg 276/2 der DFG mit Herrn Prof. Dr. Bertram Wiedenmann als Sprecher danke ich für den fachlichen Austausch und die finanzielle Förderung.

Herrn Dr. Christian Scholz danke ich die gute Zusammenarbeit.

Herrn Dr. Gerald Willimsky danke ich für den fachlichen Austausch.

Ein dickes Dankeschön an Antje Richter für ihre Hilfe bei zahlreichen Experimenten.

Den Herren Dr. Frank Essmann, Dr. Bernhard F. Gillissen und Dr. Oliver Ebenhoeh danke ich für das Korrekturlesen der Arbeit.

Ich danke allen übrigen Arbeitskollegen für ihre Hilfe und die gute Zeit die wir im Labor hatten.

Meiner Frau danke ich für ihre Liebe und Geduld.

Danke!

Ich widme diese Arbeit meinen Eltern, die mich immer unterstützten  
und mir dieses Studium ermöglichten

---

# Contents

INDEX OF FIGURES .....	9
INDEX OF ABBREVIATIONS .....	10
1. Introduction .....	13
1.1. Costimulatory signals regulate T cell proliferation and apoptosis.....	13
1.1.1. Costimulatory members of the CD28 receptor family .....	14
1.1.1.1. CD28.....	15
1.1.1.2. CTLA-4 (CD152) .....	15
1.1.1.3. ICOS .....	16
1.1.2. Costimulatory members of the tumour necrosis factor receptor family .....	17
1.1.2.1. 4-1BB (CD137).....	18
1.1.2.2. OX40 (CD134) .....	19
1.1.3. Apoptosis .....	20
1.1.3.1. Caspases .....	21
1.1.3.2. Extrinsic death pathway .....	22
1.1.3.3. Intrinsic death pathway .....	23
1.1.4. Activation-induced cell death .....	25
1.2. Tumour immunology .....	25
1.2.1. Cancer vaccines .....	27
2. Material.....	30
2.1. Chemicals and reagents .....	30
2.2. Antibodies .....	30
2.3. Primers .....	31
2.4. Inhibitors.....	31
2.5. Suppliers.....	31

---

3.	Methods .....	33
3.1.	Centrifugation .....	33
3.2.	Cell culture .....	33
3.3.	Generation of transfectants .....	33
3.4.	T cell culture and activation .....	34
	3.4.1. Ficoll separation .....	34
	3.4.2. T cell enrichment .....	34
	3.4.3. T cell stimulation .....	35
3.5.	Quantification of apoptotic and necrotic cell death .....	35
	3.5.1. Single cell measurement of genomic DNA fragmentation .....	35
	3.5.2. Annexin-V-FITC .....	36
3.6.	Measurement of T cell proliferation .....	36
3.7.	Measurement of T cell cytotoxicity .....	36
3.8.	Immunoblotting .....	37
	3.8.1. Sample preparation .....	37
	3.8.2. Determination of protein concentration .....	37
	3.8.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) .....	38
	3.8.4. Electroblotting .....	38
	3.8.5. Immunodetection of proteins .....	39
3.9.	Immunocytometry .....	39
3.10.	Immunocytochemistry .....	40
4.	Results .....	41
4.1.	Purification of primary human T cells .....	41
4.2.	Culture and stimulation of primary human T cells .....	42
4.3.	4-1BB ligand costimulates proliferation of activated T cells and inhibits AICD .....	43
4.4.	Bcl-x <sub>L</sub> and c-FLIP <sub>5</sub> are up-regulated upon costimulation through 4-1BB .....	45
4.5.	Inhibition of PI3 kinase interferes with 4-1BB-mediated inhibition of AICD and hampers Bcl-x <sub>L</sub> and c-FLIP <sub>5</sub> up-regulation .....	46
4.6.	Inhibition of AKT phosphorylation decreases 4-1BB-mediated proliferation and inhibition of apoptosis as well as Bcl-x <sub>L</sub> and c-FLIP <sub>5</sub> up-regulation .....	51

---

4.7.	Tumour cell irradiation does not influence transgene expression.....	54
4.8.	Irradiation does not affect target cell lysis .....	55
4.9.	Induction of T cell proliferation is not affected by tumour cell irradiation ..	56
4.10.	Inhibition of activation-induced cell death is not affected by irradiation .....	57
4.11.	Necrotic cell death abolishes the capacity of CD80 expressing tumour cells to costimulate T cell proliferation .....	58
4.12.	Irradiation of tumour cells induces necrosis depending on the cell line assessed.....	61
5.	Discussion .....	63
5.1.	Costimulatory signals regulate T cell proliferation and apoptosis.....	63
5.1.1.	Costimulatory signalling through 4-1BB.....	63
5.1.2.	Costimulatory signalling through CD28.....	66
5.1.3.	Costimulatory signalling and AICD inhibition .....	67
5.2.	Costimulatory signalling in tumour immunology .....	70
6.	References .....	74
7.	Appendix.....	94
7.1.	Curriculum Vitae.....	94
7.2.	Publications.....	95
8.	Zusammenfassung.....	96
9.	Summary .....	98



## Index of figures

1. T cell activation .....	14
2. Structural similarities between caspase 8 and FLIP .....	22
3. Two pathways of apoptosis .....	24
4. Purification of T cells.....	41
5. Activated T cells in prolonged culture.....	42
6. Expression of costimulatory molecules on stably transfected tumour cells.....	43
7. Costimulation induces T cell proliferation and inhibition of AICD.....	44
8. Costimulation mediates up-regulation of Bcl-x <sub>L</sub> and FLIP <sub>S</sub> .....	45
9. Kinetics of Bcl-x <sub>L</sub> and FLIP <sub>S</sub> up-regulation.....	46
10. T cell proliferation and AICD inhibition are PI3 kinase-dependent.....	48
11. AKT phosphorylation upon T cell restimulation is PI3 kinase-dependent .....	49
12. Costimulation mediated up-regulation of FLIP <sub>S</sub> and Bcl-x <sub>L</sub> is PI3 kinase-dependent.....	50
13. T lymphocyte proliferation and AICD inhibition are AKT-dependent .....	51
14. Costimulation mediated up-regulation of FLIP <sub>S</sub> and Bcl-x <sub>L</sub> is AKT-dependent .....	52
15. CD80 expression on stably transfected TE671 cells following irradiation. ....	54
16. T cell cytotoxicity .....	55
17. Induction of T cell proliferation.....	56
18. Inhibition of activation-induced cell death.....	57
19. Costimulatory capacity of TE671 cells inactivated by irradiation or freeze and thaw treatment. ....	59
20. Morphology and CD80 expression in necrotic TE671 cells .....	60
21. Irradiation-mediated apoptosis and necrosis of tumour cell lines.....	61

---

## Index of abbreviations

A	ampère(s)
aa	amino acid(s)
AICD	activation-induced cell death
AIDS	acquired immune deficiency syndrome
AIF	apoptosis inducing factor
Apaf	apoptosis associated factor
APC	antigen presenting cell
APS	ammoniumperoxodisulfate
ASK-1	apoptosis signal-regulating kinase 1
CAD	caspase activated DNase
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CARD	caspase recruitment domain
caspase	cysteinyl aspartate-specific protease
CD	cluster of differentiation
c-FLIP	cellular FLICE inhibitory protein
CHAPS	3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate
CHUK	conserved helix-loop-helix ubiquitous kinase
Ci	curie
cpm	counts per minute
CTLA-4	cytolytic T lymphocyte associated antigen-4
Da	Dalton
dATP	desoxyadenosin triphosphate
DC	dendritic cell
DD	death domain
DED	death effector domain
DISC	death inducing signaling complex
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DR	death receptor

---

DTT	dithiothreitol
ECL	enhanced chemoluminescence
EDTA	ethylenediamine tetraacetic acid
ERK	extracellular regulated kinase
FACS	fluorescence activated cell sorting
FADD	fas associated protein with death domain
FasR	fas-receptor
FITC	fluorescein-5-isothiocyanate
FLICE	fas-associated death-domain-like IL-1 $\beta$ -converting enzyme
FLIP	FLICE inhibitory protein
FOXO3a	forkhead box O 3a
g	gram
g	gravitational constant (9.81 ms <sup>-2</sup> )
h	hour
HRP	horseradish peroxidase
HTLV-1	human T lymphotropic virus-1
ICOS	inducible co-stimulator
Ig	immunoglobulin
IL	interleukin
JNK	c-Jun N-terminal protein kinase
kb	kilobase
KS	Kaposi's sarcoma
LCMV	lymphocytic choriomeningitis virus
M	molar
mAb	monoclonal antibody
MAP kinase	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MEK	MAP kinase kinase
MHC	major histocompatibility complex
NF- $\kappa$ B	nuclear factor kappa B
NGF	nerve growth factor

---

NIK	NF- $\kappa$ B inducing kinase
NK cell	natural killer cell
pAb	polyclonal antibody
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDTC	1-pyrrolidinecarbodithioic acid
PE	phycoerythrin
PGE-2	prostaglandin E-2
PI3 kinase	phosphatidylinositol 3 kinase
PIP <sub>3</sub>	phosphatidylinositol-3,4,5-triphosphate
PKC	protein kinase c
PND	paraneoplastic neurologic degeneration
PTLD	post-transplant lymphoproliferative disorders
rpm	rotations per minute
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate
SLP	SH2-domain leukocyte protein
STAT	signal transducers and activators of transcription
TAM	tumour associated macrophage
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis inducing ligand
V	volt
VDAC	voltage-dependent anion channel
VEGF	vascular endothelial growth factor
v-FLIP	viral FLICE inhibitory protein