

Chemotactic signals released during Burkitt's lymphoma cell death

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Abstract

Tumour-associated macrophages (TAMs) have been shown to play an important role in tumour survival and progression. Thus, high numbers of macrophages in the tumour tissue are often associated with a poor prognosis. Identification of factors responsible for recruiting macrophages to the sites of different types of tumours might help to develop more effective cancer treatment.

Burkitt's lymphoma (BL) is characterised by uncontrolled cell proliferation, high rate of spontaneous apoptosis and significant macrophage infiltration. Although BL cells undergo extensive apoptosis, *in situ* their corpses are cleared very effectively by macrophages infiltrating the tumour. It is now widely believed that dying cells are themselves able to release chemotactic molecules to ensure macrophage chemotaxis and subsequent clearance of their site of death. Previous work carried out in this laboratory identified fractalkine/CX3CL1 (FKN) released from dying BL cells to be an important player in macrophage chemotaxis to BL. Yet, these results have also indicated that FKN may not be the only chemokine involved in this process.

Following from those observations, the first part of this work focused on examination of the potential role of monocyte chemoattractant protein-1 (MCP-1) in macrophage recruitment to BL. Despite the initial promising results, careful analysis of the data obtained by various techniques led to the conclusion that MCP-1 is, probably, not expressed by BL cells.

Subsequently, effort was concentrated on understanding mechanisms regulating FKN processing during cell death. The studies performed before in this laboratory identified a new form of FKN to be present in apoptotic BL cells and showed that this is the form that is, most likely, responsible for mediating macrophage migration. Here, this apoptosis-related 60 kDa FKN was found to be a likely caspase-3 cleavage product. Moreover, it was demonstrated that FKN and active caspase-3 are released together in apoptotic BL cell-derived microparticles, suggesting that the proteolytic events could take place also extracellularly.

In the final results chapter the differences between BL cell lines in the way they process FKN during cell death were revealed and a new cell death-associated 55 kDa FKN was observed. Through several lines of evidence, this new form was identified to be a possible product of calpain-mediated proteolysis.

To conclude, this work provides the first evidence for a possible direct participation of the two major cell death executioner proteases – caspases and calpains, in production of ‘find me’ signals for macrophages and thus, ensuring effective clearance of dying cells. These results indicate that FKN cleavage and release might be of key importance during cell death. Moreover, the studies presented here contribute to better understanding of the process of FKN secretion.

Declaration

This thesis has been composed by myself and it has not been submitted in any previous application for a degree. The work reported within was executed by myself, unless otherwise stated.

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Table of contents

Abstract	1
Declaration	3
Acknowledgements	4
Table of contents	5
List of figures	8
List of tables	9
List of abbreviations.....	10
Chapter 1 Introduction	16
1.1 Burkitt's lymphoma	16
1.1.1 General characteristics	16
1.1.2 Cellular origin	17
1.1.3 Role of c-Myc in BL	18
1.1.4 Role of Epstein-Barr virus in BL	19
1.2 Cell death	21
1.2.1 Apoptosis	21
1.2.2 Necrosis.....	23
1.2.3 Proteases in cell death	25
1.2.3.1 Caspases	25
1.2.3.2 Calpains.....	27
1.2.3.3 Cathepsins	30
1.2.4 Clearance of dying and dead cells.....	31
1.2.4.1 Clearance of apoptotic cells	31
Signals mediating apoptotic cell recognition and engulfment	31
"Find me" signals.....	33
1.2.4.2 Clearance of necrotic cells	34
1.2.4.3 Immunological consequences of cell death.....	35
1.2.5 Tumour-associated macrophages (TAMs).....	37
1.3 Chemokines.....	39
1.3.1 MCP-1	40
1.3.2 Fractalkine (CX3CL1)	42
1.3.2.1 Structure and functions of fractalkine	42
Mechanism of fractalkine-mediated cell adhesion.....	43
Generation and functions of soluble fractalkine	45
1.3.2.2 The role of fractalkine in the central nervous system	46
1.3.2.3 The role of fractalkine in disease	47
1.4 Aims of the project.....	48
Chapter 2 Materials and Methods	49
2.1 Cells	49
2.1.1 Primary cells isolation and culture	49
2.1.1.1 Monocytes and HMDMs.....	49
2.1.1.2 Mouse germinal centre cells.....	50
2.1.1.3 Human germinal centre cells.....	50

2.1.2 Cell lines maintenance	51
2.2 Induction and evaluation of apoptosis.....	51
2.2.1 Inhibitor studies.....	52
2.3 Microparticles isolation and analysis	53
2.4 Immunostaining	54
2.5 Immunohistochemistry.....	54
2.6 ELISA	58
2.6.1 Sandwich ELISA.....	58
2.6.2 Direct ELISA	58
2.7 Transmigration assay	59
2.8 Immunoblotting.....	60
2.8.1 Whole cell lysate preparation.....	60
2.8.2 Supernatants preparation.....	61
2.8.3 Electrophoresis and Western blotting	61
2.9 <i>In vitro</i> cleavage assays.....	62
2.9.1 Caspase cleavage.....	62
2.9.2 Calpain cleavage	62
2.10 Caspase-3 activity fluorogenic assay	63
2.11 Confocal microscopy	63
2.12 Time lapse video microscopy.....	64
2.13 Molecular biology techniques	64
2.13.1 Reverse transcription PCR.....	64
2.13.2 Caspase-3 knock-down	66
2.13.3 Site-directed mutagenesis (SDM)	67
2.14 List of chemicals, reagents and equipment	68
Chapter 3 Role of MCP-1 in attracting mononuclear phagocytes to Burkitt's lymphoma.....	74
3.1 Introduction	74
3.2 MCP-1 expression in BL.....	76
3.3 MCP-1 release form apoptotic BL2 cells.....	77
3.3.1 Induction and measurement of BL2 cells apoptosis	78
3.3.2 Measurement of MCP-1 release from apoptotic BL2 cells by ELISA	79
3.4 Role of MCP-1 in mononuclear phagocyte migration to BL.....	80
3.4.1 Human monocyte migration to recombinant human MCP-1	81
3.4.2 Human monocyte-derived macrophage (HMDM) migration to supernatants from BL2 cells.....	81
3.5 MCP-1 immunodetection in the supernatants from apoptotic BL cells.....	83
3.6 Analysis of BL cells expression of MCP-1 mRNA	85
3.7 Analysis of HL-60 cell-free supernatants by MCP-1 ELISA	87
3.8 Conclusions and Discussion.....	88
Chapter 4 FKN processing in apoptotic BL cells.....	93
4.1 Introduction	93
4.2 Immunodetection of FKN in BL cells.....	94
4.3 The role of apoptosis programme in FKN modulation in BL cells	97
4.4 Loss of FKN from the surface of apoptotic BL cells.....	98
4.5 Searching for a protease that cleaves FKN during apoptosis of BL cells.....	102
4.5.1 The role of metalloproteinases	102
4.5.2 Effect of broad-spectrum inhibitors	103

4.5.3 Role of caspases	103
4.5.3.1 Specific caspase inhibitors	103
4.5.3.2 Cleavage studies	108
4.5.3.3 Down-regulation of caspase-3 in BL2 cells	110
4.5.3.4 Introducing point mutation into the FKN gene sequence	113
4.6 Role of apoptosis-derived microparticles (MPs) in the release of FKN from BL cells	114
4.6.1 FKN and caspase-3 co-release in BL2-derived MPs	114
4.6.2 Characterisation of BL cell-derived MPs.....	117
4.6.2.1 Time lapse imaging of MP release from apoptotic BL cells.....	117
4.6.2.2 Morphological characteristics of vesicles produced during BL cell death	117
4.7 FKN localisation in apoptotic BL cells.....	121
4.8 Release of FKN by germinal centre B cells	124
4.9 Conclusions	126
Chapter 5 Role of calpain in FKN cleavage in dying Burkitt's lymphoma cells.....	127
5.1 Introduction	127
5.2 Expression of FKN in group I BL lines	127
5.3 Involvement of calpain in cleavage of FKN	130
5.4 Ionomycin-induced death of BL2 cells	134
5.5 Ionomycin-induced death of Mutu I cells	136
5.6 Effect of calpeptin and Z-VAD-FMK on the FKN processing in ionomycin-treated BL cells	136
5.7 Calpain expression in BL cells.....	139
5.8 Calpain activation in BL cells	143
5.9 Monitoring FKN release from late apoptotic BL cells.....	146
5.10 Searching for FKN C-terminal cleavage products	147
5.11 Conclusions	148
Chapter 6 Discussion of the results	150
6.1 Introduction	150
6.2 FKN processing and role of differently cleaved fragments	151
6.2.1 Origin and function of 90 kDa form of FKN	154
6.2.2 Origin of 25 (and 70) kDa FKN.....	155
6.2.3 Role of caspases in FKN proteolysis	156
6.2.4 Role of calpain in FKN proteolysis.....	161
6.2.4.1 Regulation of calpain activity in BL cells.....	163
6.2.5 Possible cooperation of caspases and calpain in FKN cleavage.....	166
6.3 Mechanism of FKN release.....	168
6.4 Role of FKN in cell death	170
6.5 Role of FKN in BL.....	172
Appendix A Introducing point mutation into the FKN gene sequence.....	175
Appendix B Time lapse imaging of MP release from apoptotic BL cells	183
Appendix C Publication arising	184
CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis	184
Abstract	184
References	185

List of figures

Figure 1.1 Schematic picture of fractalkine	43
Figure 2.1 Schematic diagram of transmigration assay	60
Figure 3.1 Expression of MCP-1 in BL cells.....	77
Figure 3.2 Expression of MCP-1 in tumour tissue in situ.....	78
Figure 3.3 Staurosporine is a potent inducer of apoptosis.	79
Figure 3.4 Release of MCP-1 from apoptotic BL cells.....	81
Figure 3.5 Migration of mononuclear phagocytes to recombinant and BL cell-derived MCP-1	82
Figure 3.6 MCP-1 detection by Western blotting in BL and HL-60 cells.	84
Figure 3.7 Analysis of MCP-1 RNA transcripts present in HL-60 and BL cells.....	86
Figure 3.8 Release of MCP-1 from HL-60 cells.....	88
Figure 4.1 FKN detection in BL2 cells by Western blotting.	95
Figure 4.2 Role of apoptosis in FKN processing in BL2 cells.....	99
Figure 4.3 Effect of experimental conditions on FKN detection in BL cell samples	100
Figure 4.4 Loss of surface FKN during apoptosis of BL2 cells.....	101
Figure 4.5 Effect of selected inhibitors with various specificity for MMP and ADAM protease families on FKN processing in apoptotic BL2 cells.....	104
Figure 4.6 Effect of broad spectrum inhibitors on FKN processing in apoptotic BL2 cells.	105
Figure 4.7 Effect of peptide caspase inhibitors on processing of FKN in apoptotic BL2 cells.	107
Figure 4.8 Effect of peptide caspase inhibitors on viability of BL2 cells.....	108
Figure 4.9 Recombinant human caspase-3 cleaves FKN to generate 60 kDa form.	109
Figure 4.10 Examining capability of caspase-7 and caspase-10 to cleave FKN	110
Figure 4.11 Down-regulation of caspase-3 in BL2 cells.....	112
Figure 4.12 Presence of FKN and active caspase-3 in MPs from BL2 cells	116
Figure 4.13 Time lapse imaging of MP release from apoptotic BL cells	119
Figure 4.14 Flow cytometric analysis of vesicles derived from apoptotic BL cells.....	120
Figure 4.15 FKN and active caspase-3 localisation in apoptotic BL cells.....	122
Figure 4.16 FKN staining of non-permeabilised BL cells	123
Figure 4.17 FKN release from GC B cells.....	125
Figure 5.1 Expression of FKN in group I BL lines.....	129
Figure 5.2 Effect of different apoptosis induction methods on the FKN processing in Mutu I cells	130
Figure 5.3 Involvement of calpain in FKN cleavage	131
Figure 5.4 Effect of protease inhibitors on FKN cleavage in BL2 cell lysates.....	133
Figure 5.5 Ionomycin-induced death of BL2 cells.....	135
Figure 5.6 Ionomycin-induced death of Mutu I cells.....	137
Figure 5.7 Effect of calpeptin and Z-VAD-FMK on the FKN processing in ionomycin-treated BL cells	139
Figure 5.8 Calpain I expression in BL cells	141

Figure 5.9 Calpain II expression in BL cells.....	142
Figure 5.10 Calpain I activation in BL cells	145
Figure 5.11 Monitoring FKN release from late apoptotic BL2 cells	147
Figure 5.12 Searching for FKN C-terminal cleavage products.....	148
Figure A.1. The positions of Asp (orange) residues in the human FKN mucin stalk amino acid sequence.	176
Figure A.2. The location of aspartic acid codons (bold) and nucleotides targeted for mutation (red) in the human FKN nucleotide sequence.....	177
Figure A.3. Schematic picture of TrueORF pCMV6-Entry vector.....	178
Figure A.4. Introduction of the point mutations in the FKN sequence.....	179

List of tables

Table 2-1 List of inhibitors and used concentrations.....	52
Table 2-2 List of antibodies and used concentrations.....	56

List of abbreviations

ADAM	a disintegrin and metalloproteinase
Ala	alanine
AP24	24 kDa apoptotic protease
Apaf-1	apoptotic protease activating factor-1
Asp	aspartic acid
ATP	adenosine triphosphate
AxV	annexin V
BART	BamHI A rightward transcript
Bad	Bcl-2-associated agonist of death
BAFF	B cell-activating factor of the TNF family
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2-associated X protein
Bcl	B cell lymphoma/leukaemia gene
BH3-only	Bcl-2-homology domain 3 only
Bid	BH3-nteracting domain death agonist
BL	Burkitt's lymphoma
BSA	bovine serum albumin
C (1q; 5a)	complement component (1q; 5a)
CaM	calmodulin
CaMBP	calmodulin-binding protein
CaMBS	calmodulin-binding site
CANP	calpain
CASP	caspase
CARD	caspase recruitment domain
CD	cluster of differentiation
CHAPS	[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CrmA	cytokine response modifier A
CSF-1	colony stimulating factor-1
CTL	cytotoxic T lymphocyte

DAB	3,3'-diaminobenzidine
DAMP	danger-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DED	death effector domain
DEPC	diethylpyrocarbonate
DISC	death-inducing signalling complex
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-64	epoxysuccinyl-leucylamido-(4-guanidino) butane
EBV	Epstein-Barr virus
EBER	EBV-encoded RNA
EBNA-1	Epstein-Barr virus nuclear antigen-1
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epithelial growth factor
ELISA	enzyme-linked immunosorbent assay
EMAP II	endothelial monocyte-activating polypeptide II
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	foetal bovine serum
FCS	foetal calf serum
FKN	fractalkine
FITC	fluorescein isothiocyanate
FLIP	FLICE inhibitory protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gas6	growth arrest-specific 6
GC	germinal centre
Glu	glutamic acid
GPCR	G protein-coupled receptor

HBSS	Hank's buffered salt solution
HEPES	N-2-hydroxyethylpiperazine- N-2-ethanesulfonic acid
HIF-1 α	hypoxia-inducible factor-1 α
His	histidine
HIV	human immunodeficiency virus
HMDM	human monocyte-derived macrophage
HMGB1	high-mobility group box 1 protein
HO-1	heme oxygenase-1
HRK	activator of apoptosis harakiri
HRP	horse radish-peroxidase
IAP	inhibitor of apoptosis
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
I κ B α	inhibitor of NF- κ B type α
IL	interleukin
Ile	isoleucine
IMDM	Iscove's Modified Dulbecco's Medium
Iono	ionomycin
iPLA ₂ β	calcium-independent phospholipase
kDa	kilodalton
LB	Lysogeny broth
LDS	lithium dodecyl sulphate
Leu	leucine
LMP	lysosomal membrane permeabilisation
LOX-1	lectin-like oxidized LDL receptor 1
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
MACS	magnetic cell sorting
MALT	mucosa-associated lymphoid tissue
Mcl-1	myeloid cell leukaemia sequence 1
MBL	mannose-binding lectin
MCP-1	monocyte chemotactic protein-1
MES	4-morpholineethanesulfonic acid

MFG-E8	milk fat globule-EGF factor 8
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MNC	mononuclear cell
MP	microparticle
mRNA	messenger ribonucleic acid
nAb	neutralising antibody
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer (cell)
NO	nitric oxide
O-GlcNAc	β -O-linked N-acetylglucosamine
OX-LDL	oxidised low-density lipoprotein
PAF	platelet activating factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	phycoerythrin
PEST	proline, glutamic acid, serine, threonine enriched (sequence)
PGE2	prostaglandin E2
PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PS	phosphatidylserine
PTM	post-translational modification
PTX	pertussis toxin
PTX3	pentraxin 3
PUMA	p53-upregulated modulator of apoptosis
PVDF	polyvinylidene fluoride
RAIDD	RIP-associated Ich-1/CED homologous protein with death domain
RIP	death domain-containing receptor-interacting protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium

S1P	sphingosine-1-phosphate
SCID	severed combined immunodeficiency
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
shRNA	small hairpin ribonucleic acid
SIRP α	signal-regulatory protein α
SR-A	scavenger receptor-A
SRBC	sheep red blood cell
Stauro	staurosporine
SUMO	small ubiquitin-like modifier
TAM	tumour associated macrophage
TCA	trichloroacetic acid
TGF	transforming growth factor
Thr	threonine
TIMP-1	tissue inhibitor of metalloproteinases-1
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRADD	tumour necrosis factor receptor type 1-associated death domain protein
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
TRAIL-R	tumour necrosis factor-related apoptosis-inducing ligand receptor
Tris	Tris(hydroxymethyl)aminomethane
Trp	tryptophan
TSP-1	thrombospondin-1
Tyr	tyrosine
UTP	uridine triphosphate
UV	ultraviolet
Val	valine
VEGF	vascular endothelial growth factor
vMIP	viral macrophage inflammatory protein
WB	Western blot
XIAP	X-linked inhibitor of apoptosis protein

XV	X-Vivo
Z-AEVD-FMK	N-benzyloxycarbonyl-Ala-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone
Z-DEVD-FMK	N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone
Z-IETD-FMK	N-benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone
Z-Leu-Leu-CHO	N-benzyloxycarbonyl-Leu-leucinal
Z-LEED-FMK	N-benzyloxycarbonyl-Leu-Glu(OMe)-Glu(OMe)-Asp(OMe)-fluoromethyl ketone
Z-LEHD-FMK	N-benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethyl ketone
Z-LEVD-FMK	N-benzyloxycarbonyl-Leu-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone
Z-VAD-FMK	N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone
Z-VDVAD-FMK	N-benzyloxycarbonyl-Val-Asp(OMe)-Val-Ala-Asp(OMe)-fluoromethyl ketone
Z-VEID-FMK	N-benzyloxycarbonyl-Val-Glu(OMe)-Ile-Asp(OMe)-fluoromethyl ketone
Z-WEHD-FMK	N-benzyloxycarbonyl-Trp-Glu(OMe)-His-Asp(OMe)-fluoromethyl ketone
Z-YVAD-FMK	N-benzyloxycarbonyl-Tyr-Val-Ala-Asp(OMe)-fluoromethyl ketone

Chapter 1

Introduction

1.1 Burkitt's lymphoma

1.1.1 General characteristics

Burkitt's lymphoma (BL) is an aggressive, B-cell, non-Hodgkin's neoplasm. Originally characterised by Burkitt in children in equatorial Africa (Burkitt 1972), this malignancy occurs also in immunodeficient (e.g. HIV-carrying) individuals (Carbone & Gloghini 2005; Grogg et al. 2007). Although much less common, it is reported as well in immunocompetent individuals in other parts of the world (Banthia 2003). In its "endemic" African form, the malignancy is highly associated with Epstein-Barr virus (EBV) infection, with over 95 per cent of cases being EBV-positive (Bornkamm 2009a). Additionally, infection with malaria-causing *Plasmodium falciparum* has been shown to be an important co-factor in the endemic form pathogenesis (Rochford et al. 2005; Chene et al. 2009). Unlike endemic form, only 10 – 20 per cent of "sporadic" and 30 – 50 per cent of HIV-associated BL cases are EBV-positive (Brady et al. 2007; Allday 2009; Bornkamm 2009).

All BL types share the same morphological and phenotypical characteristics. It is a monoclonal neoplasm, consisting of monomorphic medium-sized B cells with a diffuse pattern of growth (Swerdlow 2008). The rate of BL cell division is among the highest observed in tumours, with over 95% of cells being positive for Ki-67 (Cooper et al. 1966; Hecht & Aster 2000). BL cells are also characterised by their increased susceptibility to apoptotic cell death (Gregory et al. 1991). The rate of cell death is reflected by substantial level of macrophage infiltration observed *in situ*. The infiltrating macrophages, often containing apoptotic cell debris, produce the characteristic "starry sky" appearance (Berard et al. 1969).

Phenotypically, BL cells are typified by expression of B cell markers (CD19⁺, CD20⁺, IgM⁺) and markers characteristic of germinal centre (GC) B cells: CD10⁺, CD23⁻, CD39⁻, CD77⁺ as well as Bcl-6⁺ and Bcl-2⁻ (Gregory et al. 1987; Gregory et al. 1991; Henderson et al. 1991). BL-derived cell lines with such a surface phenotype (biopsy-like) were classified as group I BL lines (Rowe et al. 1987).

Recent studies carried out in this laboratory have revealed that BL cells can actively regulate immune cell infiltration of the tumour and therefore, the immunological response directed towards the tumour. Apoptotic lymphoma cells have been shown to prevent tumour infiltration of potentially harmful neutrophils by releasing lactoferrin, an agent with neutrophil-repulsion properties (Bournazou et al. 2009). At the same time, fractalkine – a chemokine with the property of attracting mononuclear phagocytes has been found to be released from dying BL cells and to be involved in mediating macrophage infiltration of the tumour (Truman et al. 2008).

1.1.2 Cellular origin

Although the pattern of surface markers expressed by BL cells suggests they originate from germinal centre B cells (Gregory et al. 1987), controversy still exists over the B cell developmental stage from which BL cells derive. Close analysis of immunoglobulin gene rearrangements revealed the existence of differences between EBV-positive and EBV-negative BL cases as to the range of mutations. The higher percentage of hypermutation found in EBV-positive cases (when compared with EBV-negative ones) indicating a later differentiation stage and thus, probably late germinal centre origin of these cells (as opposed to early GC origin of EBV-positive BL cases) (Chapman et al. 1995; Bellan et al. 2005). Other observations made by Bellan et al. (on ongoing mutation and signs of antigen selection) suggested further differences between EBV-positive and –negative cases and would indicate that while the virus-negative tumours may derive from early centroblasts, virus-associated cases would origin from either late germinal centre cells (with completed mutation process) or from memory B cells (Bellan et al. 2005; Thorley-Lawson & Allday 2008). It has to be noted however, that these observations were not in a full agreement with results previously presented by others suggesting that both types of

BL may derive from germinal centre cells (Chapman et al. 1995; Chapman et al. 1996).

1.1.3 Role of c-Myc in BL

Irrespective of the associated factors and origin of the B-cell population, the three forms of BL (endemic, sporadic and HIV-associated) are characterised by chromosomal translocation of *Myc* proto-oncogene into one of immunoglobulin (Ig) loci. In 80 per cent of cases, *Myc* (from chromosome 8) is juxtaposed to the enhancer region of Ig heavy chain on chromosome 14. The majority of the remaining cases contain translocation to one of Ig light chain regions (chromosome 2 or 22). In mature B cells, Ig enhancers are active and thus, the juxtaposition leads to gross overexpression of c-Myc in mutated cells (Klein 1983; Allday 2009). This has a profound effect on the cell fate, as a transcription factor c-Myc is known to be (directly or indirectly) involved in regulation of around 15 per cent of all human genes and can affect various cellular functions, including growth, division, metabolism, motility and death (Dang et al. 2006; Schlee et al. 2007). As the constitutive activation of c-Myc results in increased rate of cell growth and division, it can eventually lead to development of cancer (Coller et al. 2000; Hecht & Aster 2000; Allday 2009). On the other hand, c-Myc upregulation is known to enhance cell susceptibility to apoptotic cell death (Evan et al. 1992; Harrington et al. 1994; Juin et al. 2002; Hoffman & Liebermann 2008). The increased rate of proliferation and apoptosis observed in BL has also been shown to be driven by excessive expression of c-Myc (Milner et al. 1993).

Studies aiming at developing murine models of BL provided further evidence of the oncogenic role of c-Myc. Kovalchuk and colleagues managed to generate transgenic mice spontaneously developing BL-like tumours by fusing the *Myc* gene with control regions of Ig λ chain (Kovalchuk et al. 2000). Zhu et al. achieved similar effects by mimicking the translocation most commonly found in human BL (t(8:14)) by placing *Myc* under control of Ig heavy chain enhancer (Zhu et al. 2005). Although the histological appearance of these murine tumours resembled that of human BL, unlike in human, the malignant cells have been shown to originate from naïve BL cells. Additionally, in the more recently established model, containing cells with an

immunophenotype reminiscent of that of human BL cells, a role for antigen stimulation in development of BL-like tumours from c-Myc overexpressing cells has been demonstrated (Refaeli et al. 2008).

While c-Myc translocation and subsequent protein overexpression are seen as the key events in tumourigenic transformation of B cells, it is assumed that they have to be accompanied by other types of dysregulation for BL to develop. Alteration of expression of many apoptosis-regulating proteins (e.g. p53, Bim, MDM2 or p14ARF) has been demonstrated, and it is believed these allow the c-Myc-directed apoptosis induction to be overcome (Gaidano et al. 1991; Thorley-Lawson & Allday 2008; Allday 2009). On the other hand, upregulation of the E2F1 transcription factor has recently been identified in BL tumour cells (Molina-Privado et al. 2009). It has been suggested that E2F1 can contribute to BL development by supporting cell cycle progression of, potentially arrested in G2 phase of the cycle, c-Myc overexpressing cells.

1.1.4 Role of Epstein-Barr virus in BL

EB virus, a member of the γ -herpesvirus group, was discovered during analysis of a cell line derived from endemic BL tumour (Epstein et al. 1964). It was initially thought to be the main factor involved in BL pathogenesis. Although this view has been modified by the subsequent discovery of EBV-negative BL tumours as well as identification of c-Myc deregulation as the main feature of all BL tumours, the fact that EBV-positive endemic BL tumours have a much higher prevalence than the sporadic form, suggested that EBV infection gives some advantage to the potential tumour cells. On the other hand, as EBV infection is extremely common (affecting over 95 % of human population worldwide), it appeared that additional factors are required for EBV to exert its tumourigenic function. Malaria (in the endemic form of BL) and HIV (in the immunodeficiency-associated) have been identified as the co-factors. Both malaria and HIV infection lead to massive B cell activation (Brady et al. 2007; Bornkamm 2009; Rowe et al. 2009). *P. falciparum* infection can constrain host immunological response by inhibiting cytotoxic T cells activation, thus, indirectly enabling EBV expansion (Rochford et al. 2005). Moreover, *P. falciparum*

erythrocyte membrane protein 1 (PfEMP1) has been shown to induce EBV lytic cycle (Chene et al. 2009).

It is now believed that EBV, which has a capacity for inhibiting death of infected cells and inducing cell immortality, is mainly responsible in BL for providing anti-apoptotic signals to B cells that have acquired a c-Myc mutation during the activation process driven by malaria or HIV infection (Bornkamm 2009; Rowe et al. 2009). The important role of EBV infection on BL cell survival rate has been clearly demonstrated in the studies performed by Kelly and colleagues, who observed that EBV latent gene expression effectively, suppressed c-Myc-mediated death of BL cells (Kelly et al. 2006).

In various EBV-associated pathologies, different sets of the EBV latent genes are known to be expressed. In the majority of cases of BL, the expression is limited to only one viral protein – EBNA-1 (Epstein-Barr virus nuclear antigen-1). Additionally, viral non-coding RNA fragments: EBERs (EBV-encoded RNAs) and BARTs (BamHI A rightward transcripts) are detected. This restricted mode of EBV expression has been classified as a type I latency programme (Rowe et al. 2009).

In accordance with the hypothesised anti-apoptotic role of EBV during BL development, both EBNA-1 and EBERs have been shown to have potency to suppress B cell death triggered by various stimuli. Kennedy and colleagues have demonstrated that the inhibition of EBNA-1 results in death of EBV-positive BL cells and that introduction of EBNA-1 into EBV-negative cells improves their survival (Kennedy et al. 2003). EBERs have been found responsible for protecting BL cells from death mediated by IFN- α (Nanbo et al. 2002). Also, EBV-expressed microRNA BART5 has been implied to suppress the expression of pro-apoptotic protein PUMA (Choy et al. 2008).

All the BL cell lines used during the studies presented in this thesis belong to group I BL lines in respect to their surface marker expression. As for viral gene expression, Mutu I and SAV BL cells are characterised by type I latency, while BL2 and L3055 cells are EBV-negative.

1.2 Cell death

Two major types of cell death are generally discriminated, apoptosis and necrosis. While these two types death are characterised by sets of fairly distinct features, it should be noted that in very many experimental systems, cell death possessing attributes of both apoptosis and necrosis have been observed (Blomgren et al. 2007; Kroemer et al. 2009). Apart from these two major types of cell death, several less well understood mechanisms of cell demise have been described, e.g. autophagy, mitotic catastrophe, anoikis, paraptosis, pyroptosis, pyronecrosis or entosis (Kroemer et al. 2009). However, many of these forms of death seem to be characteristic only for some very specific physiological processes and often the features by which they were typified overlap with each other and with those of apoptotic or necrotic death. The current state of knowledge of these processes is such that it is unclear whether any of them truly is a distinct type of cell death.

1.2.1 Apoptosis

Apoptosis is regarded as a genetically regulated, quiet suicidal form of death, serving to remove unwanted and damaged cells. The term apoptosis was first proposed by Kerr and colleagues in 1972. Cells undergoing apoptosis were defined by shrinkage of organelles and cytoplasm, fragmentation of the nucleus and release of their contents in the form of variously sized vesicles called apoptotic bodies (Kerr et al. 1972). Later, this list has been expanded to include DNA fragmentation or exposure of the membrane lipid phosphatidylcholine (PS) to the outer leaflet of the plasma membrane (Kroemer et al. 2009).

Insight into the molecular mechanism of apoptosis was made by studies on genetic regulation of programmed cell death in *Ceanorhabditis elegans*. Three main genes – *ced-3*, *ced-4* and *ced-9* have been shown to regulate this process in nematodes (Yuan & Horvitz 2004). Identification of human homologues of these genes has allowed for identification of the key mediators of mammalian apoptosis. The caspase family of proteases has been shown to be homologous to *ced-3*, apoptosome-forming protein Apaf-1 to *ced-4* and anti-apoptotic members of the Bcl-2 family to *ced-9* (Degterev et al. 2003; Yuan & Horvitz 2004). Subsequent studies have revealed the enormous

complexity of the various interplaying mechanisms that drive the process of death by apoptosis. The key mechanisms will be introduced below. It should be mentioned however, that in the numerous studies on the molecular mechanisms of apoptotic death, several additional players have been described and huge variation observed as to which proteins and mechanisms operate in different cell types and under various death-inducing stimuli.

At the very centre of all apoptotic mechanisms lies the activity of caspases. Normally present in cells as inactive pro-enzymes, they become activated under death stimuli and regulate and execute the demise process (Taylor et al. 2008). Two major canonical apoptosis-inducing pathways that lead to caspase activation have been characterised. The extrinsic, “death receptor pathway” is responsible for transmitting death signals (for example, TNF α , FasL or TRAIL) from the cell environment. The signalling is mediated through the death receptors (e.g. TNFR1 and 2, Fas, TRAIL-R). Upon binding with their substrates, these death domain-containing receptors multimerise and, together with many adaptor proteins (FADD, TRADD, DAXX, RIP, RAIDD and FLIP) and multiple molecules of caspase-8 (and/or caspase-10), form death inducing signalling complex (DISC). DISC-formation results in caspase-8 (10) molecule interactions and their autoproteolytic activation, which in turn can activate executioner caspases -3, -6 and -7 (Kischkel et al. 2001; Sprick et al. 2002; Degterev et al. 2003).

Another apoptosis-inducing pathway, “the mitochondrial pathway” is the cell response to intracellular signals like DNA damage, reactive oxygen species or cytotoxic drugs. These signals are transduced by various pro-apoptotic BH3-only proteins (Bad, Bid, Bik, Bim, HRK, NOXA or PUMA) which promote the release of cytochrome c from the mitochondrial intermembrane space. BH3-only proteins enable oligomerisation of Bcl-2 family members Bak and Bax and thus, formation of pores in the outer mitochondrial membrane through which cytochrome c can be released to the cytosol (Taylor et al. 2008). The process of Bak and Bax oligomerisation has been found to be an important regulatory point. The interaction of anti-apoptotic members of Bcl-2 family (Bcl-2 itself as well as Bcl-XL, Bcl2A1, Bcl-B, Bcl-W or Mcl-1) with BH3-only proteins may result in the blockade of

Bak/Bax oligomerisation and so, cytochrome c release from mitochondria (Danial & Korsmeyer 2004; Lomonosova & Chinnadurai 2008).

The release of cytochrome c drives the formation of the apoptosome – complex formed by oligomerised Apaf-1 (apoptotic protease activating factor-1) and caspase-9. Caspase-9 interactions with Apaf-1 result in its activation. Like active caspase-8, activated caspase-9 mediates cleavage of effector caspases -3, -6 and -7 (Bao & Shi 2007).

It is noteworthy that, under certain circumstances, during activity of the extrinsic pathway of apoptosis, active caspase-8 may amplify apoptotic signals by cleaving BH3-only protein Bid and consequently, activating the mitochondrial pathway (Degterev et al. 2003; Taylor et al. 2008). In addition, Bid has been identified as a substrate of other caspases as well as of calpains or cathepsins and so, shown to be an important mediator coupling mitochondrial death signalling with various death stimuli (Billen et al. 2008).

One of less common apoptotic pathways is dependent on granzyme B. This serine protease can be released from NK cells or cytotoxic T cells and directly activate apoptosis in target cells by cleaving effector caspases or Bid (Sutton et al. 2000).

Activated executioner caspases mediate cellular breakdown selectively cleaving a vast number of their specific substrates. These processes eventually lead to the development of the morphology that was described by Kerr et al. (Kerr et al. 1972; Taylor et al. 2008).

1.2.2 Necrosis

From the morphological perspective, necrotic death is discriminated by the swelling of cytoplasm and organelles as well as by the rupture of lysosomes, early disruption of plasma membrane integrity and leakage of the cell content, which can potentially induce inflammatory reactions and damage of the surrounding cells (Kroemer et al. 2009). While necrosis progression is independent of caspase activation, in many instances calpains and cathepsins, which become activated as a consequence of increase of intracellular Ca^{2+} concentration, may be required for mediating necrosis progression (Golstein & Kroemer 2007). A decrease in the level of cellular ATP and

overproduction of reactive oxygen species (ROS) are also observed in cells dying by necrosis (Festjens et al. 2006; Kroemer et al. 2009).

Necrosis was originally proposed to be a cellular reaction to irreversible injuries of various origins and was seen as an uncontrolled process. However, substantial evidence has emerged demonstrating that at least part of cell death with morphological features of necrosis is in fact governed by tightly regulated mechanisms.

This programmed necrosis (sometimes also called necroptosis) may, like apoptosis, be induced via death domain receptors (Fas, TNFR1, TRAIL-R). This mechanism has been shown to be in operation particularly in the apoptosis-incompetent cells i.e. having defects in apoptosis activation pathways or under treatment with Z-VAD-FMK (e.g. Vercaemmen et al. 1998; Holler et al. 2000; Denecker et al. 2001). Although both necrosis and apoptosis can be induced by signalling through the death domain receptors, unlike in apoptosis, in necrosis this does not result in the involvement of Bcl-2 family members or in cytochrome c release from mitochondria (Degterev et al. 2005). Death domain receptor-interacting kinase RIP1 has been found to play a central role in directing cells to the necrosis pathway after signalling through the death domain receptors (Holler et al. 2000; Kalai et al. 2002; Degterev et al. 2008). Specific RIP1 inhibitors – necrostatins, have been shown to inhibit the progression of necrotic death, or even redirect cells to the apoptotic death pathway (Degterev et al. 2005; Degterev et al. 2008; Han et al. 2009). Also RIP1-regulating kinase RIP3 is believed to play a central role in regulating cell reaction to TNF death stimulus and promoting necrosis (Cho et al. 2009; Declercq et al. 2009; Zhang et al. 2009; Christofferson & Yuan 2010).

Interestingly, induction of necrosis has also been observed after stimulation of Toll-like receptors 3 and 4, treatment with *Clostridium* α -toxin or as a consequence of viral infection (Festjens et al. 2006; Cho et al. 2009; Kennedy et al. 2009).

Programmed necrosis is recently gaining recognition for its role in various clinical conditions. It has been reported to contribute to ischemia reperfusion injury or progression of various neurodegenerative disorders (Degterev et al. 2005; Festjens et al. 2006; Rosenbaum et al. 2010).

1.2.3 Proteases in cell death

Proteases are the major players in the induction phase of cell death, in the regulation of the process and in the effector phase, when the cell content is being broken down and life processes stopped. Although caspases are postulated to be in the centre of these events, other families of proteases, particularly calpains and cathepsins, appear to also be involved and in some systems can in fact drive apoptosis in the absence of caspases (e.g. Fettucciari et al. 2006). The latter two families, unlike caspases, seem to be particularly prominent during necrotic death (Golstein & Kroemer 2007). Additionally, other, less characterised serine proteases, like Omi (HtrA2), AP24 or granzymes A and B have been found to contribute to cell death progression (Garrido & Kroemer 2004; Vandenabeele et al. 2005; O'Connell & Stenson-Cox 2007).

1.2.3.1 Caspases

Caspases are a family of evolutionary highly conserved cysteine-dependent aspartate-directed proteases. They are known to specifically cleave their substrates after an aspartic acid (Asp; D) residue. The specificity for an Asp residue appears to be up to four orders of magnitude higher than for a glutamic acid (Glu; E) residue (Timmer & Salvesen 2007). The initial studies on caspase specificity resulted in identification of the canonical caspase-3 cleavage motif: P₄-DXXD-P₁, with X being any amino acid (Talanian et al. 1997). However, more recently it has been appreciated that other surrounding amino acids, like one in the position P₁' are as important in the recognition of the target protein (Timmer & Salvesen 2007). The importance of distant recognition sites – exosites, is now hypothesised (Timmer & Salvesen 2007). In fact, a growing number of caspase-3 substrates have been reported to be cleaved in the non-canonical sites (Nicholson 1999; Fischer et al. 2003). This might support the possibility that the exosites play an important role in the recognition process. Equally, the position of, perhaps less specific, cleavage motifs on the solvent-exposed parts of protein, where they can be more easily accessed by the proteases might be responsible for the observed discrepancy (Timmer & Salvesen 2007; Pop & Salvesen 2009).

The differences in the substrate specificity between different members of the caspase family have been shown to depend, at least partly, on the composition of amino acids

adjacent to the scissile bond (Thornberry et al. 1997). However, the degree of overlap in specificity, especially in the *in vitro* studies, seems to be high. Caspase-3 is generally considered to be the most potent of all the caspases, being likely to win the competition with other family members over the shared substrates (McStay et al. 2007; Walsh et al. 2008).

The human family consists of twelve members, which can be divided into groups based on their chemical structure. Caspases -2, 8, 9, 10 have long N-terminal prodomains. In case of caspase -8 and -10 these are two death effector domains (DED). Caspases -2 and -9 contain caspase recruitment domains (CARDs). The prodomains are indispensable for the recruitment of these proteins to apoptosis activation complexes (apoptosome, DISC) and their subsequent activation. Conversely, caspases -3, 6, 7 and -14 do not contain prodomains and require proteolytic cleavage for their activation (Kumar 2007; Taylor et al. 2008).

Caspases -8, 9, 10 (and to a lesser extent -2) are involved in the initiation phase of apoptosis. Once proteolytically active, they amplify apoptotic signal by directing the proteolysis of effector caspase (-3, 6, 7) zymogens (Kumar 2007).

Cleavage of the specific substrates by executioner caspases may have a dual effect; the target protein may become either inactivated or deactivated (Timmer & Salvesen 2007). The list of substrates that were identified as being processed during the effector phase of apoptosis contains several hundreds of proteins and is growing rapidly thanks to the application of high-throughput analysis methods (Luthi & Martin 2007; Dix et al. 2008; Mahrus et al. 2008). Most apoptotic caspase substrates have been shown to belong to a few main pathways or functional groups, for example regulating transcription, cell adhesion, those responsible for DNA repair or anti-apoptotic or cytoskeletal proteins (Fischer et al. 2003; Mahrus et al. 2008).

Although mediation of apoptosis is the best-recognised function of caspases, they are also known to serve other functions. Caspases -1, 4, 5, 11, 12 and -14 are associated mainly with non-apoptotic functions. However, caspase -3, 8, 9 and 10 have also been reported to operate in non-apoptotic conditions (Lamkanfi et al. 2007; Chowdhury et al. 2008). The best recognised out of these non-apoptotic caspase roles are the maturation of pro-inflammatory cytokines, activation of NF- κ B and the

regulation of cell cycle and differentiation (Lamkanfi et al. 2007; Chowdhury et al. 2008).

As caspases have a critical role in deciding on cell fate, their activity has to be closely regulated. Unsurprisingly, several cellular caspase inhibitors have been identified. CrmA (cytokine response modifier A), one of the best characterised inhibitors, has been found to inhibit caspases -8 and -10 as well as caspase-1. It has been reported to successfully block the induction of apoptosis via death receptors. Additionally, it is capable of inhibiting apoptosis resulting from forced overexpression of caspase-1 *in vitro*. Furthermore, XIAP, a member of the IAP (inhibitors of apoptosis) family, using its different domains, can block the activity of both caspases -3, 7 and caspase-9. Another well-characterised cellular inhibitor of caspases, FLIP, contains death effector domains (DEDs) and can competitively bind to FEDD, therefore, inhibiting caspase-8 association with DISC and activation. It is noteworthy that, most of the described inhibitors were first identified when viral mechanisms of inhibiting host cell apoptosis were studied (Callus & Vaux 2007; Kumar 2007; Pop & Salvesen 2009).

1.2.3.2 Calpains

A family of calcium-dependent cysteine proteases – the calpains consists of two ubiquitous and fairly well characterised major isozymes (calpain I and II or μ and m , respectively) and several less-described tissue-specific forms (n-caspases). The ubiquitous forms of calpain are heterodimers consisting of a common small (28 kDa) subunit and different large (80 kDa) subunits. They differ as to the Ca^{2+} concentrations required for their activity. Whilst for calpain I low-millimolar concentrations were sufficient, calpain 2 needed almost micromolar Ca^{2+} concentrations *in vitro*. In the event of Ca^{2+} influx, calpains undergo autoproteolytic cleavage. This is known to be a two-step process, in the case of calpain I initially leading to generation of a 78 kDa (79 kDa for calpain II) fragment of the large subunit and in the next stage, the 78 kDa-sized subunit is turned into a 76 kDa one (78 kDa for calpain II) (Zimmerman & Schlaepfer 1991; Brown & Crawford 1993). The small subunit is processed in three steps from 28 kDa to 18 kDa (McClelland 1989). Autoproteolysis has initially been shown to reduce the Ca^{2+} concentration

required for calpain activity (Zimmerman & Schlaepfer 1991; Brown & Crawford 1993; Li et al. 2004) . However, the true role of these autolytic steps remain a source of the controversy, as several studies have shown that even the 80 kDa unprocessed large subunit may be proteolytically active (Johnson & Guttman 1997; Li et al. 2004).

In addition to Ca^{2+} concentration, calpain activity can be regulated by a ubiquitous, specific, cellular inhibitor of calpains – calpastatin (Wendt et al. 2004; Melloni et al. 2006). The initial studies on the two proteins interactions indicated that calpastatin can bind calpain (and so, inhibit its action) when Ca^{2+} levels are elevated (Barnoy et al. 1999). The results presented more recently by Melloni et al. have indicated that this interaction can take place also at physiological Ca^{2+} levels (Melloni et al. 2006). Additionally, calpain activity has been found to be modulated by local redox conditions (Guttman & Johnson 1998) or by specific phosphorylation (either of calpain itself or of the substrate) (Shiraha et al. 2002; Smith et al. 2003; Chen et al. 2010).

Calpains hydrolyse their substrates at a limited number of sites and it is believed that the cleavage serves to modify the substrate function rather than to cause its degradation (Chan & Mattson 1999; Goll et al. 2003). Given that calpain selectivity seems to be so tightly regulated, it would be expected that these proteases have a high requirement for a specific cleavage motif. Perhaps surprisingly, no consensus cleavage sequence has been found (Tompa et al. 2004; Cuerrier et al. 2005). Amino acid preferences seemed to be only partly responsible for substrate recognition and it has therefore been suggested that the primary structural characteristic of the scissile bond-adjacent area might be involved in the recognition (Tompa et al. 2004; Cuerrier et al. 2005). Calpain cleavage sites have been found to be more likely situated in the unstructured regions of their target proteins (Tompa et al. 2004).

Calpains have a well-recognised role in necrosis. Under some types of death stimulus, they appeared to be essential for the death process to take place (Golstein & Kroemer 2007). It is the Ca^{2+} overload, often observed in cells that have suffered an insult, that is believed to drive calpain overactivation and consequently, cellular demolition (Goll et al. 2003). During this process cytoskeletal (e.g. actin-binding

proteins), membrane (ion transporters, growth factor receptors, adhesion molecules) or signalling (various kinases, phosphatases, and transcription factors) proteins are targeted by calpains (Rami 2003). Furthermore, after the calpain-mediated permeabilisation of the lysosomal membrane, the cathepsins are released into the cytosol and contribute to cell breakdown (Yamashima 2000). Although the mechanism has not yet been clarified, calpains are also considered responsible for the early plasma membrane rupture observed in necrotic cells (Liu et al. 2004). This type of calpain activity is implicated in various pathologies, including acute processes like ischemic injury or chronic disorders, like Alzheimer's, Huntington's and Parkinson's diseases (Rami 2003).

Although calpain activity has mainly been associated with necrotic death, it plays an important role in various mechanisms involved in apoptosis. Amongst many others, calpain inhibitors have been found to block T cell apoptosis triggered by anti-CD3 antibodies (Rami 2003). Calpain involvement in apoptotic processes seems to be finely regulated. It has been demonstrated that caspases can have a role in activating calpains e.g. calpastatin is known to be hydrolysed not only by calpains but also by caspases during apoptosis (Wang et al. 1998; De Tullio et al. 2000; Shi et al. 2000). In fact, calpain-caspase crosstalk is even more complex, as, under some death conditions, calpain has been reported to activate caspases (Ruiz-Vela et al. 1999; Blomgren et al. 2001; Altnauer et al. 2004). On the other hand, it can cause caspase degradation and inactivation (Chua et al. 2000). Like caspases, calpains can regulate the process of apoptosis by directly modulating both pro-apoptotic: Bid, Bax (Chen et al. 2001; Cao et al. 2003) and anti-apoptotic: Bcl-2, Bcl-XL (Nakagawa & Yuan 2000; Gil-Parrado et al. 2002) members of Bcl-2 family.

Remarkably, a considerable number of proteins that can be cleaved by both caspases and calpains have been identified (Chan & Mattson 1999; Wang 2000; Liu et al. 2006), suggesting that these enzymes can have complementary functions. Among these substrates, many cytoskeletal, plasma membrane and signal-transducing proteins have been found. Even more interestingly, during experimental transient brain injury, where both the proteases are activated, they have been shown to cleave their substrates simultaneously (Liu et al. 2006). In many of the dually cleaved

proteins, caspases- and calpain-driven cleavage leads to the production of similarly sized products (Wang 2000).

1.2.3.3 Cathepsins

Cathepsins are a heterogeneous group of lysosomal proteases, consisting of aspartic – cathepsins D, E; serine – A, G; and several cysteine proteases – cathepsins B, L, H, plus others (Conus & Simon 2008). Recent years have brought ample evidence for their role in various death-mediating mechanisms. The involvement of cathepsins in death progression starts when they become released from lysosomes into the cytosol as a result of lysosomal destabilisation or lysosomal membrane permeabilisation (LMP) (Stoka et al. 2007). This has been found to be caused by Fas, TNF α or TRAIL signalling (Deiss et al. 1996; Nagaraj et al. 2006; Nagaraj et al. 2007), oxidative stress (Roberg & Ollinger 1998), the direct action of Bax (through its insertion into lysosomal membrane) (Kågedal et al. 2005) as well as by several chemical lysosomotropic agents (Stoka et al. 2007; Conus & Simon 2008). The diversity of the factors and mechanisms inducing cathepsin release from lysosomes shows that they can be primary players in apoptosis induction (death signals – Fas, TNF α , TRAIL or oxidative stress) or may have a secondary role in cell death, perhaps being part of a death signal enhancing loop, when apoptosis was induced by other stimuli (Bax-driven LMP).

Once in the cytosol, cathepsins are believed to act mainly by mediating Bid cleavage and activating mitochondrial pathway of apoptosis (Stoka et al. 2001; Cirman et al. 2004; Blomgran et al. 2007). More recent advances have revealed, however, that the role of cathepsins might be more complex than this, as most cysteine cathepsins have been found to be able to cleave anti-apoptotic members of Bcl-2 family – Bcl-2, Mcl-1, Bcl-XL and even more interestingly, cellular caspase inhibitor – XIAP (Droga-Mazovec et al. 2008).

In addition to being active in apoptosis, cathepsins have an important function during necrotic cell death. In fact, they seem to be indispensable for some forms of necrosis to take place (Golstein & Kroemer 2007). The extent of the lysosomal damage induced by a death trigger seems to regulate whether apoptotic (after moderate damage) or necrotic (after serious damage) death mechanisms will be switched on

(Li et al. 2000). For example, in the necrotic insults where intracellular Ca^{2+} levels are significantly increased, activated caspases can mediate lysosomal disruption and subsequent cathepsin release (Yamashima 2000). In addition, recent evidence suggests that cathepsin Q is a direct product of p53 and that DNA damage triggers p53-dependent activation of this cathepsin, which in turn (together with ROS) is responsible for mediating necrosis (Tu et al. 2009).

1.2.4 Clearance of dying and dead cells

Dying cells are known to produce signals that allow for their recognition and removal. Moreover, the way in which a dying cell interacts with the cell that engulfs it is thought to modulate the immune response (Savill & Fadok 2000).

1.2.4.1 Clearance of apoptotic cells

Signals mediating apoptotic cell recognition and engulfment

The mechanisms involved in the clearance of cells dying by apoptosis have been studied extensively and numerous molecules, which take part in the process of recognition and engulfment of these cells, have been identified.

Thus far, the rearrangement of the plasma membrane phospholipids and translocation of phosphatidylcholine (PS) to the outer leaflet of the membrane appears to be the best characterised event that is responsible for labelling of apoptotic cells for phagocytic uptake (“eat me” signal) (Fadok et al. 1992; Ravichandran & Lorenz 2007). PS translocation takes place in the early stage of the apoptotic process and it has been thought to be a caspase-dependent phenomenon (Martin et al. 1996; Fadok et al. 2001), however, some later studies have demonstrated that in cells deficient in caspase-3, PS has also been externalised during apoptosis and cell clearance seemed unimpaired (Fadok et al. 1998; Zheng et al. 1998; Turner et al. 2003).

Various molecules have been shown to participate in phagocyte detection of PS on the surface of apoptotic cells. Among them, several soluble “bridging” molecules have been found to be involved in PS binding. PS-associated milk fat globule protein MFG-E8 mediates apoptotic cell recognition through binding to $\alpha_v\beta_{3(5)}$ integrins (Hanayama et al. 2002). In a similar manner, Gas6 (growth arrest-specific 6) molecule can indirectly contribute to PS detection by binding to receptor tyrosine

kinase MER on the phagocytic cell (Nagata et al. 1996; Nakano et al. 1997) and complement component C1q can act through binding to CD91/calreticulin or, possibly, CD93 on the phagocyte membrane (Ogden et al. 2001; Vandivier et al. 2002; Norsworthy et al. 2004; Gardai et al. 2005; Païdassi et al. 2008). Protein S has also been found to be bound to PS on apoptotic cells and to have a role in ingestion of these cells (Anderson et al. 2003). Furthermore, macrophage scavenger receptor CD36 mediates phagocyte recognition of PS on the apoptotic cells. This may require the formation of a complex with thrombospondin/ $\alpha_v\beta_3$ integrin (Savill et al. 1992) or might take place directly (Tait & Smith 1999; Greenberg et al. 2006). Huge progress has been made in recent years in identifying direct receptors for PS. Tim4, BAI1 and stabilin-2 were found to be capable of directly binding PS (Miyanishi et al. 2007; Park et al. 2007; Park et al. 2008). While downstream signalling leading to phagocytosis has been well characterised for BAI1 and stabilin-2, the capability of Tim4 to signal directly has recently been questioned (Park et al. 2009).

Although PS appears to be in the centre of all signalling events leading to the apoptotic cell clearance, it has been demonstrated that PS exposure alone is not sufficient for apoptotic cell uptake to take place (Devitt et al. 2003). In addition to PS, other molecular interactions are implicated. Phagocyte scavenger receptors SR-A, LOX-1 and CD36 interaction with their common ligand - oxidised low-density lipoprotein (OX-LDL) on the surface of apoptotic cells is known to play a role in the phagocytosis of the apoptotic cells (Endemann et al. 1993; Platt et al. 1996; Oka et al. 1998; Chang et al. 1999). An important role in apoptotic corpse disposal has also been described for the pattern recognition receptor CD14 (Devitt et al. 1998). ICAM-3, which expression on the plasma membrane has been found to be altered in apoptotic cells, might serve as the CD14 substrate (Moffatt et al. 1999). Moreover, exposure of calreticulin on the surface of the dying cell and its subsequent binding to the scavenger receptor CD91 on the phagocyte, participate in dead cell uptake (Ogden et al. 2001; Gardai et al. 2005). Two members of the collectin family, C1q and mannose-binding lectin (MBL), which bind to the surface of dying cells and can mediate signalling via calreticulin/CD91, have also been shown to have an important role in the process of dead cell ingestion (Ogden et al. 2001; Stuart et al. 2005;

Païdassi et al. 2008) . Finally, annexin 1, like PS, has been found to translocate to the surface of apoptotic cells and to mediate their ingestion (Arur et al. 2003).

In addition to the positive “eat me” signals described above, the negative regulation of some signals (“don’t eat me”) displayed by target cells are as important for binding and phagocytosis of apoptotic cell corpses. Surface molecule CD31, which has a role in leukocyte detachment from phagocytes, has also been shown to become disabled in apoptotic cells, therefore allowing for apoptotic cell binding and subsequent engulfment by a phagocyte (Brown et al. 2002). In a similar manner, CD47 (on target cells) interaction with SIRP α (on phagocytes) is disrupted as a consequence of apoptosis due to the altered CD47 expression on the dying cells. Under normal circumstances CD47-SIRP α cooperation is known to prevent cell uptake by phagocytes (Oldenborg 2000; Oldenborg et al. 2001; Gardai et al. 2005) and the interruption to this signalling has been demonstrated to enable calreticulin-CD91-mediated phagocytosis (Gardai et al. 2005).

“Find me” signals

Although in the majority of cases apoptotic cells are engulfed by their neighbours, when death occurs in high numbers, help from specialised phagocytic cells might be required (Kerr et al. 1972; Gregory & Devitt 2004). Molecules having chemotactic properties for phagocytes, so called “find me” signals, are therefore released from dying cells. Thus far, only a few chemoattractants that are released from apoptotic cells have been identified.

Studies performed by Knies and colleagues have revealed that in murine embryos, elevated expression of the inflammatory cytokine and chemoattractant for mononuclear phagocytes - EMAP II, was associated with areas of intense apoptosis and substantial macrophage infiltration (Knies et al. 1998). The processing and release of mature EMAP II was initially demonstrated to be caspase-dependent and consecutive studies have implicated caspase-7 in this process (Knies et al. 1998; Behrendorf et al. 2000). Interestingly, it has also been shown that EMAP II processing under hypoxic conditions was mediated by proteases other than caspase (Matschurat et al. 2003). Some studies, however, have questioned the capability of caspases to cleave pro-EMAP II (Zhang & Schwarz 2002; Liu & Schwarz 2006).

A role in attracting phagocytes to sites of apoptosis has also been attributed to the ribosomal protein S19 dimer (Horino et al. 1998). Horino et al. observed its release from heat-treated HL-60 cells and found it was responsible for macrophage migration to the site of injection of these cells *in vivo*. G protein-coupled receptor for C5a (CD88) is responsible for mediating S19-dependent migration of macrophages (Nishiura et al. 1998).

In the course of further investigations, phospholipid lysophosphatidylcholine (LPC) has been shown to mediate the migration of THP-1 monocytic cells to UV-irradiated MCF-7 cells (Lauber et al. 2003). The release of this phospholipid is dependent on caspase-3-driven activation of phospholipase A₂β (iPLA₂β). LPC is known to mediate chemoattraction of mononuclear phagocytes also via G-protein coupled receptor, G2A (Yang et al. 2005; Peter et al. 2008).

Later, another lipid factor, sphingosine-1-phosphate (S1P), was also shown to be released from Jurkat and U937 cells in an apoptosis-dependent manner to induce migration of mononuclear phagocytes (Gude et al. 2008).

Finally, Elliott et al. reported that nucleotides ATP and UTP are released from apoptotic T cells to mediate chemotaxis of phagocytes. Here again, the liberation of chemotactic factor has been shown to be caspase inhibitor-sensitive (Elliott et al. 2009).

1.2.4.2 Clearance of necrotic cells

The mechanisms of phagocyte recognition and uptake of cells undergoing necrosis remain far less characterised. Interestingly, necrotic cells have been shown, just like apoptotic cells, to externalise PS in response to death stimuli (Cocco & Ucker 2001; Krysko et al. 2004) and the exposed PS has been reported to contribute to the engulfment of necrotic cells (Cocco & Ucker 2001; Hirt & Leist 2003; Brouckaert et al. 2004; Böttcher et al. 2006) . However, while in the report from Hirt and Leist, PS appeared to be the main component responsible for necrotic cell phagocytosis, the studies by Cocco et al. and Böttcher et al. rather suggested that PS is only partly responsible for necrotic cell recognition and uptake (Cocco & Ucker 2001; Hirt & Leist 2003; Böttcher et al. 2006) .

Some other molecules that have previously been implicated in apoptotic cell clearance are often found to be involved in recognition and phagocytosis of primary or secondary necrotic cells in addition to, or instead of their role in clearance of apoptotic cells. For instance, studies of Nauta and colleagues on mannose-binding lectin ability to bind to dying cells revealed that it had a preference for (secondarily) necrotic cells (Nauta et al. 2003). Similarly, components of the classical complement pathway, which were associated mainly with the engulfment of apoptotic cells (Ogden et al. 2001; Païdassi et al. 2008), have been shown in other studies to assist in the clearance of both primary and secondary necrotic cells (Gaipf et al. 2001; Gullstrand et al. 2009). Blume et al. have reported that annexin 1, previously found to be externalised on apoptotic cells (Arur et al. 2003), is associated mainly with secondary necrotic cells and is responsible for inhibition of the phagocyte pro-inflammatory response (Blume et al. 2009). Likewise, C-reactive protein, apart from binding apoptotic cells (Gershov et al. 2000) can opsonise secondary necrotic cells (Hart et al. 2005). An effect of the latter protein binding on the rate of dying cell uptake by macrophages, however, has not been observed (Hart et al. 2005). Further, Rovere et al. have demonstrated that in addition to C-reactive protein, another pentraxin - PTX3, could bind to both apoptotic and necrotic cells and that its binding could affect dying cell phagocytosis by dendritic cells (Rovere et al. 2000).

Despite involvement in the clearance of apoptotic and necrotic cells of several common molecules, the two types of death have been shown to elicit different modes of uptake by macrophages. While during the internalisation process of cells dying by apoptosis formation of classical phagosome can be observed and cells are engulfed in a “zipper-like” mechanism (Krysko et al. 2006), necrotic cells have been shown to be uptaken using less efficient micropinocytic mechanism (Krysko et al. 2006).

1.2.4.3 Immunological consequences of cell death

Perhaps the most essential difference between the cells dying by apoptosis and necrosis occurs in the immunological consequences of their clearance. In most situations, cells dying by apoptosis are able to drive an anti-inflammatory response of phagocytes (Fadok et al. 1998) whereas necrotic cells in the majority of cases are known to elicit a release of pro-inflammatory factors from the phagocytic cells

(Festjens et al. 2006). However, under some conditions both apoptotic-mediated inflammatory reactions and a “quiet” death by necrosis have been observed (Gregory & Devitt 2004; Green et al. 2009).

Evidence accumulated that apoptotic cell interactions with phagocytes can elicit an anti-inflammatory response. It was demonstrated that the presence of apoptotic cells led to the secretion of IL-10, TGF β , PAF or PGE₂ from mononuclear phagocytes (Voll et al. 1997; Fadok et al. 1998; Freire-de-Lima et al. 2000). Moreover, the anti-inflammatory effects of apoptotic cells are strong enough to inhibit inflammatory cytokine release from LPS-treated phagocytes (Voll et al. 1997; Fadok et al. 1998). Further analysis has implicated PS and signalling through CD36 in this process (Voll et al. 1997; Huynh et al. 2002). In addition to these mechanisms, the secretion of anti-inflammatory agents has been shown to be a part of the process of apoptosis - IL-10, TGF β and HO-1 release from the apoptosing cells have been detected (Gao et al. 1998; Chen et al. 2001; Weis et al. 2009).

Strikingly, despite the fact that antigens from these cells are presented in the context of MHC class I by immature dendritic cells (Albert et al. 1998), the immunological response is not triggered. The observation that following apoptotic cell ingestion immature dendritic cells could only present antigens to CD8⁺ T cells but not to CD4⁺ T cells (Griffith et al. 2007) helped to propose another mechanism partly responsible for apoptotic cell-mediated immunosuppression. It has been assumed that CD8⁺ T cells, which become activated in the absence of active CD4⁺ T cells and thus, lack of priming from CD4⁺ T cell-activated DCs, differentiate into “helpless” cytotoxic T lymphocytes (Green et al. 2009). These otherwise functional cells, upon re-encounter of the apoptotic cell-derived antigen could release the death molecule, TRAIL, causing death of other helpless CTLs and possibly active CD4⁺ T cells and therefore, induce tolerance (Griffith et al. 2007; Green et al. 2009).

In case of necrotic cells, it is believed that the main process responsible for their immunogenicity is the rapid leakage of their contents, including molecules described as “danger signals” or DAMPs (danger-associated molecular patterns) (Festjens et al. 2006; Kono & Rock 2008; Green et al. 2009). Several molecules have been implicated, among them high-mobility group box 1 protein (HMGB1), various heat

shock proteins, uric acid, S100 proteins, nucleosomes or ATP (Kono & Rock 2008). Being mostly ligands for various TLRs and scavenger receptors, these factors can very effectively induce inflammation and adaptive immune responses (Kono & Rock 2008; Mosser & Edwards 2008). Most of these damage signals are not released from apoptotic cells until they lose plasma membrane integrity. Nevertheless, the existence of a protective mechanism, which neutralises the damage signals has been observed. Inhibition of HMGB1 activity is achieved by its oxidation. This caspase- and ROS-dependent process has been found indispensable for apoptotic cells induction of immunological tolerance (Kazama et al. 2008).

1.2.5 Tumour-associated macrophages (TAMs)

Macrophages are among the immune cells most commonly found in the tumour tissues (Solinas et al. 2009). Although under various danger stimuli they are known to produce pro-inflammatory factors and induce adaptive immunological responses, as resident cells tumour associated macrophages are mainly linked with the phenotype described as “M2-like”, “alternatively activated” or “regulatory” and anti-inflammatory and pro-tumour actions (Mantovani et al. 2002). As a consequence, a high number of tumour infiltrating macrophages is often considered to be a poor prognostic sign (Qian & Pollard 2010).

TAMs are postulated to derive predominantly from peripheral blood monocytes that were attracted to the tumour tissue (Mantovani et al. 2002). Several chemokines that are produced by cells from various tumours have been found to take part in this process; MCP-1 (CCL2) and CCL5 have been implicated in several types of neoplasms but CCL3, CCL4, CCL8, CXCL18, CCL22 or CXCL8 are among others involved (Lamagna et al. 2006; Solinas et al. 2009; Mantovani & Sica 2010). Aside from chemokines, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1) were also found to be important (Mantovani et al. 2002; Solinas et al. 2009). Furthermore, various cleaved extracellular matrix components e.g. fibronectin or fibrinogen might serve as additional chemotactic stimuli (Solinas et al. 2009). A contribution from the factors secreted by dying or damaged tumour cells, for example EMAP II, has also been implied (Murdoch et al. 2004).

It is the cooperation of various tumour environment components that is believed to lead to macrophage acquisition of the “regulatory” and pro-tumour phenotype. Some tumour-secreted cytokines have been directly implicated. For instance, MCP-1, in addition to its role in macrophage attraction to the tumour site, has also been reported to mediate TAM survival and their shift towards “regulatory” phenotype (Roca et al. 2009). Similarly, Burkitt’s lymphoma cells are known to release IL-10, which can directly mediate macrophage drift towards a “regulatory” phenotype (Stewart et al. 1994; Ogden et al. 2005).

Additionally, hypoxic conditions, which are common in many tumours, can markedly influence the pattern of genes expressed by TAMs. Increased production of IL-10 and PGE2 by macrophages in hypoxic areas has been detected (Biswas et al. 2008). Moreover, induction of hypoxia-induced factor-1 α (HIF-1 α) and consequently, an increased expression of pro-angiogenic factors like VEGF, have also been observed (Murdoch et al. 2004; Biswas et al. 2008). HIF-1 α upregulation in the tumour is commonly seen as a poor prognostic indicator for the anti-tumour treatment outcome (e.g. Theodoropoulos et al. 2004; Chai et al. 2008;).

As it is a case with other macrophages, encounter of apoptotic cells by tumour macrophages should result in the induction of anti-inflammatory response. It has been demonstrated that apoptotic tumour cells were able to suppress a macrophage-mediated anti-tumour response (Reiter et al. 1999). Subsequent studies by Weigert et al. have revealed that apoptotic tumour cell-derived sphingosine-1-phosphate mediates downregulation of macrophage production of TNF α and IL-12 as well as increasing production of IL-10 and IL-8 (Weigert et al. 2007).

In an attempt to identify other tumour-derived factors that help to induce TAMs pro-tumour properties, Kuang and colleagues have shown that hyaluronan produced by tumour cells can also be involved (Kuang et al. 2007). Their studies demonstrated that intermediate-sized fragments of hyaluronan can alter macrophage expression profiles, reduce the production of typical “classical activation”-associated molecules such as CD64, CD86, HLA-DR or TNF α and instead, stimulate the production of IL-10. In other models, similar effects on macrophage phenotype shift have been reported for IgG or released nucleotides (Mosser & Edwards 2008).

Altogether, the release of anti-inflammatory cytokines (IL-10, PGE2) and pro-angiogenic factors has a profound impact on the tumour environment, suppressing anti-tumour immune responses as well as supporting development of the vasculature and consequently, improving nutrient supply to the tumour cells. However, TAMs' effects on the tumour reach even further as they have been shown to secrete survival and proliferative agents too. Importantly in the context of this work, in BL, TAMs have been shown to release BAFF (B cell-activating factor of the TNF family), a survival factor for B lymphoma cells (Levens et al. 2000; Ogden et al. 2005).

Moreover, in various tumours milieu TAMs have been shown to release proteolytic enzymes, particularly of the MMP family, which engage in extracellular matrix degradation and can contribute to basement membrane destruction, facilitating metastasis. MMPs, particularly MMP-9, activity have an equally important role in the process of neo-vascularisation (Mantovani et al. 2009; Qian & Pollard 2010).

Finally, the environment-driven induction of macrophage chemoattractants (e.g. MCP-1, CCL5 or CXCL12) secretion by TAMs themselves can result in further magnification of the effect of TAMs on the tumour progression process (Solinas et al. 2009).

1.3 Chemokines

Chemokines are a group of small-molecular weight, chemoattractant cytokines. As of today, the human family consists of 46 chemokine members for which there are 19 identified functional receptors (Zlotnik et al. 2006). Based on the position of the conserved cysteines in their amino acid sequence, chemokines are subdivided into 4 groups: XC, CC, CXC, and CX3C. The largest subfamily, the CC group (CCL1 - CCL28), consists of proteins in which the first two of four cysteines are located next to each other. In the second biggest group, CXC (CXC1-CXC17), the first two cysteines are separated by one amino acid. Fractalkine (CX3CL1), the only protein of the CX3C group contains three amino acids between its cysteines whereas the only two members of XC subfamily have only two of the four cysteines (Zlotnik et al. 2006).

Despite the sequence differences, most chemokines are similarly sized secreted proteins (60-125 amino acids; 8-15 kDa) with similar three-dimensional structure (Olson & Ley 2002; Onuffer & Horuk 2002). The exceptions are fractalkine (CX3CL1) and CXCL16, much bigger transmembrane proteins, in which the classically built chemokine fragment is located atop a long, mucin-like stalk (Ludwig & Weber 2007).

Chemokine signalling is mediated via a seven-transmembrane subfamily of G-protein coupled receptors (CCR1 - CCR10, CXCR1 - CXCR7, XCR1, and CX3CR1) (Zlotnik et al. 2006). In addition to the functional receptors involved in signal transduction, regulatory, non-signalling chemokine “interceptors” have been identified (D6, Duffy antigen, and possibly also CXCR7) (Graham 2009). Unusually, the network of chemokine ligand - receptor interactions is highly complex, with some ligands binding to more than one receptor and some of the receptors having several ligands (Gerard & Rollins 2001; Zlotnik et al. 2006).

This elaborate system allows chemokines to tune a great number of physiological processes. Chemokine signalling has mainly been associated with regulating diverse aspects of leukocyte trafficking, including directing the migration of various subsets of cells at different stages of immunological responses as well as their development and maturation processes (Moser & Willmann 2004). However, chemokines also exert their effects on other types of cells and their function in angiogenesis (mainly CXC chemokines) or neuron-microglial interactions has been recognised (Harrison et al. 1998; Belperio et al. 2000). Not surprisingly therefore, dysregulated chemokine expression is a common feature of multiple pathologies, often significantly contributing to their development and progression (Gerard & Rollins 2001; Jin et al. 2008).

1.3.1 MCP-1

MCP-1 (Monocyte chemoattractant protein-1; CCL2), a member of a CC chemokine subfamily, is one of the most abundantly expressed and so, studied, chemokines (Van Coillie et al. 1999). This 76 amino acid-long protein exists in several 8 – 15 kDa forms depending on the state of its glycosylation and the presence of the signalling sequence (Ishii et al. 1995; Proost et al. 1998).

Secretion of MCP-1, either constitutive or upon certain stimulation, is a common feature of numerous types of cells. It was reported for instance by mononuclear, epithelial and endothelial cells, fibroblasts, smooth muscle cells, chondrocytes, microglia or eosinophils (Van Coillie et al. 1999). In addition, various neoplastic cells have been found to release this chemokine (Mantovani et al. 2002).

To exert its function MCP-1 binds to the CCR2 receptor. The best-characterised role of MCP-1 is to induce chemotaxis of cells carrying CCR2 in the direction of higher concentrations of MCP-1, allowing them to transmigrate from the blood stream to the tissues. This chemokine is mainly seen as a strong chemoattractant for mononuclear phagocytes but it can also act on T cells, B cells and natural killer cells (Allavena et al. 1994; Qin et al. 1996; Corcione et al. 2002). The effect of MCP-1 on the migratory properties of neutrophils is, however, very weak (Rollins et al. 1991). On top of its ability to regulate trafficking of leukocytes, MCP-1 has also been found to induce chemotaxis of endothelial cells, vascular smooth muscle cells, neuronal progenitors and hepatic stellate cells (Marra et al. 1999; Widera et al. 2004; Arefieva et al. 2005; Ma et al. 2007).

Although the mediation of chemotaxis is perhaps the most important function of MCP-1, other direct effects on its target cells have been described. In monocytes, upon MCP-1 binding an increase in the intracellular Ca^{2+} level and respiratory burst have been observed (Rollins et al. 1991). In addition, Jiang and colleagues have demonstrated that MCP-1 binding affects cytokine and adhesion molecule expression pattern in monocytes (Jiang et al. 1992). Moreover, an increase in NK and T cell cytotoxicity might be a result of MCP-1 signalling (Taub et al. 1996). MCP-1-driven proliferation of vascular smooth muscle cells has been reported by Seltzman et al. whereas Roca with co-workers have provided evidence for its role in promoting monocyte survival and mediating their differentiation towards M2-like phenotype (Seltzman et al. 2002; Roca et al. 2009).

An increased MCP-1 expression is characteristic for many disorders, in particular inflammatory conditions like atherosclerosis, rheumatoid arthritis or Crohn's disease (Van Coillie et al. 1999; Deshmane et al. 2009). Moreover, MCP-1 is believed to be an important player in the process of tumorigenesis. It is one of the most abundantly

expressed chemokines in tumours (Mantovani et al. 2002). Tumour cell-derived MCP-1 is believed to substantially contribute to macrophage infiltration. In fact, a correlation has been observed between the amount of MCP-1 produced by the tumour and the number of TAMs (Zhang et al. 1997; Nesbit et al. 2001; Murdoch et al. 2004). As was discussed above, MCP-1 can support the malignant progression by stimulating angiogenesis, for example by driving the chemoattraction and proliferation of vascular smooth muscle cells (Salcedo et al. 2000; Selzman et al. 2002; Ma et al. 2007). Pro-tumour effects of MCP-1 can also be achieved through its contribution to survival and M2-like-polarisation of tumour macrophages (Roca et al. 2009).

1.3.2 Fractalkine (CX3CL1)

1.3.2.1 Structure and functions of fractalkine

Fractalkine (FKN) the only chemokine in the CX3C group, is distinct from most other chemokines by its complex structure and plasma membrane exposure. The conserved chemokine fragment (76 amino acid-long) is placed at the NH₂-terminal end of the protein and makes up less than one third of its size (Bazan et al. 1997). A highly glycosylated mucin-like stalk constitutes the biggest central part of the chemokine while a transmembrane domain and intracellular tail are located at the COOH-end of the protein (Bazan et al. 1997) (Figure 1.1).

Fractalkine is expressed by many cell types, including neurons, epithelial and endothelial cells, mature dendritic cells, smooth muscle and vascular smooth muscle cells as well as B and non-B lymphocytes (Harrison et al. 1998; Harrison et al. 1999; Papadopoulos et al. 1999; Lucas et al. 2001; Ludwig et al. 2002; Truman et al. 2008). CX3CR1, the only identified receptor for FKN, has been detected on microglia, other mononuclear phagocytes, neuronal cells, dendritic cells, NK cells, subsets of T cells, platelets and smooth muscle cells (Imai et al. 1997; Combadiere et al. 1998; Nishiyori et al. 1998; Lucas et al. 2003; Schafer et al. 2004).

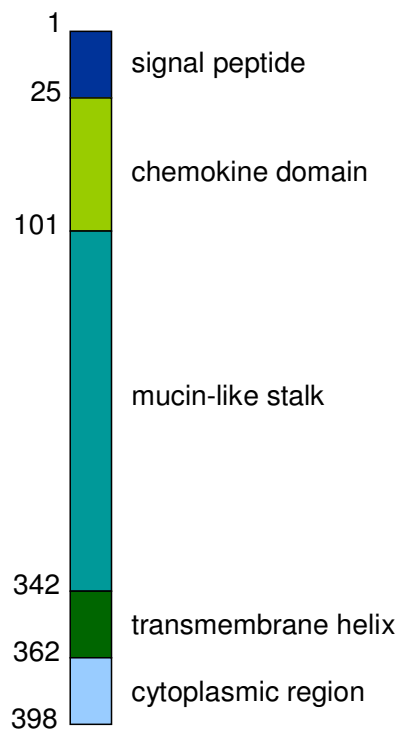


Figure 1.1 Schematic picture of fractalkine

Mechanism of fractalkine-mediated cell adhesion

The structural complexity of FKN is reflected in the complexity of its functions and their regulation. Surface exposed FKN's interaction with CX3CR1 may result in a firm adhesion of receptor-carrying cells (Imai et al. 1997). In order to perform a classical chemokine role in directing chemotaxis FKN needs to be liberated from the surface by proteolytic cleavage (Imai et al. 1997). Interestingly, FKN/CX3CR1-mediated adhesion, unlike FKN/CX3CR1-driven chemotaxis, seems to be independent of further receptor signalling that involves G-protein activation (Imai et al. 1997). This has been observed both in studies where pertussis toxin was used to uncouple the receptor from G proteins and in experiments involving mutated non-signalling forms of CX3CR1 (Fong et al. 1998; Haskell et al. 1999). In some studies, however, FKN/CX3CR1 binding was able to induce Ca^{2+} flux in the adherent cells but the response was weaker than that stimulated by the soluble form of FKN (Harrison et al. 1998). An interaction between NK cells and the membrane-anchored FKN seems to be an exception for this rule as it is known to mediate NK cell activation (Robinson et al. 2003; Yoneda et al. 2003).

It has been demonstrated that it is the FKN chemokine domain that is responsible for adhesion. No difference in the binding efficacy has been observed in studies with proteins in which the FKN chemokine domain was placed on top of other protein stalks (Fong et al. 2000). Nevertheless, exposure of the chemokine domain on the long projection has been found to be indispensable, as the immobilised chemokine domain by itself was much less efficient than full length FKN in mediating capture and adhesion of cells under flow (Fong et al. 2000). More recent analysis revealed that for effective adhesion surface-exposed FKN has to form aggregates (Hermand et al. 2008). However, Haskell and colleagues who analysed the affinity with which chimeric proteins comprised of various chemokines fused to the FKN mucin-like stalk, mediated adhesion have observed that FKN chemokine domain was the most efficient (Haskell et al. 2000). This led to the conclusion that this is not only the exposure on the mucin stalk but also the special type of FKN chemokine domain/CX3CR1 interaction that is important for the strength of FKN-dependent adhesion (Haskell et al. 2000).

The 95-100 kDa-sized (Bazan et al. 1997; Garton et al. 2001) membrane-anchored FKN is known to be cleaved and shed. This process, mediated by two proteases from the ADAM family – ADAM-10 and ADAM-17, is believed to regulate FKN-dependent adhesiveness (Garton et al. 2001; Tsou et al. 2001; Hundhausen et al. 2003; Hundhausen et al. 2007). ADAM-10 has been found responsible for the constitutive shedding of FKN, while ADAM-17 activity, is observed after cell stimulation, for example with PMA (Garton et al. 2001; Tsou et al. 2001; Hundhausen et al. 2003). Proteolysis, which generates a 90-95 kDa fragment of FKN, is known to take place in a membrane-proximal area of the chemokine within the last 45 amino acids of the mucin stalk (Garton et al. 2001; Hundhausen et al. 2007).

Evidence has accumulated that regulation of FKN-driven cell-cell adhesion can also be mediated through its internalisation. Liu et al. found that FKN is in constant movement between the plasma membrane and a poorly characterised endocytic compartment (Liu et al. 2005). A recent report from the same group has shown that the process of internalisation is clathrin/dynamin-dependent (Huang et al. 2009).

Furthermore, the critical role of the FKN C-terminus in this process has been demonstrated (Andrzejewski et al. 2010).

Generation and functions of soluble fractalkine

FKN proteolysis can also be mediated by proteases other than ADAM-10 and ADAM-17. Cathepsin S has been found to be involved in the process of release of an as yet unidentified fragment of FKN from the neuronal cells under neuropathic conditions (Clark et al. 2009). Additionally, MMP-2 has also been implicated (Dean & Overall 2007; Bourd-Boittin et al. 2009). Dean and Overall identified FKN as a potential MMP-2 substrate using a degradomic approach and have provided confirmation that MMP-2 is capable of cleaving FKN. The potential cleavage site for MMP-2 is situated at the boundary between the FKN chemokine domain and its mucin stalk (Dean & Overall 2007). This would be expected to give rise to a FKN fragment within the size-range of other small secreted chemokines (i.e. 8-15 kDa). However, the existence of such a small N-terminal fragment of FKN has never been confirmed in either *in vivo* or *ex vivo* studies.

Various scientific publications have reported on the chemoattractant properties of soluble FKN for mononuclear phagocytes, NK cells, T cells, microglia and smooth muscle cells (e.g. Bazan et al. 1997; Imai et al. 1997; Lucas et al. 2003). However, substantial discrepancies exist between different studies on the ability of soluble FKN to attract mononuclear phagocytes. While in some of these studies FKN was found to attract these cells very effectively (e.g. Bazan et al. 1997; Harrison et al. 1998; Chapman et al. 2000; Lucas et al. 2003), in others the FKN effect appeared to be rather poor: (Imai et al. 1997; Pan et al. 1997; Umehara et al. 2001; Ludwig et al. 2002; Vitale et al. 2004). Unfortunately, in most of these reports limited information is available on the source or length of the tested FKN fragment and therefore, explanations for the observed differences cannot be easily identified.

In a manner similar to that previously described here for MCP-1, soluble FKN has also been shown to play roles other than mediation of chemotaxis. For instance, there is a growing evidence for FKN acting as a survival signal for monocytic phagocytes. Boehme et al. have observed that the presence of FKN strongly supports cultured microglia survival, even when they are induced to undergo death by Fas ligand

binding (Boehme et al. 2000). FKN has also been shown to support the survival of cultured monocytes (Landsman et al. 2009).

Moreover, Landsman and colleagues observed that *in vivo*, FKN or CX3CR1 knock out resulted in apoptosis and depletion of a Gr1^{low} CX3CR1^{high} subset of monocytes (Landsman et al. 2009). FKN has also been found to serve as a cell survival factor for intestinal epithelial cells (Brand et al. 2002).

It has also been demonstrated that soluble FKN may provide support for the process of neovascularisation. This can be exerted by mediation of endothelial cell proliferation and new vessel formation (Lee et al. 2006). FKN is believed to act as an anti-apoptotic and mitogenic signal for vascular smooth muscle cells as well (White et al. 2010).

In addition, FKN seems capable of modulating gene expression and so, type of immunological response of microglial cells. Studies on LPS or IFN- γ -treated microglia have revealed that addition of FKN results in the inhibition of the release of TNF α , IL-6 and NO (Zujovic et al. 2000; Mizuno et al. 2003). These observations have been further confirmed by Cardona et al. who showed that CX3CR1-deficient mice suffered from excessive neurotoxicity after LPS injection (Cardona et al. 2006). Furthermore, FKN can promote apoptotic cell clearance by macrophages by inducing the production of MFG-E8. This process can further contribute to macrophage shift towards M2-like phenotype (Miksa et al. 2007).

Interestingly, the outcome of FKN signalling seems to be dependent on the concentration of FKN present in the target cell environment. Mizutani and colleagues tested the effect of different concentrations of soluble FKN on the amount of TNF α released from LPS-treated macrophages and found that only low concentration of FKN resulted in reduction of secretion of the inflammatory agent. Conversely, high concentrations of FKN caused additional release of TNF α and IL-23 (Mizutani et al. 2007).

1.3.2.2 The role of fractalkine in the central nervous system

In the central nervous system, FKN is abundantly and constitutively expressed by neurons whereas its receptor, CX3CR1 is found on microglial cells (Harrison et al.

1998). FKN/CX3CR1 interactions have been found to be an important, although very complex, method of neuron-microglia communication.

Initial studies demonstrated that FKN would function in the brain during acute events, as a chemoattractant and activator for microglia (Pan et al. 1997; Maciejewski-Lenoir et al. 1999; Chapman et al. 2000). The fact that FKN-deficient mice have been shown to be partly protected from ischemia-reperfusion-driven injury strongly supports this notion (Soriano et al. 2002).

At the same time, FKN has been found to play an important role in inhibiting microglial-mediated neurotoxicity. Such an effect of FKN has been observed both in the previously described, *in vitro* experiments on LPS and IFN- γ -treated microglia (Zujovic et al. 2000; Mizuno et al. 2003) and in a convincing *in vivo* study on CX3CR1-deficient mice (Cardona et al. 2006). The effect's dependence on the chemokine concentration may again be an explanation for the observed dual role of FKN (Maciejewski-Lenoir et al. 1999).

On top of that, FKN release from neurons (perhaps cathepsin S-dependent) has been implicated in mediation of pathological pain (Milligan et al. 2004; Clark et al. 2007).

1.3.2.3 The role of fractalkine in disease

In most normal cells, FKN expression is induced by inflammatory agents. IL-1, IFN- γ , TNF α and LPS have been implicated (Harrison et al. 1999; Fujimoto et al. 2001; Ludwig et al. 2002). Not surprisingly therefore, its elevated expression has been found in most inflammatory diseases (e.g. atherosclerosis, rheumatoid arthritis, inflammatory bowel disease and glomerulonephritis), where it is believed to considerably contribute to the disease progression (Muehlhoefer et al. 2000; Furuichi et al. 2000; Volin et al. 2001; Combadiere et al. 2003).

FKN's role in tumorigenesis has also been investigated. In most tumours in which FKN expression was detected, it appeared to correlate with better prognosis (Ohta et al. 2005; Matsubara et al. 2007; Yu et al. 2007; Blum et al. 2008; Hyakudomi et al. 2008). Interestingly, it has been observed that high but not low FKN concentrations had an anti-tumour effect (Matsubara et al. 2007). This seemed to be in agreement with the findings of Mizutani et al. (discussed above) on the high concentration-

dependence induction of inflammation and immune tolerance depending on low concentrations of FKN (Mizutani et al. 2007). Consequently, the anti-tumour action of FKN has been demonstrated to rely on the activation of NK cells, cytotoxic T cells and DCs (Guo, Chen, et al. 2003; Guo, Zhang, et al. 2003).

These propensities of FKN have been found useful when tested as a potential anti-cancer therapy. When colon carcinoma, melanoma or lymphoma cell lines were modified to express FKN, they were shown to have a reduced capability for forming tumours *in vivo* (Lavergne et al. 2003; Xin et al. 2005; Vitale et al. 2007).

In addition, CX3CR1 expressed by tumour cells has been found to home to sites of FKN expression during the process of metastasis (Shulby et al. 2004; Marchesi et al. 2008).

1.4 Aims of the project

Given the potential that tumour-associated macrophages may have in mediating tumour progression (Mantovani et al. 1992), the broad aim of this project was to increase our understanding of mechanisms that lead to macrophage infiltration of Burkitt's lymphoma. As BL cells in the tumour are known to undergo spontaneous apoptosis at a high rate (Gregory et al. 1991) and apoptotic cells have previously been shown to be a source of chemoattractant agents for macrophages (e.g. Horino et al. 1998; Knies et al. 1998; Lauber et al. 2003; Gude et al. 2008), the focus was on chemotactic factors released from dying BL cells.

Following on from work carried out in the past in this laboratory by Truman, who identified fractalkine as a factor released from apoptotic BL cells responsible for macrophage chemotaxis, and also showed that another classical chemokine might be involved in this process (Truman 2005), initial studies were carried out to identify that chemokine. Later, the attention was transferred to fractalkine. The process of cell death-specific modulation of FKN and mechanisms of its release after death-stimuli were investigated in detail.

Chapter 2

Materials and Methods

2.1 Cells

2.1.1 Primary cells isolation and culture

2.1.1.1 Monocytes and HMDMs

Primary cells were obtained from peripheral blood of healthy volunteer donors. Mononuclear cells were isolated from the peripheral blood by Ficoll gradient (Ficoll-Paque Plus; GE Healthcare). In a 50 ml Falcon tube (BD Biosciences), 35 ml of blood diluted with sterile PBS was layered on 15 ml of room temperature Ficoll. Cells were centrifugated at 900 x g, at room temperature for 20 min, with the break off. The cell pellet was collected from Ficoll/serum interface, transferred to a fresh tube and washed in PBS (900 x g, room temperature, 10 min, break on). The washing procedure was repeated three times. Monocytes were sorted by positive selection from isolated cells incubated with anti-CD14 magnetic micro beads (Miltenyi Biotec). 20 µl of beads was added to each 80 µl of MACS buffer (PBS, 2 mM EDTA, 0.5% BSA) containing 1×10^7 cells. Reaction tubes were incubated on ice for 15 min. Cells were washed and re-suspended in 500 µl of MACS buffer, then passed through a MACS MS column (Miltenyi Biotec) placed in a magnetic field. CD14⁺ cells were eluted in MACS buffer after the column was removed from the magnetic field. Monocyte purity was monitored by CD14 labelling. Over 90% of separated cells were CD14-positive.

To obtain human monocyte-derived macrophages (HMDMs), cells pelleted in the last PBS-wash step were re-suspended in IMDM (PAA) (containing L-glutamine (2mM) (PAA), 25mM HEPES, 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen)) at 4×10^6 /ml concentration. 3 ml of cells were seeded per well of 6-well plates. After 1 hr incubation (37°C, 5% CO₂) medium and non-adherent cells

were removed and IMDM containing L-glutamine, HEPES, penicillin/streptomycin and supplemented with 10% autologous serum was instead added to the wells. Cells were fed at day 3. HMDMs were collected at day 6 by washing with cold PBS and incubating for 5 min with detachment buffer (HBSS (without Ca and Mg), 5 mM EDTA, 0.2% BSA). Subsequently, a cell lifter was used to detach the cells. Cells were washed twice to remove excess of EDTA.

2.1.1.2 Mouse germinal centre cells

Germinal centre and resting, follicular B cells were separated from spleens of wild-type mice immunised with sheep red blood cells (SRBCs). The reaction was boosted after 14 days and the spleens were harvested after a further 12 days. In order to obtain germinal centre B cells, splenocyte suspensions were incubated with rat anti-IgD antibodies (BD Biosciences). Subsequently, IgD-expressing cells were depleted using magnetic separation with goat anti-rat IgG microbeads (Miltenyi Biotec). Then, anti-mouse B220 antibodies (eBioscience) were used to select IgD-negative B cells (Truman et al. 2008). Further control analysis using GL7 antibodies (eBioscience) demonstrated that more than 65% of the obtained IgD-negative B cells expressed high levels of Ly-77 and less than 10% of the IgD-positive follicular B cell fraction expressed high levels of Ly-77.

2.1.1.3 Human germinal centre cells

Human germinal centre B cells were isolated from tonsils within approximately 15 hrs after tonsillectomy. Tonsils were transferred to a Petri dish containing RPMI with antibiotics and cut into pieces to release cells. Cells were recovered from the supernatant after sedimentation at 1 x g then washed in fresh RPMI. Mononuclear cells (MNC) were isolated by density gradient centrifugation on Ficoll-Paque Plus at 800 x g for 20 min. MNCs harvested from the media interface were then washed twice in RPMI. T cells were depleted by rosette formation with AET-treated SRBCs. T cell rosettes were then removed by sedimentation on Ficoll-Paque Plus as above. Resting B cells were obtained from T cell-depleted MNCs by depletion of residual T cells and activated B cells including germinal centre B cells by magnetic cell separation using mouse anti-human CD14, 61D3, mouse anti-human CD38, OKT10 (Birmingham University), mouse anti-human CD3, OKT3 (Birmingham University)

antibodies and goat anti-mouse IgG-coated MACS microbeads (Miltenyi Ltd). For isolation of germinal centre B cells, residual T cells and resting B cells were depleted by magnetic cell separation using Dynabeads (DynaL Ltd). T cell-depleted MNCs were incubated with mouse anti-human CD14, mouse anti-human CD39, AC2 (Birmingham University), mouse anti-human CD3, OKT3 (Birmingham University) and mouse anti-human IgD, HJ9 (Becton Dickinson) antibodies and with Dynabeads coupled with sheep anti-mouse IgG and subsequently separated on a magnet. Recovered cells were subjected to a second round of magnetic depletion as above. Remaining germinal centre B cells were then washed twice in RPMI.

2.1.2 Cell lines maintenance

Group I (biopsy-like) EBV-negative (BL2, BL2/Bcl-2, L3055) and EBV-positive (Mutu I, SAV) Burkitt's lymphoma cell lines were used in the studies. Apoptosis resistant BL2/Bcl-2 cells were previously generated in this group from BL2 cells by stable transfection with *bcl-2* (using pEFbcl2-MC1neopA vector, following formerly established protocol (Wang et al. 1996)). All BL cell lines as well as HL-60 cells (human promyelocytic leukaemia cells) were cultured in suspension in RPMI 1640 (Invitrogen) supplemented with 10% foetal calf serum (PAA), 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

2.2 Induction and evaluation of apoptosis

BL cells from confluent cultures were washed 3 x 5 min (220 x g) in serum-free RPMI 1640 (with L-glutamine and penicillin/streptomycin), counted and re-suspended in the same medium at 10×10^6 /ml. Apoptosis was induced in BL cells by treatment with 1µM staurosporine (Calbiochem), 2 µM ionomycin (Calbiochem) or by UVB irradiation (100mJ/cm²). Cells were kept in cell culture incubators (37°C, 5%CO₂) for indicated period of time (1-24 hrs). The level of apoptosis was evaluated by staining with Annexin V (AxV)-FITC and propidium iodide (PI) (Bender

Medsystems), using Beckton XL flow cytometre. In short, 1×10^6 cells were placed in a FACS tube, spun down and re-suspended in 100 μ l of Annexin V binding buffer (10 mM HEPES/NaOH (pH 7.4); 140 mM NaCl; 2.5 mM CaCl_2). 1 μ l of Annexin V-FITC was added per tube and incubated for 10 min on ice. Cells were next washed and re-suspended in 500 μ l of binding buffer. 10 μ l of propidium iodide was added to each tube prior to FACS measurement.

2.2.1 Inhibitor studies

BL cells were prepared as described above. Protease inhibitors (at the concentrations indicated in Table 2-1) were added to cell culture 30 min prior to induction of cell death.

Table 2-1. List of inhibitors and used concentrations

inhibitor	Specificity	working concentration	reference
Z-VAD-FMK	poly-caspase	25-100 μ M	Mills et al. 1998
GM6001	metalloproteinases: MMPs, ADAMs (ADAM-17)	10-50 μ M	Garton et al. 2001; Litterst et al. 2007
TIMP-1	metalloproteinases: MMPs, ADAM-10	10 μ M	Ludwig et al. 2002; Ito et al. 2004
TAPI-2	metalloproteinases: MMPs, ADAM-17	5-20 μ M	Tsou et al. 2001; Ito et al. 2004
chymostatin	cysteine and serine proteases (cathepsin A, B, D, chymotrypsin)	100 μ M	Marchetti et al. 2003
leupeptin	cysteine and serine proteases (cathepsin B, trypsin, plasmin)	100 μ M	Marchetti et al. 2003
E-64-d	cysteine proteases (cathepsin B,H,L)	10 μ M	Rofstad et al. 2006
aprotinin	serine proteases (trypsin, chymotrypsin, plasmin)	0.5 μ M	Goselink et al. 2006
Z-Leu-Leu-CHO	Calpains	25 μ M	Tsubuki et al. 1996
calpeptin	Calpains	25 μ M	Goñi-Oliver et al. 2007

Z-YVAD-FMK	caspase-1	25 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-VDVAD-FMK	caspase-2	25 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-DEVD-FMK	caspase-3 (-6,-7,-8)	25-100 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-LEVD-FMK	caspase-4 (-5)	25 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-WEHD-FMK	caspase-5 (-1,-4,-8)	25 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-VEID-FMK	caspase-6	25 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-IETD-FMK	caspase-8 (granzyme B)	25 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-LEHD-FMK	caspase-9 (-8)	25 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-AEVD-FMK	caspase-10 (-6,-8)	25-100 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008
Z-LEED-FMK	caspase-13	25 μ M	Garcia-Calvo et al. 1998; Pereira and Song 2008

2.3 Microparticles isolation and analysis

For isolation of MPs, BL cells from confluent cultures were washed 3 x 5 min (220 x g) in serum-free RPMI 1640 (supplemented with L-glutamine and penicillin/streptomycin). Cells were resuspended at 1×10^7 /ml and induced into apoptosis using 1 μ M staurosporine. After two hrs, cells were pelleted by centrifugation (5 min, 300 x g). Supernatants were transferred to fresh tubes and centrifuged again at (1400 x g) for 10 min to remove bigger cellular debris. MPs were isolated by ultracentrifugation of the obtained supernatants (placed in polyallomer tubes (Beckman Coulter)) using an Optima TLX ultracentrifuge (Beckman Coulter) (100 000 x g, 4°C, 30 min). Supernatants were discarded and particle pellets were re-suspended and analysed further. For FACS analysis, MPs were re-suspended in PBS. MP size was assessed by flow cytometry by comparison with 50 nm (Miltenyi Biotec) and 1 μ m (Invitrogen) diameter beads. For Western blot analysis MPs were re-suspended in lysis buffer (1 mM EDTA, 10 mM HEPES, 1% Triton-X-100) and incubated for 30 min on ice prior to addition to LDS sample

buffer (NuPAGE, Invitrogen). For confocal microscopy studies, MPs were re-suspended in fixation medium from Fix&Perm cell permeabilisation kit (Invitrogen). All reagents used for MP isolation and studies were passed through 0.22 μm filters.

2.4 Immunostaining

1×10^6 of BL cells were placed in each FACS tube. For surface staining, cells were incubated for 30 min with 5% normal goat serum in PBS. After washing in 1% BSA/PBS, cells were re-suspended in 100 μl of 1% BSA/PBS containing antibodies of choice (or corresponding isotype control antibodies) and incubated for 30 min at 4°C. Next, cells were washed in 1% BSA/PBS and then incubated in 100 μl of 1% BSA/PBS containing matching secondary FITC- or PE-conjugated antibodies (30 min, 4°C). For intracellular staining, before incubation with antibodies, cells were fixed and permeabilised according to the manufacturer's protocol using Cytofix/Cytoperm kit (BD Pharmingen) or Fix&Perm cell permeabilisation reagents (Invitrogen). Cells were analysed immediately or were kept in 1% formaldehyde/1% BSA/PBS at 4°C prior to analysis. Antigen expression was assessed using Beckton XL flow cytometre. Concentrations of antibodies are shown in Table 2-2. Post-measurement analyses were performed in FlowJo (Tree Star). Histograms of unfixed (surface-stained) cells were drawn only from cells that fell into a "viable" gate based on their forward versus side scatter.

2.5 Immunohistochemistry

Slides containing 5 μm formalin-fixed, paraffin-embedded tumour sections obtained from xenograft tumours, generated by subcutaneous injection of 10×10^6 BL2 cells into SCID mice, were stained with mouse monoclonal antibodies anti-human MCP-1 (R&D Systems, MAB2791). Slides were first dewaxed in histoclear (2 x 5 min), dehydrated in dilutions of ethanol (100% - 90% - 70%) (5 min in each) and next heated in Antigen Retrieval Solution (Vector Laboratories) in a microwave oven

(3 x 5 min). After cooling in tap water (~20 min), slides were incubated with 2% hydrogen peroxide in order to block endogenous peroxidase activity (15 min). After washing in PBS (2 x 3 min) and transferring to Sequenza chambers (Thermo Scientific) slides were blocked with serum-free Protein Block (Dako Cytomation) (10 min). MAB2791 antibodies diluted in ChemMate Antibody Diluent (Dako Cytomation) were then added to the chambers and incubated overnight at 4°C. Next, anti-mouse-biotin-conjugated antibodies diluted in ChemMate Diluent were added to chambers for 30 min (room temperature). Vectastain Elite ABC reagent (Vector Laboratories) was then added to amplify signal (30 min) and next the enzyme substrate 3,3'-diaminobenzidine (DAB) (Vector Laboratories) was used (5 min). Chambers were washed in PBS between the staining steps. Slides were counterstained with haematoxylin (3 min). No primary antibodies were added in the control slides.

Table 2-2 List of antibodies and used concentrations

antibodies	catalogue no	Producer*	used concentration**				
			WB	IF	IHC	ELISA	N
mouse monoclonal anti-human MCP-1 (clone 23002)	MAB2791	RDS	4 µg/ml	15 µg/ml	25 µg/ml		
mouse monoclonal anti-human MCP-1 (clone 23007)	MAB679	RDS	2 µg/ml			1 µg/ml	
goat polyclonal anti-human MCP-1	AF279-NA	RDS	1 µg/ml				2µg/ml
mouse monoclonal anti-human FKN (clone 81513)	MAB3651	RDS	2 µg/ml	15 µg/ml			
goat polyclonal anti-human FKN C-terminus	sc-7225	SCB	0.5 µg/ml				
rabbit polyclonal anti-mouse FKN	TP-233	TPB	1 µg/ml				
rabbit polyclonal anti-caspase-3 antibodies	9662	CST	1:500				
rabbit polyclonal anti-active caspase-3 antibodies	9661	CST		1:50			
rabbit polyclonal anti-active caspase-3 antibodies	PC679	C	0.5 µg/ml				
rabbit polyclonal anti-human calpain I large subunit	2556	CST	1:500	1:25			
rabbit polyclonal anti-human calpain II large subunit	2539	CST		1:25			
mouse monoclonal anti-β-actin (clone AC-15)	A5441	SA	1:5000				

Table 2-2 continued

antibodies	catalogue no	Producer	used concentration			
mouse IgG1	M9269	SA		20 µg/ml		
mouse IgG2	M9144	SA		20 µg/ml		
sheep anti-mouse IgG-HRP	NXA931	GEH	1:2000			1:1000
goat anti-mouse IgG-biotin	BA-9200	VL			1:250	
goat anti-mouse IgG-FITC	F0257	SA		1:100		
goat anti-mouse IgG-PE	P9287	SA		1:100		
goat anti-mouse IgG-AF488	A11001	I		1:50		
goat anti-rabbit IgG-HRP	PO448	DC	1:2000			
goat anti-rabbit IgG-FITC	F0382	SA		1:100		
goat anti-rabbit IgG-PE	P9795	SA		1:100		
goat anti-rabbit IgG-AF594	A11012	I		1:50		
rabbit anti-goat IgG-HRP	PO449	DC	1:2000			

* RDS=R&D Systems; SCB= Santa Cruz Biotechnology; TPB= Torrey Pines Biolabs; CST= Cell Signaling Technology; C= Calbiochem; SA= Sigma Aldrich; GEH= GE Healthcare; VL= Vector Laboratories; I= Invitrogen; DC= DakoCytomation;

** WB=Western blot; IF=Immunofluorescence; IHC=Immunohistochemistry; N=Neutralisation

2.6 ELISA

2.6.1 Sandwich ELISA

MCP-1 release was assessed by sandwich ELISA of supernatants collected from overnight cultures of HL-60 cells (5×10^6 /ml in culture medium, alone or stimulated with LPS (1 ng/ml)) as well as supernatants collected from BL2, BL2/Bcl-2 and Mutu I cells treated with 1 μ M staurosporine for 3 hrs to induce apoptosis (1×10^7 /ml in serum-free RPMI). ELISA DuoSet (R&D Systems) for human MCP-1 was used according to the manufacturer's protocol. In short, ELISA plates (Nunc) were coated with goat anti-MCP-1 antibodies (1 μ g/ml in PBS; 50 μ l/well) and incubated overnight at 4°C. Plates were washed 3 times with 0.05% Tween-20/PBS and blocked using 2% FCS/PBS (1 hr/room temperature). Plates were washed and cell supernatants were added to the wells (100 μ l/well) and incubated for two hrs at room temperature. Next, supernatants were removed, plates were washed thoroughly and detection mouse anti-MCP-1 antibodies were added (0.5 μ g/ml; 100 μ l/well). Plates were incubated for two hrs at room temperature and washed again before adding biotinylated anti-mouse antibodies (0.5 μ g/ml; 100 μ l/well) for 1 hr at room temperature. Following washing, plates were incubated with streptavidin-HRP (diluted 1:200, 100 μ l/well) for 20 min at room temperature. After the final washing step Tetramethylbenzidine/H₂O₂ (Sigma-Aldrich) substrate was added to the wells (100 μ l/well) and incubated for ~20 min. Reaction was stopped with 2N H₂SO₄ (50 μ l/well). Plate was measured on plate reader at 450 nm. All conditions were set up in triplicate. Negative control wells were coated with assay buffer (serum-free RPMI 1640 with 2mM L-glutamine and 100 IU/ml penicillin and 100 μ g/ml streptomycin) or blocking solution (4% BSA/PBS).

2.6.2 Direct ELISA

96-well ELISA plates were coated directly with cell-free supernatants from staurosporine-treated BL cells (cultured at 1×10^7 /ml for 1 – 3 hrs) (100 μ l/well). Plates were incubated for 4 hrs at 37°C, and subsequently blocked with 4% BSA in PBS at 37°C for 30 min. MCP-1 was detected using mouse monoclonal

anti-human MCP-1 antibodies (R&D Systems, MAB679) (1 hr incubation at room temperature) and visualised by incubation with goat anti-mouse IgG-HRP-conjugated antibodies (1 hr at room temperature) (see Table 2-2 for antibody concentrations). Plates were washed 3 times with 0.05% Tween-20/PBS between the incubation steps. Tetramethylbenzidine mix with H₂O₂ was used to develop the plates. Reactions were stopped with 2N H₂SO₄. Results were measured on a plate reader at 450 nm. All conditions were set up in duplicate. Negative control wells were coated with assay buffer (serum-free RPMI 1640 with 2mM L-glutamine and 100 IU/ml penicillin and 100 µg/ml streptomycin) or blocking solution (4% BSA/PBS).

2.7 Transmigration assay

Transmigration assays were performed by using modified Boyden chambers (Figure 2.1). Polycarbonate membrane transwells with 5 µm pore size (Costar Corning) were used in the assays. RPMI containing 0.1% low-endotoxin BSA (Sigma-Aldrich) was used as the assay medium. Monocytes (2 x 10⁶/ml) and 6-day-old HMDMs (1 x 10⁶/ml) were placed in the upper chamber. Recombinant human MCP-1 (1 - 100 ng/ml; R&D Systems); cell-free supernatants from UV-treated BL2 cells, collected 3 hrs post UV-exposure; C5a - positive control (6.25 ng/ml; R&D Systems) or assay medium - negative control, were placed in the lower chamber. When indicated, mouse polyclonal neutralising antibodies anti-human MCP-1 (R&D Systems, AF-479-NA) were also added in the lower chamber to counteract the effect of MCP-1. Monocyte migration assays were carried out for 90 min and HMDM for 240 minutes. Subsequently, cells were removed from the inside of transwells with a cotton bud and transwells were fixed in 100% methanol for 10 min, followed by staining with Diff-Quik (Reagen) (20 min). Number of migrating cells was calculated by counting cells on the underside of the transwell membrane using an inverted microscope. Ten high power fields (x 400) were counted per transwell. All conditions were set up in duplicate.

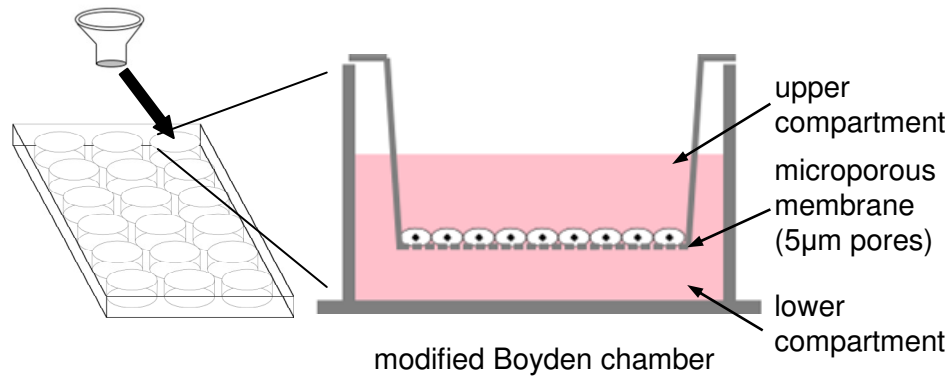


Figure 2.1 Schematic diagram of transmigration assay

2.8 Immunoblotting

2.8.1 Whole cell lysate preparation

For preparation of whole cell lysate samples, cells were collected from the experimental cultures, spun down to remove the supernatants and subsequently re-suspended in lysis buffer (1 mM EDTA, 10 mM HEPES, 1% Triton-X-100) (12.5 µl of the buffer per 1×10^6 cells of initial culture). Samples were vortexed intensively and incubated on ice for 30 min. Next, samples were spun at $18\,000 \times g$, 4°C for 10 min. Supernatants, free of cellular debris, were transferred to fresh tubes and kept at -20°C for further analysis. Protein concentration in whole cell lysate samples was assessed using Bradford dye reagent (Bio-Rad). Dilutions of lysate samples were mixed with the dye and colour concentration was measured on plate-reader at 595 nm wavelength. Protein concentration was calculated based on the readings from serial dilution of human IgG samples of known concentration, which were analysed in parallel with whole cell lysate samples. For each sample 30 µg of proteins was mixed with NuPAGE LDS sample buffer (Invitrogen) containing NuPAGE reducing agent (Invitrogen). Samples were denatured for 10 min at 70°C prior to loading into the gel.

2.8.2 Supernatants preparation

In order to analyse proteins released from cells (1×10^7 cells/ml) during experiments, proteins were first precipitated from supernatants using trichloroacetic acid (TCA). For each sample analysed on the gel, 1 ml of supernatant was mixed with 100 μ l of 100% TCA and incubated on ice for 30 min. Samples were then spun down at 18 000 x g, 4°C for 10 min. Supernatants were discarded and protein precipitates were washed with 1 ml of ice-cold acetone and spun down as before. Acetone was discarded and precipitates were let to dry and subsequently, were re-suspended in NuPAGE LDS sample buffer with NuPAGE reducing agent. Samples were denatured for 10 min at 70°C prior to loading into the gel.

2.8.3 Electrophoresis and Western blotting

Electrophoretic sample separation and their transfer to nitrocellulose membranes were performed using the Invitrogen NuPAGE system and reagents, according to the manufacturer's protocol. Samples, prepared as described above, were loaded into NuPAGE pre-cast 4-12% Bis-Tris gels. SeeBlue Plus2 pre-stained standard (Invitrogen) was used as molecular weight indicator. Protein separation was performed in NuPAGE MES SDS running buffer at 200 V for approximately 35 min. Subsequently, proteins were transferred on to PVDF membranes in NuPAGE transfer buffer, at 30 V for 1 hr. The membranes were blocked at 4°C overnight in 0.1% Tween-20/PBS containing 5% milk. Next, the membranes were incubated with antibodies of choice diluted in 0.1% Tween-20/PBS containing 1% milk (see Table 2-2 for antibody concentrations) for 1 hr at room temperature, washed thoroughly in 0.1% Tween-20/PBS for 1 hr and then incubated with matching HRP-conjugated secondary antibodies (diluted in 0.1% Tween-20/PBS/1% milk). After final washing in 0.1% Tween-20/PBS, bands were developed using ECL reagents (GE Healthcare). Chemiluminescence was detected on photographic film (GE Healthcare).

For the purpose of multiple staining with different antibodies membranes were washed in stripping buffer (100 mM Glycine, 150 mM NaCl, pH 2.6) for 2 hrs and washed in 0.1% Tween-20/PBS prior to re-labelling.

2.9 *In vitro* cleavage assays

Protease capability of cleaving FKN was studied *in vitro* as follows:

2.9.1 Caspase cleavage

75 ng of recombinant human FKN (rh FKN) (R&D Systems) and 300 – 500 ng of recombinant human caspases (-3, -7, -10) (R&D Systems) were diluted in caspase reaction buffer (25 mM HEPES pH 7.5, 10 mM DTT, 0.1% CHAPS) (final volume: 100 µl) and were incubated for 2 hrs at 37°C. When indicated, 2 – 3 µg of caspase inhibitor (Z-VAD-FMK or Z-DEVD-FMK) (R&D Systems) were also added to the samples. Rh FKN incubated in caspase reaction buffer alone served as a control.

100 µg of total protein from untreated BL2 whole cell lysates and 300 – 500 ng of recombinant human caspases (-3, -7, -10) were diluted in caspase or ADAM (25 mM Tris pH 9.0, 2.5 µM ZnCl₂, 0.005% Brij 35) reaction buffers (final volume: 100 µl) and were incubated for 2 hrs at 37°C. When indicated, 2 – 3 µg of caspase inhibitor (Z-VAD-FMK or Z-DEVD-FMK) was also added to the samples. 100 µg of total protein from BL2 cell lysates incubated in caspase or ADAM reaction buffer alone served as a control.

2.9.2 Calpain cleavage

75 ng of recombinant human FKN and 240 ng of active human calpain 1 (BioVision) were diluted in calpain reaction buffer (30 mM Tris-HCl pH 7.5, 1.5 mM DTT, 750 µM CaCl₂) (final volume: 100 µl) and were incubated for 30 min at 37°C. When indicated, 40 ng of calpeptin (Alexis Biochemicals) was also added to the samples. Rh FKN incubated in calpain reaction buffer alone served as a control.

100 µg of total protein from untreated BL2 cell whole lysates and 240 ng of active human calpain 1 were diluted in ADAM reaction buffer (25 mM Tris pH 9.0, 2.5 µM ZnCl₂, 0.005% Brij 35) (final volume: 100 µl) and were incubated for 30 min at 37°C. When indicated, 40 ng of calpeptin was also added to the samples. 100 µg of total protein from BL2 cell lysates incubated in ADAM reaction buffer alone served as a control.

2.10 Caspase-3 activity fluorogenic assay

100 µg of total protein from whole cell lysates of untreated or apoptotic (2 hrs post-staurosporine) or 50 µl of MP lysate (prepared from MPs released from 5×10^7 BL2 cells during 2 hr-incubation with staurosporine; ~90 µg total protein) was diluted in ADAM reaction buffer containing 1.5 µg of caspase-3 fluorogenic substrate (Z-DEVD)₂-Rh110-bisamide (Calbiochem) (total volume: 100 µl). Samples containing only ADAM reaction buffer or lysis buffer combined with 1.5 µg of substrate served as controls. All conditions were set up in duplicate. Reactions were carried out for 2 hrs at room temperature. A fluorescent plate reader was used to measure the outcome (excitation ~485 nm; emission ~535 nm).

2.11 Confocal microscopy

For analysis of intracellular localisation of FKN and active caspase-3 1×10^6 of untreated or staurosporine-treated BL2 cells (2 hrs post-treatment) were fixed and permeabilised using Fix&Perm cell permeabilisation reagents (Invitrogen) according to the manufacturer's protocol. Surface FKN expression was analysed by firstly fixing untreated BL cells or MPs isolated by centrifugation from supernatants of 6×10^7 apoptotic BL cells (2 hrs post-staurosporine) using fixation medium from the Fix&Perm kit. Next, cell and MP samples were blocked in 5% normal goat serum/PBS for 30 min on ice. After washing in 1% BSA/PBS, cells or MPs were re-suspended in 1% BSA/PBS containing mouse monoclonal anti-human FKN antibodies (R&D Systems; MAB3651) and rabbit anti-human active caspase-3 antibodies (Cell Signaling Technology; 9661) (or the corresponding isotype control antibodies) and incubated for 30 min at 4°C. Subsequently, samples were washed in 1% BSA/PBS and then incubated in 1% BSA/PBS containing secondary anti-mouse IgG AF488-conjugated or anti-rabbit IgG AF594-conjugated antibodies (30 min, 4°C, in the dark). After the final wash, samples were re-suspended in 2 drops of ProLong Gold antifade reagent (containing DAPI) (Invitrogen). 15 µl of sample was spread on a microscope slide and covered with a cover slip (thickness 1). Slides were

left overnight (in the dark) for mountant to solidify. Samples were analysed on a Leica SP5C confocal laser scanning microscope. Blue diode (405 nm excitation line), argon (488 nm) and HeNe 2 (594 nm) lasers and 63 x oil objective were used for image acquisition. Huygens Professional software (SVI) was used for spectral deconvolution. Merged images were created in ImageJ software.

Antibody concentration details are collected in Table 2-2.

2.12 Time lapse video microscopy

1 ml of 1×10^6 /ml BL2 cells in 25 mM HEPES-buffered RPMI 1640 + 10% foetal calf serum medium (which was saturated with 5 % CO₂ prior to starting the experiment) were induced into death with 1 μ M staurosporine and placed in a POC mini chamber (PeCon) on a heated microscope stage (37°C) of an Olympus widefield inverted microscope equipped with an Olympus F-View camera. A low-magnification image (10 x objective) of the cells was collected every 15 sec, for 150 min, starting 30 min after the cells were exposed to staurosporine. Cell-P software (Olympus) was used to operate the system.

2.13 Molecular biology techniques

2.13.1 Reverse transcription PCR

RNA was isolated from cells using RNeasy Mini kit (Qiagen), according to the manufacturer's protocol. The concentration of eluted RNA was measured using a NanoDrop (Thermo Scientific).

cDNA was synthesised using reverse transcriptase by mixing 2 μ g RNA, 1 μ l oligo dT (Promega), 1 μ l dNTP mix (Promega) and DEPC-treated H₂O up to 13 μ l and incubating at 65°C for 5 min before placing on ice to cool down. The following reagents were then added to each sample: 4 μ l of 5 x First strand buffer (Invitrogen), 1 μ l 0.1M DTT (Invitrogen), 1 μ l RNase inhibitor (Invitrogen) and 1 μ l reverse transcriptase (SuperScript) (Invitrogen). The reagents were mixed and incubated at

50°C for 75 min, followed by 10 min incubation at 90°C. Samples were cooled down on ice, centrifuged briefly and used immediately for the PCR reactions or stored at -20°C.

Primers used in the reactions:

MCP-1 (from Ohta et al. 2003):

forward 5'-CTGTGCCTGCTGCTCATA-3'

reverse 5'-ATTTCCCCAAGTCTCTGT-3'

Caspase-3 (from Winter et al. 2001):

forward 5'-TTCAGAGGGGATCGTTGTAGAAGTC-3'

reverse 5'-CAAGCTTGTCGGCATACTGTTTCAG-3'

GAPDH:

forward 5'-CGACAGTCAGCCGCATCTTCTTTTGCGTCG-3'

reverse 5'-GGACTGTGGTCATGAGTCCTTCCACGATAC-3'

All primers were sequenced by Eurofins MWG Operon.

Each reaction mixture contained: 2 µl of cDNA, 5 µl 10 x reaction buffer (Promega), 3 µl of MgCl₂ (Promega), 1 µl of dNTPs (Promega), 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), 0.5 µl of Taq polymerase (Promega) and 36.5 µl of DEPC-treated H₂O.

PCR reactions were carried out in thermocycler as follows:

for MCP-1: step 1 - 94°C for 7 min, step 2 - 94°C for 1 min, step 3 - 56°C for 1 min, step 4 - 72°C for 1 min, repeat steps 2 - 4 for 40 cycles, step 5 - 72°C for 5 min.

for caspase-3: step 1 - 94°C for 5 min, step 2 - 94°C for 1 min, step 3 - 52°C for 1 min, step 4 - 72°C for 45 sec, repeat steps 2 - 4 for 28 cycles, step 5 - 72°C for 5 min.

for GAPDH: step 1 - 94°C for 7 min, step 2 - 94°C for 1 min, step 3 - 50°C for 1 min, step 4 - 72°C for 45 sec, repeat steps 2 - 4 for 35 cycles, step 5 - 72°C for 5 min

Reaction products were analysed on 1% agarose gels containing ethidium bromide.

2.13.2 Caspase-3 knock-down

Small interfering RNA (MISSION shRNA; Sigma-Aldrich) was used to silence *caspase-3* in BL2 cells. Five bacterial clones carrying lentiviral plasmids (pLKO.1-Puro) with different anti-caspase-3 constructs from supplied stocks were plated out onto LB (Lysogeny broth) agarose plates containing 100 µg/ml of ampicillin (Sigma-Aldrich). Plates were incubated overnight at 37°C. Colonies picked from each plate were amplified in 3 ml of LB broth (with 100 µg/ml ampicillin) for 8 hrs (on shaker). 0.5 ml of each culture was then used to inoculate 500 ml LB broth for overnight culture (37°C, shaking).

Plasmids were purified from 300 ml of cultures using Plasmid Maxi kit (Qiagen), following the manufacturer's instructions precisely.

Purified plasmids and ViraPower lentiviral packaging mix (pLP1, pLP2, pLP-VSV-G; Invitrogen) were later used for co-transfection of 293FT cells. For each lentiviral plasmid a mix was prepared containing 3 µg of plasmid, 9 µl of the lentiviral packaging mix in 1.5 ml Opti-MEM (Invitrogen). Each of these mixtures was combined with a mix of 36 µl of lipofectamine 2000 (Invitrogen) in 1.5 ml Opti-MEM and incubated for 20 min at room temperature. Each combined plasmid/lipofectamine mix was then added to a well of a 6-well plate containing 6×10^6 of 293FT cells in 10 ml of DMEM supplemented with 5% FCS. After 24 hrs cell medium was replaced with DMEM supplemented with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin (10 ml/well). Supernatants containing virus particles were collected after 48 hrs, passed through 0.45 µm filters and stored at -80°C.

For transduction of BL2 cells, 5-fold serial dilutions of the supernatants in BL cell culture medium were prepared. 1 ml of undiluted supernatant and 1 ml of 7 successive dilutions were placed in wells of a 48-well plate. 0.6×10^6 of BL2 cells in 300 µl culture medium were added per well. Puromycin at a final concentration of 2 µg/ml was added after 48 hrs to select transduced cells. MISSION non-target shRNA control vector (Sigma-Aldrich) was used as a negative control.

2.13.3 Site-directed mutagenesis (SDM)

TrueORF pCMV6-Entry vector containing C-terminal Myc- and DDK-tagged human *fkn* (Figure A.4) was purchased from Origene. Point mutations in Asp (D) residues in *fkn* gene were introduced using the following mutagenic primers:

D122 forward 5'- CCGGGGGAATGGAGGAGTCTGTGGTCC - 3'

reverse 5'- GGACCACAGACTCCTCCATTCCCCCGG -3'

D177 forward 5'- GCCAAAGGCTCAGGAAGGAGGGCCTGTGGG -3'

reverse 5'- CCCACAGGCCCTCCTTCCTGAGCCTTTGGC -3'

D223 forward 5'- CGTCCACCCAGGAGCCCTCCACCCAGG -3'

reverse 5'- CCTGGGTGGAGGGCTCCTGGGTGGACG - 3'

D273 forward 5'- CCAGCGCACACGGAAGCCTTCCAGGACTGG -3'

reverse 5'- CCAGTCCTGGAAGGCTTCCGTGTGCGCTGG -3'

D277 forward 5'- GCCTTCCAGGAGTGGGGGCCTGGC -3'

reverse 5'- GCCAGGCCCCCACTCCTGGAAGGC -3'

D320 forward 5'- CCATGCCACCATGGAGCCCCAGAGGCTGG -3'

reverse 5'- CCAGCCTCTGGGGCTCCATGGTGGCATGG -3'

D333 forward 5'- CACTCCTGTCCCTGAGGCCAGGCTGCC -3'

reverse 5'- GGCAGCCTGGGCCTCAGGGACAGGAGTG -5'

Primers were synthesised by Eurofins MWG Operon.

Each SDM reaction mixture contained: 1 µl of TruORF plasmid (200 ng), 1 µl of primer forward (125 ng), 1 µl of primer reverse (125 ng), 5 µl of 10 x reaction buffer (Stratagene), 1 µl 10 mM dNTPs (Promega), 40 µl DEPC H₂O and 1 µl PfuUltra high-fidelity polymerase (Stratagene). Control reactions without primers and without template were also prepared.

Reactions were carried out in thermocycler as follows: step 1 - 94°C for 2 min, step 2 - 94°C for 1 min, step 3 - 55°C for 1 min, step 4 - 68°C for 9 min, repeat steps 2 - 4 for 18 cycles, step 5 - 68°C for 30 min.

Parental copies of the plasmid were then removed from the digestion reaction using Dpn I restriction enzyme (Promega). 1 µl of Dpn I was added to each 50 µl SDM reaction and samples were incubated at 37°C for 3 hrs.

Consequently, the reactions were used for bacterial transformation. 250 µl of DH5α competent cells (Invitrogen) were incubated for 30 min on ice with 10 µl of Dpn I digest. Next, cells were heat-shocked for 2 min at 42°C. After 2 min incubation on ice, 500 µl of SOC medium (Super optimal broth with catabolite repression (supplemented with glucose)) was added. Cells were then shaken for 1 hr at 32°C and subsequently, plated out onto LB agar plates containing 25 µg/ml of kanamycin (Sigma Aldrich). Plates were incubated overnight at 37°C. 2 colonies were picked from each plate. Each colony was amplified in 3 ml of LB broth containing 25 µg/ml of kanamycin for 8 hrs, at 37°C, with intense shaking. 0.5 ml of culture was next transferred to 500 ml of LB broth (with kanamycin). Culture bottles were incubated overnight at 37°C with shaking.

Plasmids were purified from 300 ml of cultures using Plasmid Maxi kit (Qiagen), following the manufacturer's instructions precisely.

Introduction of point mutations was confirmed by plasmid sequencing (DNA Sequencing & Services, University of Dundee). TrueORF pCMV6-Entry vector sequencing primers (Origene) were used:

- VP1.5 (forward) - 5'-GGACTTTCCAAAATGTCG-3'
- XL39 (reverse) - 5'-ATTAGGACAAGGCTGGTGGG-3'

2.14 List of chemicals, reagents and equipment

Antibodies – see Table 2-2

Cell culture reagents

DMEM - PAA

FCS -PAA

IMDM - PAA

L-glutamine - PAA

Penicillin/streptomycin - Invitrogen

RPMI-1640 - Invitrogen

X-Vivo - Lonza

Immunohistochemistry

DAB - Vector Laboratories

ChemMate antibody diluent - Dako Cytomation

Histo-Clear II – National Diagnostics, USA

Serum-free protein block - Dako Cytomation

Vectastain Elite ABC reagent - Vector Laboratories

Inhibitors

Aprotinin – Sigma-Aldrich

Calpeptin – Alexis Biochemicals

Chymostatin – Sigma-Aldrich

E64 – Sigma-Aldrich

GM6001 – Calbiochem

Leupeptin – Sigma-Aldrich

TAPI-2 – Calbiochem

TIMP-1 - Calbiochem

Z-AEVD-FMK - BioVision, R&D Systems

Z-DEVD-FMK - BioVision, R&D Systems

Z-IETD-FMK – BioVision

Z-LEED-FMK - BioVision

Z-LEHD-FMK - BioVision

Z-Leu-Leu-CHO - BioVision

Z-LEVD-FMK - BioVision

Z-VAD-FMK – BioVision, R&D Systems

Z-VDVAD-FMK – BioVision

Z-VEID-FMK - BioVision

Z-WEHD-FMK - BioVision

Z-YVAD-FMK - BioVision

Molecular biology reagents

100bp DNA Ladder - Promega

Agarose – Invitrogen

Ampicillin – Sigma-Aldrich
dNTP mix - Promega
Ethidium bromide – Sigma-Aldrich
GoTaq Flexi DNA Polymerase - Promega
GoTaq Flexi PCR buffer (5x) - Promega
Library Efficiency DH5 α Competent Cells – Invitrogen
Lipofectamine 2000 – Invitrogen
Magnesium chloride (25 mM) - Promega
Mission shRNA glycerol stock (against human caspase-3) – Sigma-Aldrich
Oligo(dT)₁₅ Primer – Promega
Opti-MEM - Invitrogen
PfuUltra High-Fidelity DNA Polymerase - Stratagene
Plasmid Maxi kit - Qiagen
Puromycin - Invitrogen
RNaseOUT - Invitrogen
RNeasy Mini kit - Qiagen
SuperScript III Reverse Transcriptase – Invitrogen
TrueORF pCMV6-Entry Vector (containing human *fkn*) - Origene
ViraPower lentiviral packaging mix - Invitrogen

Proteins

Active human calpain I - BioVision
Recombinant human C5a - R&D Systems
Recombinant human Caspase-3 – R&D Systems
Recombinant human Caspase-7 – R&D Systems
Recombinant human Caspase-10 – R&D Systems
Recombinant human Fractalkine (full-length) – R&D Systems
Recombinant human MCP-1 – R&D Systems

Western blotting reagents

Bradford's assay solution - Bio-Rad
Hyperfilm ECL (18x24cm) – GE Healthcare
Hybond-P membrane – GE Healthcare
NuPAGE pre-cast 4-12% Bis-Tris gels - Invitrogen
NuPAGE LDS sample buffer - Invitrogen
NuPAGE MES SDS running buffer - Invitrogen

NuPAGE reducing agent - Invitrogen
NuPAGE transfer buffer - Invitrogen
PVDF membrane - GE Healthcare
SeeBlue Plus2 pre-stained standard - Invitrogen

Others

Acetone - Fisher Scientific
Annexin-V-FITC – Bender Medsystems
Bradford dye reagent – Bio-Rad
Brij 35 solution - Sigma-Aldrich
BSA - PAA
Calcium chloride – Sigma-Aldrich
CHAPS – Sigma-Aldrich
Cytotfix/Cytoperm Kit - BD Pharmingen
Diff-Qwik - Reagent
DTT - Invitrogen
Dulbecco's PBS - PAA
Dynabeads coupled with sheep anti-mouse IgG – Dnal
EDTA - Sigma-Aldrich
ELISA DuoSet – R&D Systems
Ethanol – Fisher Scientific
Ficoll-Paque Plus - GE Healthcare
Fix&Perm Cell Permeabilisation Reagents - Invitrogen
Formaldehyde - Sigma-Aldrich
Glycine - Fisher Scientific
Hank's BSS (without Ca and Mg) - PAA
HEPES - Sigma-Aldrich
Human IgG - Sigma-Aldrich
Hydrogen peroxide - Sigma-Aldrich
Ionomycin - Calbiochem
Low-endotoxin BSA – Sigma-Aldrich
LPS – Sigma-Aldrich
Methanol - Fisher Scientific
Microbeads anti-human CD14 – Miltenyi Biotec
Microbeads goat anti-mouse IgG - Miltenyi Biotec

Microbeads goat anti-rat IgG - Miltenyi Biotec
Microspheres 1 µm - Invitrogen
Mouse anti-human CD14 antibodies, 61D3 - Birmingham University
Mouse anti-human CD3 antibodies, OKT3 - Birmingham University
Mouse anti-human CD38 antibodies, OKT10 - Birmingham University
Mouse anti-human CD39 antibodies, AC2 - Birmingham University
Mouse anti-human IgD antibodies, HJ9 - Becton Dickinson
Normal goat serum - Biosera
ProLong Gold Antifade Reagent (with DAPI) - Invitrogen
Propidium iodide – Bender Medsystems
Rat anti-IgD antibodies (clone 11-26c.2a) – BD Biosciences
Rat anti-B220 antibodies - eBioscience
Rat anti-Ly-77 (GL7) antibodies - eBioscience
Sheep red blood cells - TCS Biosciences Ltd
Sodium chloride - Fisher Scientific
Sodium hydrogen - Fisher Scientific
Staurosporine - Calbiochem
Sulphuric acid – Fisher Scientific
Trichloroacetic acid - Sigma-Aldrich
Tetramethylbenzidine – Sigma-Aldrich
Tris - Sigma-Aldrich
Tris-HCL - Sigma-Aldrich
Triton-X-100 - Sigma-Aldrich
Tween-20 - Sigma-Aldrich
(Z-DEVD)₂-Rh110-bisamide - Calbiochem
Zinc chloride – Sigma-Aldrich

Equipment

Cell culture

BS748 improved Neubauer haemocytometer - Weber
Class II microbiological safety cabinets - BIOMAT
Incubators - LEEC
Polypropylene tubes (15, 50 ml) – BD Biosciences, Greiner bio-one
Polyvinyl uncoated transwells - Costar

Tissue culture flasks - Iwaki

Tissue culture plates – Nunc

Centrifuges

Beckman GS-6R – Beckman Coulter

Optima TLX – Beckman Coulter

Rotina 46R - Hettich

Sigma 1-15K – Sigma

Microscopes

Fluorescence microscope, Axioskop 2 - Zeiss

Leica SP5C confocal laser scanning microscope – Leica Microsystems

Olympus CK2 light microscope - Olympus

Olympus widefield inverted microscope – Olympus

Zeiss Axiovert 25 - Zeiss

Others

Anthos It-2 plate reader – Anthos Labtec Instruments

Coulter EPICS XL-MCL flow cytometre – Beckman Coulter

Horizon gel tanks – Life technologies

NanoDrop - Thermo Scientific

Polyallomer tubes - Beckman Coulter

Transilluminator Uvidoc - Uvitec

XCell SureLock Mini-Cell with XCell II Blot Module – Invitrogen

Chapter 3

Role of MCP-1 in attracting mononuclear phagocytes to Burkitt's lymphoma

3.1 Introduction

Macrophages are found in almost all types of tumours and are commonly referred to as tumour-associated macrophages (TAMs). TAMs have been shown to play an important role in tumour survival and progression. Thus, high numbers of macrophages in the tumour tissue are often associated with a poor prognosis (Mantovani et al. 1992; Lewis & Pollard 2006). Characterisation of factors responsible for recruiting macrophages to the sites of different types of tumours might help to develop more effective cancer treatment.

Burkitt's lymphoma (BL) is an aggressive type of lymphoproliferative disorder characterised by uncontrolled cell proliferation, high rate of spontaneous apoptosis and significant macrophage infiltration. Although BL cells undergo extensive apoptosis, *in situ* their corpses are cleared very effectively by macrophages infiltrating the tumour tissue (Cooper et al. 1966; Berard et al. 1969; Levens et al. 2000). It is now widely believed that dying cells are themselves able to release chemotactic molecules to ensure macrophage chemotaxis and subsequent clearance of their site of death (Horino et al. 1998; Knies et al. 1998; Lauber et al. 2003; Gude et al. 2008; Truman et al. 2008; Elliott et al. 2009). So far however, mechanisms

responsible for macrophage recruitment to the BL tumour site are not fully understood.

Previous work carried out in this laboratory identified fractalkine/CX3CL1 (FKN) to be an important player in macrophage chemotaxis to BL (Truman et al. 2008). Yet, from the results presented by Truman and colleagues it can also be concluded that FKN may not be the only chemokine involved in this process. The authors have demonstrated that macrophages migrate to the supernatants collected from apoptotic BL cells. To understand the nature of this migration viral macrophage inflammatory protein II (vMIP-II) and pertussis toxin (PTX) were included in the assay. vMIP-II is a broad-range chemokine antagonist of the viral origin. It is known to efficiently block CC and CXC receptors as well as CX3CR1 - the only known FKN receptor (Chen et al. 1998; DeBruyne et al. 2000). PTX has the ability to block signal transduction through chemokine receptors by blocking G proteins to which chemokine receptors are coupled. Both vMIP-II and PTX caused considerable abrogation of macrophage chemotaxis, suggesting that it was a chemokine-driven phenomenon. However, the usage of CX3CR1-deficient macrophages in a similar type of experiment caused the drop in the chemotaxis rate by only around fifty per cent when compared with the wild type macrophages. Based on these observations a hypothesis was formulated that apart from FKN another member(s) of chemokine family might have a role in the process of macrophage recruitment to Burkitt's tumours (Truman et al. 2008). Lysophosphatidylcholine (LPC), which has been shown to be released from apoptotic MCF-7 cells and to have an ability to attract macrophages (Lauber et al. 2003), is also known to act through G protein-coupled receptor (Lin & Ye 2003; Radu et al. 2004; Yang et al. 2005; Peter et al. 2008). The experiments previously performed in this laboratory indicated, however, that LPC is not involved in macrophage chemotaxis to apoptotic BL cells (Truman, unpublished).

Monocyte chemoattractant protein-1 (MCP-1, CCL2) has been selected as a candidate factor. MCP-1 is a very potent chemotactic molecule for monocytes. It regulates the migration of monocytes from the blood into tissue where they become tissue macrophages and, very importantly, is one of the chemokines most frequently found in tumours (Graves et al. 1989; Zhang et al. 1997; Nesbit et al. 2001;

Mantovani et al. 2002). A positive correlation between the level of MCP-1 expression and the number of infiltrating macrophages has also been reported (Murdoch et al. 2004).

The studies presented in this chapter were undertaken to determine the potential role of MCP-1 in macrophage recruitment to BL.

3.2 MCP-1 expression in BL

The starting point for this study was to examine MCP-1 expression in BL cells. Two human BL cell lines were used for this purpose – BL2 and BL2/Bcl-2 (BL2 that was previously transfected with the anti-apoptotic gene bcl-2). The cells were immunofluorescently labelled with anti-MCP-1 monoclonal antibodies (MAB2791) and subsequently analysed on the flow cytometer. The level of total MCP-1 was assessed in permeabilised cells, whereas the intact cells were used for the surface expression measurements. Figure 3.1 shows a high level of MCP-1 expression in both BL2 and BL2/Bcl-2 cells (upper panel). On the lower panel it can be seen that there was no MCP-1 detected on the surface of these cells. The latter result is consistent with what was expected, knowing that MCP-1 is a classical small secreted chemokine, normally not present on cell surface.

Having demonstrated that MCP-1 is present in BL cell lines, we next wanted to confirm this result using a mouse model of BL in which SCID mice were xenografted with BL2 cells. Monoclonal anti-MCP-1 antibodies (MAB2791) were used for immunohistochemistry staining of the tumour sections. As shown in Figure 3.2 the brown staining that represents MCP-1, was abundant in these sections. Significantly, the staining seemed to be associated mainly with B cells. The macrophages scattered throughout the tissue generally remained unstained, with the exception of the apoptotic cell debris that was visible inside the vacuoles. These observations led to the conclusion that the B cells, but not the macrophages are responsible for production of MCP-1 in BL tumours.

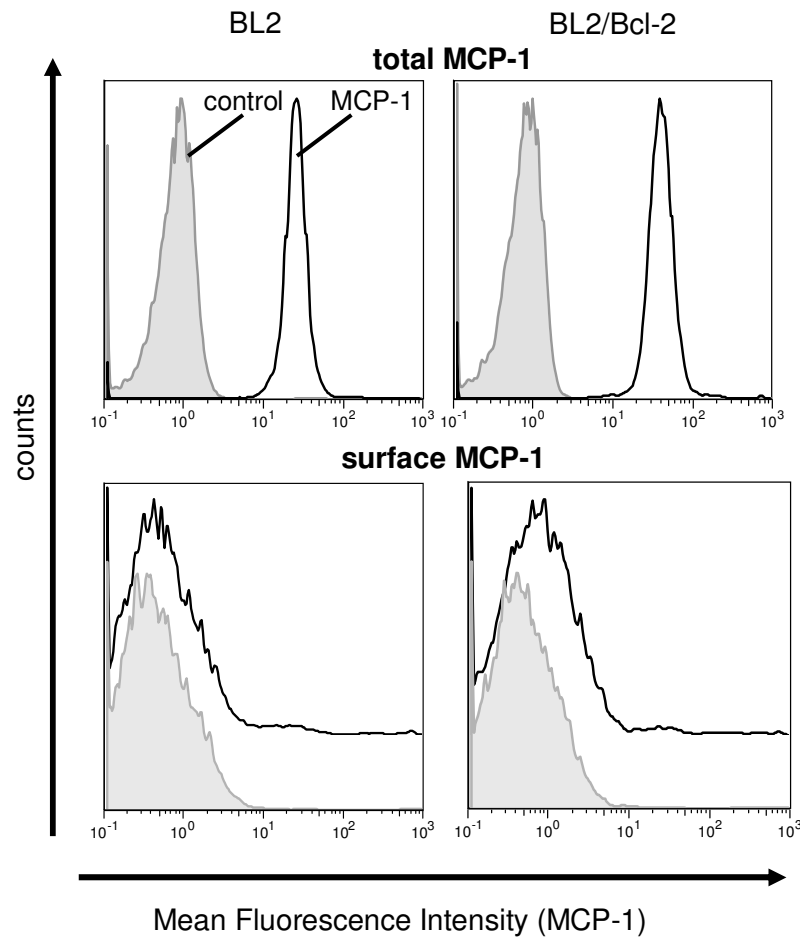


Figure 3.1 Expression of MCP-1 in BL cells.

Permeabilised (upper panels) or intact (lower panels) BL2 and BL2/Bcl-2 cells were stained for MCP-1 using a mouse monoclonal anti-human MCP-1 antibodies (MAB2791) (white), and FITC-conjugated goat anti-mouse secondary antibodies. Isotype control shows background staining (grey).

3.3 MCP-1 release from apoptotic BL2 cells

Having shown that MCP-1 is produced by BL cells, it was important to determine whether, and under what conditions MCP-1 is released from these cells.

It has previously been demonstrated that apoptotic cells release molecules that are chemotactic for phagocytes (Horino et al. 1998; Knies et al. 1998; Lauber et al. 2003). FKN, which has been shown to be involved in macrophage recruitment to BL tumours, is also released from BL cells rapidly following the induction of apoptosis

(Truman et al. 2008). The secretion of MCP-1 from apoptotic BL cells was therefore examined.

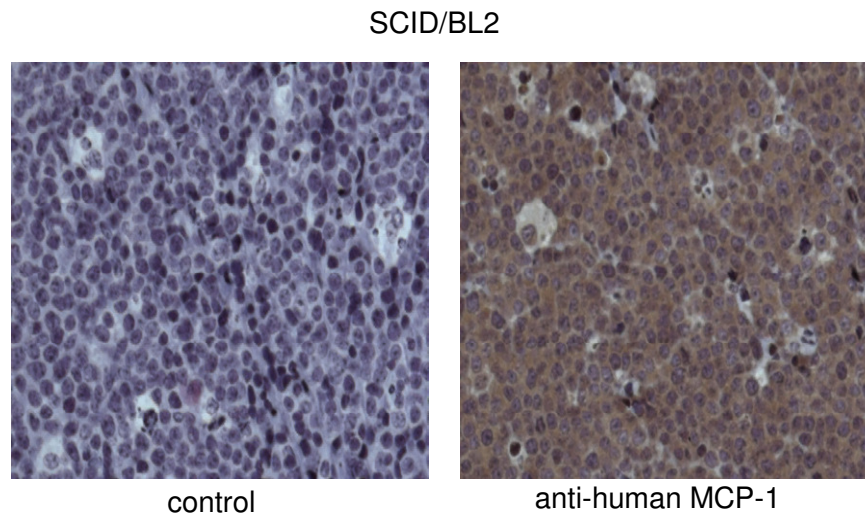


Figure 3.2 Expression of MCP-1 in tumour tissue in situ.

Immunohistochemistry of SCID/BL2 sections. Sections were labelled with mouse monoclonal anti-human MCP-1 antibodies (MAB2791). Brown staining represents MCP-1. No primary antibodies were used in the control slides. Nuclei were counter-stained blue with haematoxylin.

3.3.1 Induction and measurement of BL2 cells apoptosis

Even though group I BL lines, such as BL2 retain the ability of biopsy tumour cells to undergo spontaneous apoptosis, the usual level of apoptosis in BL2 cell culture does not exceed 10-15 per cent. Therefore it was necessary to establish an efficient and reliable method of triggering apoptosis of BL cells. The protein kinase inhibitor staurosporine was used as an apoptotic stimulus and the induction of apoptosis was assessed by staining the cells with Annexin V-FITC (AxV) and propidium iodide (PI) followed by FACS analysis. Figure 3.3 shows the typical pattern of AxV/PI staining obtained from staurosporine-treated BL2 cells in which staurosporine-exposure generates a fairly uniform population of cells displaying typical features of early apoptotic cells (PS exposure on the outer leaflet of the plasma membrane but lack of PI binding to the nuclear DNA), within only 2 hrs.

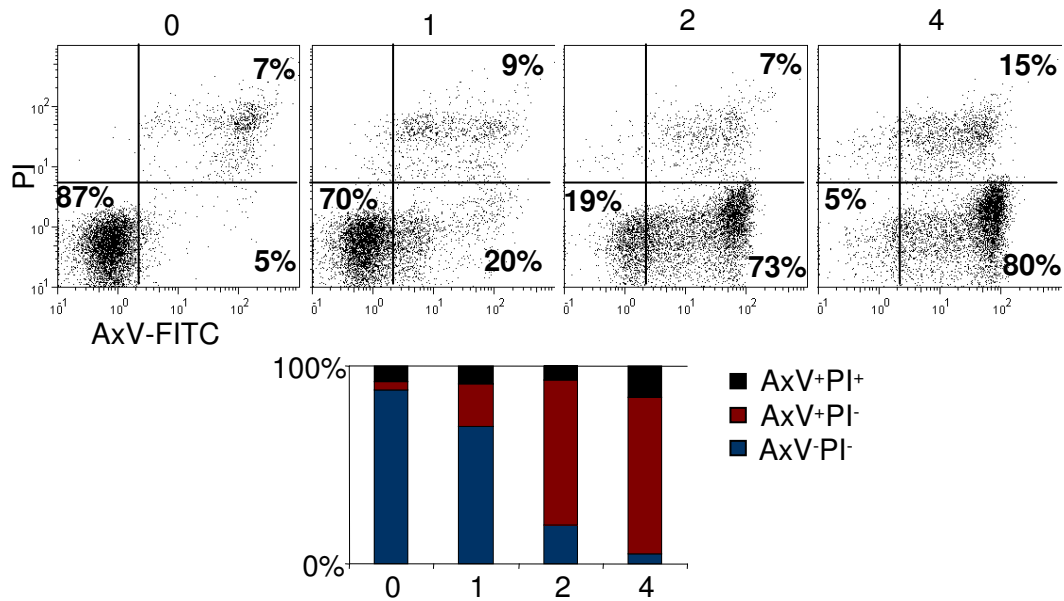


Figure 3.3 Staurosporine is a potent inducer of apoptosis.

BL2 cells were treated with 1 μ M staurosporine. Cells were harvested and stained with annexin V-FITC (AxV) and propidium iodide (PI) at the indicated times after apoptosis induction. Percentage of AxV⁺ and PI⁺ cells was measured by flow cytometry. Summary graph shows the percentage of viable (AxV-PI⁻) (blue bars), early apoptotic (AxV+PI⁻) (red bars) and late apoptotic (AxV+PI⁺) (black bars) cells at different time points (hrs) after induction of apoptosis. One representative result of more than thirty similar is shown.

3.3.2 Measurement of MCP-1 release from apoptotic BL2 cells by ELISA

Two cell lines - BL2 and BL2/Bcl-2 - were chosen for measurements of MCP-1 secretion. BL2/Bcl-2 cells, which were resistant to apoptotic stimuli, were used to compare the MCP-1 release from non-apoptotic cells to that from the early apoptotic ones. Staurosporine was added to the cells and cell-free supernatants were collected at the indicated time points following its addition. Initially, the supernatants were analysed by a sandwich ELISA kit (R&D Systems) specific for MCP-1. However, by using this technique it was not possible to detect any MCP-1 in the samples (this data is not shown, but it is visible also in Figure 3.8). Similar problems have been faced by the previous group members while they were trying to measure the amount of other chemokines in BL cell supernatants but adopting a “direct” ELISA seemed to improve the assays sensitivity (Wilkinson 2006). Thus, a “direct” ELISA using anti-MCP-1 mAb (MAB679) was set up. As presented in Figure 3.4a, MCP-1 was detected in the samples. Importantly, MCP-1 concentrations in the supernatants from

BL2 cells seemed to increase as the apoptosis programme progressed (Figure 3.4b). The level of MCP-1 increased considerably within only 2 hrs of staurosporine exposure, when the majority of cells became apoptotic but their cell membranes were still intact (AxV+PI-), suggesting that MCP-1 is actively released from early apoptotic cells.

However, the most dramatic change in the MCP-1 concentration was observed between the samples from 2 and 3 hrs post staurosporine treatment, when the rise in a number of PI positive, and thus probably leaky, cells was also detected. In contrast, in the supernatants collected from BL2/Bcl-2 cells which remained more viable throughout the course of the assay, the amounts of MCP-1 were smaller (Figure 3.4a, right panel). A slight increase was only observed at 3 hrs post-staurosporine exposure, when the number of viable cells dropped to approximately 50 per cent (Figure 3.4b). These results led to the conclusion that the secretion of MCP-1 from BL cells is an apoptosis-dependant process.

3.4 Role of MCP-1 in mononuclear phagocyte migration to BL

Having established that MCP-1 is released from apoptotic BL cells, the role of MCP-1 in macrophage migration to these cells was examined. This was made possible by the use of a Boyden chamber-based transmigration assay in which mononuclear phagocytes are placed in a transwell, on top of a pored membrane, and allowed to transmigrate to the lower compartment, following the gradient of a chemoattractant of interest (Figure 2.1).

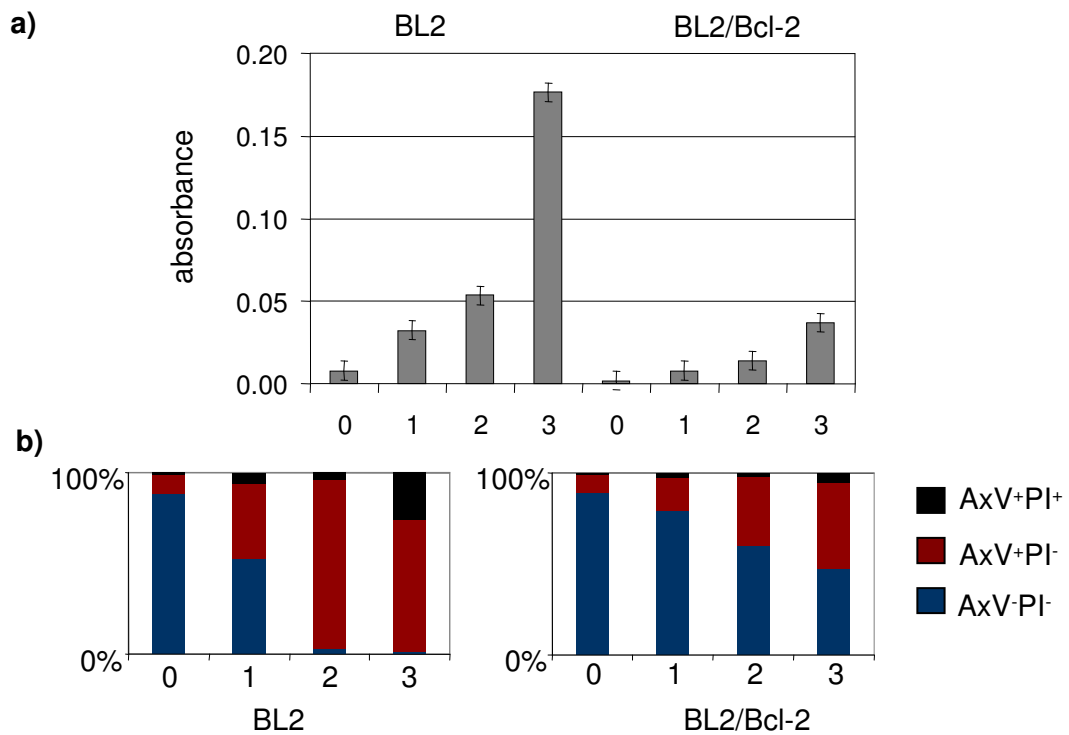


Figure 3.4 Release of MCP-1 from apoptotic BL cells.

BL2 and BL2/Bcl-2 cells were induced to apoptosis by staurosporine treatment. Cells and cell supernatants were collected at the indicated times (hrs) after apoptosis induction. **(a)** Cell-free supernatants were tested for MCP-1 presence by direct ELISA. The error bars show the pooled standard deviation (n=2). **(b)** Apoptosis was assessed by AxV/PI staining.

3.4.1 Human monocyte migration to recombinant human MCP-1

In the first instance, the chemoattractant potential of MCP-1 for mononuclear phagocytes was tested using freshly isolated human monocytes that were allowed to migrate to a range of concentrations of rh MCP-1. Rh MCP-1 proved to efficiently induce transmigration of human monocytes in a concentration dependent manner (Figure 3.5a).

3.4.2 Human monocyte-derived macrophage (HMDM) migration to supernatants from BL2 cells

To further test the hypothesis that MCP-1 has a role in macrophage migration to dying BL cells, supernatants from apoptotic BL2 cells were used as a chemoattractant in the Boyden-chamber based assay.

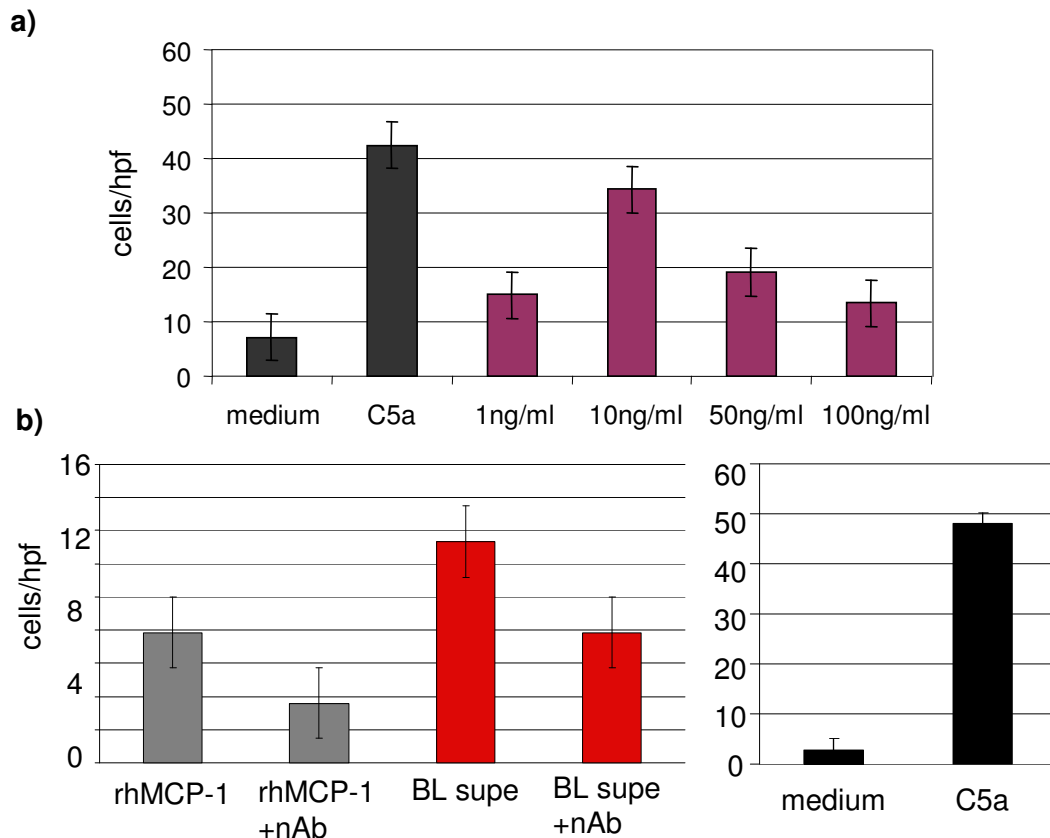


Figure 3.5 Migration of mononuclear phagocytes to recombinant and BL cell-derived MCP-1

(a) Migration of fresh CD14⁺ monocytes to a range (1-100 ng/ml) of rhMCP-1 concentrations. C5a was used as a positive control and assay medium as a negative control. The error bars show the pooled standard deviation (n=2). One representative result from three is shown. (b) Six-day-old HMDMs were migrating towards rhMCP-1 (5ng/ml) and cell-free supernatants from apoptotic BL2 cells (collected 3 hrs after UV-irradiation). Addition of mouse polyclonal neutralising anti-human MCP-1 antibodies (2µg/ml) (AF-279-NA) inhibited macrophage migration. C5a was used as a positive control and assay medium as a negative control. The error bars show the pooled standard deviation (n=2). One representative result from two is shown.

However, since staurosporine present in BL2 cell supernatants would affect the viability of tested macrophages, BL2 cells were instead induced into apoptosis by UVB exposure (Truman et al. 2008). Cell-free supernatants were collected after 3 hrs and subsequently used in a transmigration assay with six-day-old HMDMs. Rh MCP-1 was also included as a positive control in the assay. The chemotactic role of MCP-1 was examined by addition of neutralising anti-human MCP-1 antibodies (nAb) (AF279-NA). As shown in Figure 3.5b, HMDMs migrated to supernatant from apoptotic BL2 cells and rh MCP-1. The presence of nAb in the samples resulted in the impaired migration of macrophages to both rhMCP-1 and supernatants from

apoptotic BL cell, suggesting that MCP-1 is a potentially important factor in macrophage migration to dying BL cells.

Based on the simple experiments presented here it would be impossible to establish if the observed migration of macrophages was a movement along the concentration gradient and can therefore be called chemotaxis. However, the chemotactic properties of the apoptotic BL cell supernatants have been extensively analysed by the previous members of this group. In the initial studies, Truman examined if the macrophage movement to the lower chamber was truly chemotactic by placing the BL cell supernatants also in the upper chamber and therefore, abolishing the chemotactic gradient. Subsequent calculation of the difference in number of migrating cells between the wells with and without the supernatants added above the filter allowed for confirmation that the apoptotic BL cell-derived supernatants truly have chemotactic properties (Truman 2005).

3.5 MCP-1 immunodetection in the supernatants from apoptotic BL cells

In parallel with the experiments presented above, an attempt was made to detect MCP-1 in previously used, EBV-negative BL2 and BL2/Bcl-2 as well as in EBV-positive BL Mutu I cell supernatants by Western blotting. Again, BL cells were induced to death by either staurosporine or UV exposure. As these cells had previously been shown to secrete MCP-1 in a constitutive manner (Steube et al. 1999), supernatants from overnight cultures of untreated or LPS-exposed HL-60 cells were also tested in this assay and served as a positive control. Prior to the electrophoresis, cell-free supernatants were treated with trichloroacetic acid (TCA) in order to precipitate and concentrate the proteins present in the samples. Two different antibodies were used to detect MCP-1 on the nitrocellulose membranes: MAB679 – also used as a detection antibody in the direct ELISA (Figure 3.4a), and AF279-NA – the MCP-1-neutralising antibody from Figure 3.5b, both of them being recommended for Western blot analysis by the supplier. Figure 3.6a shows a blot stained with AF279-NA antibodies. Surprisingly, bands of the size of MCP-1 (9-15

kDa) (Yoshimura & Leonard 1991; Ishii et al. 1995) were only present in HL-60 but not in BL samples. Therefore, a suspicion arose that MCP-1 might after all not be expressed by BL cells and that the inhibitory effect of AF279-NA antibodies on macrophage migration, observed in the chemotaxis experiment (Figure 3.5b), could be due to reasons other than blocking of MCP-1 in the samples.

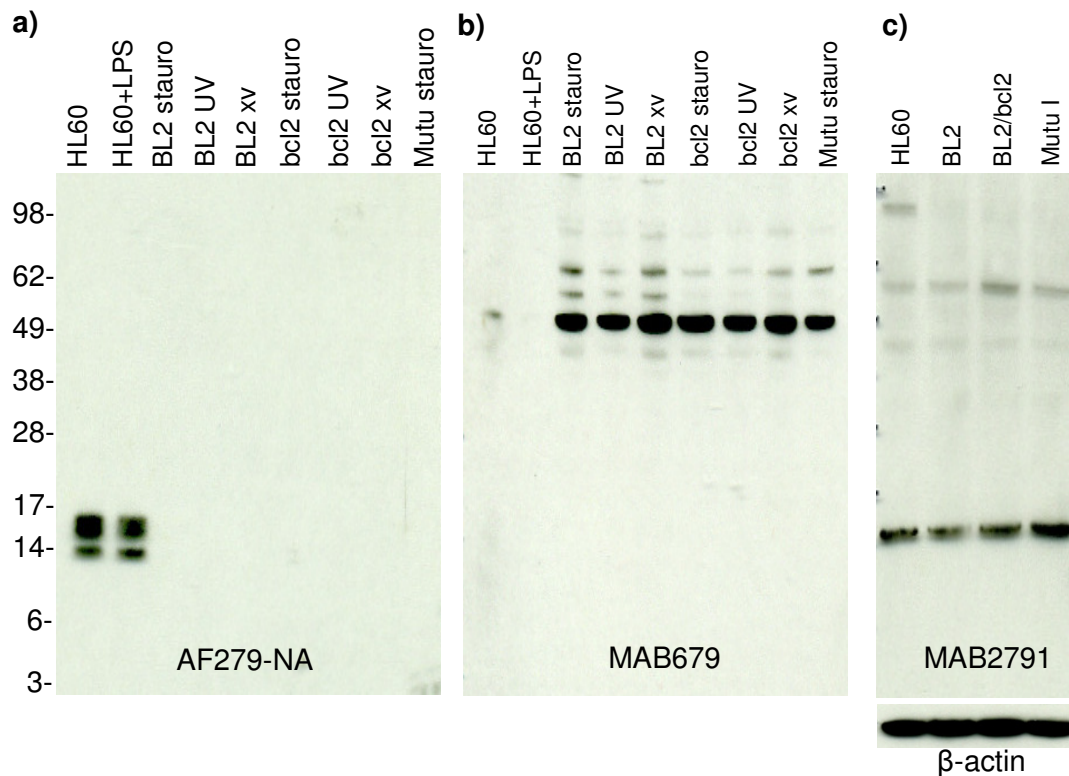


Figure 3.6 MCP-1 detection by Western blotting in BL and HL-60 cells.

(a & b) BL2, Mutu I and BL2/Bcl-2 cells grown in serum-free RPMI 1640 or in X-Vivo (xv) were induced into apoptosis using either staurosporine (stauro) or UV exposure and cell-free supernatants were collected after 3 hrs of incubation. Supernatants from overnight cultures of HL-60 or LPS-treated HL-60 cells were also collected. Proteins were precipitated from the medium by TCA and analysed by Western blotting. (a) Membrane was labelled with AF279-NA anti-MCP-1 antibodies. (b) Membrane was labelled with MAB679 anti-MCP-1 antibodies. (c) MCP-1 was detected by immunoblotting of HL-60, BL2, Mutu I and BL2/Bcl-2 whole cell lysates. MAB2791 antibodies were used to label the membrane.

Even more puzzlingly, when MAB679 antibodies were used for the labelling of the membrane, no MCP-1-size band was detected, even in the HL-60 samples (Figure 3.6b). The only clearly visible bands, detected in the BL cell supernatants exclusively, were of around 50 kDa. As MCP-1 has never been reported to be of a size other than 9 -15 KDa, typical for this group of small secretory chemokines, it

was concluded that the occurrence of the 50 kDa band might be a result of non-specific or cross-reactive binding of the antibodies. This in turn, prompted concerns that the positive result of the direct ELISA (Figure 3.4a) may be due to a cross-reactivity of the MAB679 antibodies. In conclusion, the experiments presented so far in this section failed to detect MCP-1 in the supernatants of BL cells raising questions about the validity of the results reported earlier in this chapter.

MAB2791 antibodies initially detected MCP-1 in BL cells when used to label cells for FACS analysis (Figure 3.1) or in the immunohistochemistry (Figure 3.2). The ability of these antibodies to detect MCP-1 in BL and HL-60 whole cell lysates by Western blotting was thus tested despite these antibodies not being recommended for this technique by the supplier. Nonetheless, 15 kDa (one of the possible sizes of MCP-1) bands were visible in both HL-60 and BL samples (Figure 3.6c). Western blot with MAB2791 thus supported earlier observations made in this chapter concerning MCP-1 expression in BL.

3.6 Analysis of BL cells expression of MCP-1 mRNA

Being unable to produce conclusive evidence for MCP-1 expression or its lack at the protein level, the expression of its mRNA in BL cells was assessed.

Total RNA was extracted from both viable and apoptotic BL2 cells as well as from HL-60 cells (untreated or stimulated with LPS). Next, cDNA was obtained from all the samples using reverse transcriptase and polymerase-chain reaction was carried out with MCP-1-specific primers. The reaction mixtures were analysed on the agarose gel (Figure 3.7a).

Clear and bright product bands of the expected size were visible in both of the HL-60 samples but in none of the BL2 ones. As the control reactions with the GAPDH-specific primers were also included in this experiment and the levels of the specific product bands in all the control samples were similar, it suggests that the

lack of MCP-1 band in BL2 samples is a result of MCP-1 mRNA being absent in these cells rather than being due to the analysis of poor quality cDNA.

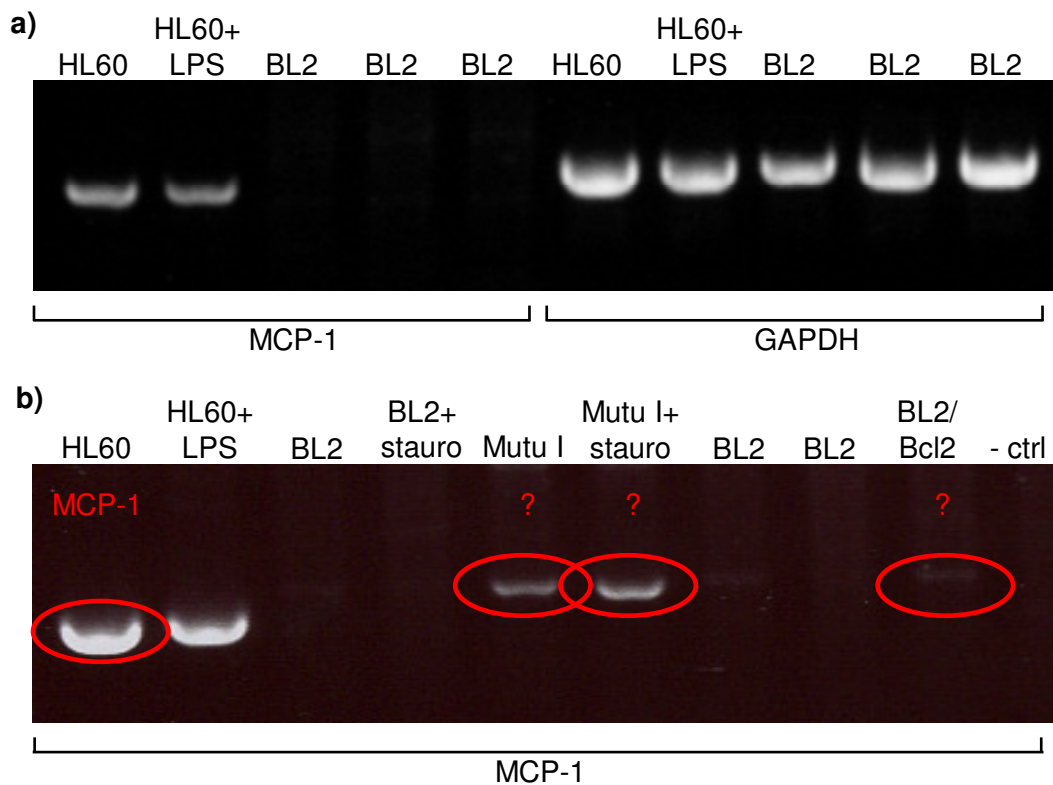


Figure 3.7 Analysis of MCP-1 RNA transcripts present in HL-60 and BL cells.

(a) Total RNA was extracted from HL-60, LPS-treated HL-60 and BL2 (x3) cells and reverse transcriptase PCR for MCP-1 was performed. Control reactions for GAPDH were also performed. (b) Total RNA was extracted from HL-60, LPS-treated HL-60, BL2 (x3), Mutu I, BL2/Bcl-2 as well as from staurosporine-treated BL2 and Mutu I cells and reverse-transcriptase PCR for MCP-1 was performed. Selected bands (circled) were extracted from the gel, DNA was purified and sequenced. The sequence of the amplification product from HL-60 sample matched the sequence of human MCP-1. The sequences obtained from the other extracted samples did not match any known gene sequences.

Although this seemed like a rather strong argument for the lack of MCP-1 expression in BL cells, the presence of the low intensity bands of the approximately MCP-1 size in some BL2 samples (not well visible in Figure 3.7a, but clear in Figure 3.7b) was still causing some concern. In order to resolve the remaining doubts, PCR reactions were performed with the previously analysed samples and with additional ones prepared from Mutu I BL cells. Some of the strong MCP-1 bands from HL-60 cell samples and faint and, incorrectly sized bands in BL cell samples (Figure 3.7b) were

extracted, purified and sent away to the University DNA sequencing facility (Ashworth Laboratories). Sequencing showed that only the sequence obtained from the HL-60 band analysis matched the MCP-1 sequence (NCBI Blast). Poor quality sequences from BL preparations did not match any of the known DNA sequences in the genome. In summary, the results presented in this section suggested that MCP-1 RNA transcripts may not be present in BL cell lines and added to the evidence suggesting MCP-1 is absent from BL.

3.7 Analysis of HL-60 cell-free supernatants by MCP-1 ELISA

Although MCP-1 mRNA has been shown in the previous section to be expressed in HL-60 cells, the results obtained in the Western blot analysis of the HL-60 supernatants (Figure 3.6a) did not provide consistent results proving the presence of MCP-1 in these cells. Since HL-60 cells were intended to serve as a positive control in the assays, a confirmation of the presence of MCP-1 in these cells was sought. For this reason, cell-free supernatants from HL-60 overnight cultures (untreated and LPS-stimulated) were analysed by sandwich ELISA (Figure 3.8). Significant levels of MCP-1 were detected in both samples whereas, consistent with the earlier observations, no MCP-1 could be found in supernatants from apoptotic BL2, BL2/Bcl-2 and Mutu I cells. Taken together, these observations strongly indicate that MCP-1 is not produced by Burkitt's lymphoma cell lines rather than there being a technical problem with the MCP-1 sandwich ELISA.

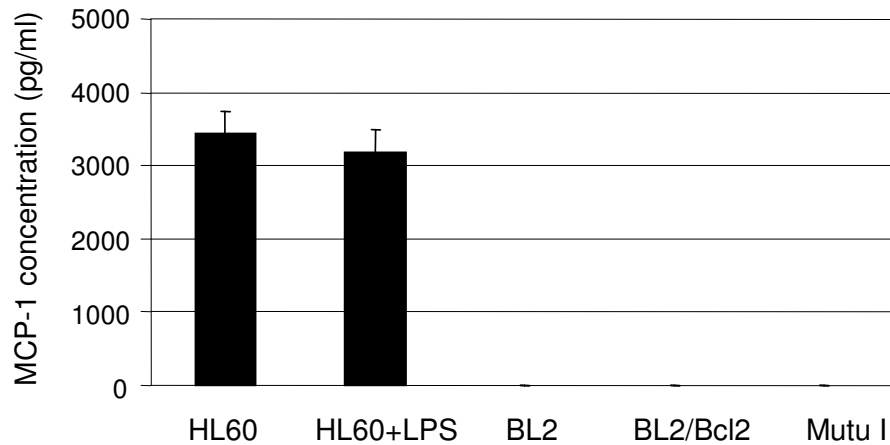


Figure 3.8 Release of MCP-1 from HL-60 cells.

Cell-free supernatants from HL-60 and LPS-treated HL-60 cells overnight cultures as well as the supernatants from BL2, BL2/Bcl-2 and Mutu I induced into death with staurosporine for 3 hrs were analysed by sandwich ELISA. The error bars represent the standard error of the mean (n=3). One representative result from two is shown.

3.8 Conclusions and Discussion

In summary, the work described in this chapter:

- confirmed previous observations (Truman 2005) that apoptotic Burkitt's lymphoma cells release factors that induce macrophage migration
- demonstrated that, although some of the tested anti-human MCP-1 antibodies bound to the epitopes present in BL cells, MCP-1 is, most likely, not expressed by these cells

MCP-1 is known to be a strong chemoattractant for monocytes. It is also one of the most commonly found chemokines of the tumour environment (Nesbit et al. 2001). MCP-1 was thus selected as a candidate chemoattractant that could work alongside fractalkine (Truman 2005) to enable macrophage infiltration in Burkitt's lymphoma. For this reason, attempts were made to assess the levels of MCP-1 in BL samples. The high intensity of the MCP-1 staining seen in both immunohistochemistry and FACS measurements (Figure 3.1 and Figure 3.2) initially seemed very promising and prompted the subsequent studies presented here. These included demonstrating the

presence of MCP-1 in BL cells using a direct ELISA (Figure 3.4a) as well as (although indirectly) using transmigration assays (Figure 3.5b). The inhibition of macrophage migration observed after the addition of anti-MCP-1 neutralising antibodies seemed consistent with the hypothesis that apoptotic BL cells release a chemokine(s) other than fractalkine to attract macrophages (Truman 2005). Taken together, these observations suggested that MCP-1 is highly expressed by, and released from, BL cells early in the apoptotic cell death programme.

However, doubts were raised as to the accuracy of this conclusion when Western blotting was used to detect MCP-1 in apoptotic BL cell supernatants. None of the antibodies previously used to test for MCP-1 presence in BL cell supernatants were able to detect MCP-1 in the samples by Western blotting. When MAB679 antibodies were used (the same as in the direct ELISA), the only strong bands that were detected were of 50 kDa in size and were of unknown identity (Figure 3.6b). The presence of these bands was limited to BL cells - they were not observed in HL-60 cell supernatants. Intriguingly, MCP-1 (band of 9 - 15 kDa) was also absent from HL-60 cell samples (these cells are known to express this chemokine (Steube et al. 1999)). These results suggest that MAB679 antibodies may not be very good at detecting 9-15 kDa MCP-1 in Western blotting and that the apparent detection of MCP-1 in BL cells using the direct ELISA may have been due to detection of the 50 kDa protein of unknown identity.

By contrast, AF279-NA antibodies were able to detect 9-15 kDa MCP-1 bands in HL-60 samples but were unable to detect these MCP-1 bands in BL cell supernatants (Figure 3.6a). These observations raise the question: how did these antibodies impair macrophage migration when added to BL supernatants, if they did not detect MCP-1 in the same samples by Western blotting despite MCP-1 bands being detectable in Western blots of HL-60 supernatants? One possible explanation is that AF279-NA antibodies blocked the action of another macrophage chemoattractant present in the apoptotic BL cell supernatants (and thus inhibited macrophage migration) but binding to this protein was not visualised by Western blotting perhaps due to the denaturation of proteins whilst TCA-precipitating the supernatants and heating the samples in preparation for Western blotting. It would be feasible to test this possibility by pre-incubating the antibodies with recombinant MCP-1 prior to testing

their inhibitory effect on the macrophage migration. If this is indeed what is happening it would be interesting to identify this protein, for example by immunoprecipitating it with the AF279-NA antibodies. However, it is equally possible that the observed inhibition of the macrophage transmigration was caused by alternative effects these antibodies could have had on macrophage migration or viability. It would therefore be important to confirm these results by using suitable isotype control antibodies in the transmigration assay. It is possible that the observed impairment of the phagocyte migratory properties was caused by a binding of antibody aggregates or antibody-antigen complexes to the Fc receptors on their surface, macrophage activation and consequently, adhesion. In order to eliminate such a risk, it would be profitable to employ in this assay the MCP-1-specific Fab (or F(ab)'₂) fragments of antibodies, which contain their antigen-binding domain but are deprived of their power to ligate receptors on the surface of phagocytes.

The use of a third anti-MCP-1 antibody in Western blotting (MAB2791 - previously used to detect MCP-1 in BL samples by immunohistochemistry and FACS analysis (Figure 3.1 and Figure 3.2) produced a different set of results with 15 kDa bands - one of the possible sizes of MCP-1 (Ishii et al. 1995) - being clearly visible in all the BL and HL-60 cell lysate samples (Figure 3.6c). This suggested that BL cells might contain MCP-1.

Having obtained such an inconsistent set of results it was important to examine MCP-1 mRNA levels in BL cell lines (Figure 3.7). By showing the absence of MCP-1 mRNA in BL cells, despite this mRNA being detectable in HL-60 cells, it seemed highly likely that MCP-1 is not produced by BL cells.

The results gathered from RT-PCR analysis, sandwich ELISA and WB analysis with AF279-NA antibodies, weighed strongly against MCP-1 presence in BL cells. The direct ELISA, WB with MAB679 antibodies and inhibition of chemotaxis by AF279-NA antibodies were shown to be unreliable because of the cross-reactivity of the antibodies. The only evidence supporting MCP-1 presence in BL cells therefore come from the experiments where MAB279 antibodies were used.

As the negative results, produced by a variety of techniques, seem to overwhelm the positive results, it is necessary to conclude that MCP-1, most likely, is not expressed

in Burkitt's lymphoma. Interpretation of the MCP-1 data has been greatly complicated by the cross-reactivities of the anti-MCP-1 antibodies (at least one – MAB679 – out of three of the antibodies used in this study appears to cross-react with other protein species).

In hindsight, the inability to detect MCP-1 in BL samples using the sandwich ELISA should have been better recognised as a strong indication that the BL cells may not release MCP-1. Since the sandwich ELISA requires two different epitopes on the same protein to be bound by two different antibodies in order for the assay to give a positive result it should not be prone to generate false positive results. This was clearly exemplified in the results – the sandwich ELISA only detected MCP-1 in the samples with clearly confirmed MCP-1 expression (HL-60) and was unable to show MCP-1 expression in samples in which the expression of MCP-1 had not been confirmed (BL cells) (Figure 3.8). By contrast, the direct ELISA (Figure 3.4a), as with other techniques based on a single type of antibody, is more likely to be impaired by antibody cross-reactivity.

Although the work presented in this chapter may look unfinished, some of the results not utterly convincing and one would wish to repeat them or include additional controls where appropriate, when the convincing proof had been obtained for the lack of MCP-1 mRNA expression in BL cell lines, the decision was made to halt all further work concerning MCP-1.

This chemokine has been detected in several types of cancer, including lymphomas, and the roles of MCP-1 in macrophage infiltration of tumours (Graves et al. 1989) and in promoting tumour progression via enhancing angiogenesis (Salcedo et al. 2000; Ma et al. 2007) are well recognised. Furthermore, Luciani and colleagues demonstrated that it is widely expressed by Hodgkin's lymphoma as well as some of the B-cell lymphoma cells (Luciani et al. 1998). However, the general experimental approach taken by the authors does not specify which cellular component of these tumours is responsible for the MCP-1 production and normally monocytes, endothelial cells and fibroblasts, but not B cells, are considered to be a source of MCP-1 (Schall 1991). A constitutive MCP-1 production is also characteristic of neoplastically transformed cells. MCP-1 expression has been studied in detail in two

types of B-cell lymphoma: in diffuse large B-cell lymphoma, malignant B cells have been found to produce MCP-1 whereas in follicular lymphoma, MCP-1 has been shown to be present in follicular dendritic cells, not in B cells (Husson et al. 2001; Kitai et al. 2007). Although data presented in this chapter shows that Burkitt's lymphoma B cells most likely do not express MCP-1, the possibility that resident TAMs do, and therefore might self-regulate the level of macrophage infiltration to the tumour, was not excluded. For just now, the identification of the chemokine that works in conjunction with fractalkine to mediate macrophage recruitment in BL (Truman et al. 2008) remains unknown. A candidate chemokine will be discussed in Chapter 6.

Chapter 4

FKN processing in apoptotic BL cells

4.1 Introduction

Fractalkine/CX3CL1 (FKN), the only member of CX3C chemokine family, is structurally unusual in that it, like CXCL16, is a transmembrane molecule. Its chemokine domain is situated at the end of long mucin-like stalk, fixed to the plasma membrane by a transmembrane domain and a cytoplasmic tail (Figure 1.1) (Bazan et al. 1997; Ludwig & Weber 2007). FKN present on the surface of cells (for example epithelial cells) plays a role in adhesion and migration of cells carrying the only known receptor for FKN – CX3CR1 (Imai et al. 1997; Lucas et al. 2001; Ludwig & Weber 2007). However, once the chemokine domain of FKN is freed it can act as a secretory chemokine - it is known to be a chemotactic factor for monocytes, T cells and natural killer cells (Tsou et al. 2001). In recent years several proteases engaged in the shedding of FKN from the cell membrane have been identified. At first, two metalloproteinases – ADAM-10 and ADAM-17 were found to be key players in this process (Garton et al. 2001; Hundhausen et al. 2003; Hundhausen et al. 2007; Schulte et al. 2007). More recently, however, cathepsin S and MMP-2 have also been shown to take part in the cleavage and release of FKN (respectively: Clark et al. 2007; Clark et al. 2009; Bourd-Boittin et al. 2009; Dean and Overall 2007).

As already mentioned in Chapter 3, previous work within the Gregory group has shown FKN to have a role in recruiting macrophages to Burkitt's lymphoma tumours (Truman 2005; Truman et al. 2008). In this study, FKN was demonstrated to be released predominantly by dying BL cells and the initial Western blot analysis revealed that FKN present in BL cells was modified (presumably truncated) when

these cells underwent apoptosis. However, the nature of this process was not further characterised (Truman 2005; Wilkinson 2006).

The studies presented in this chapter were carried out as a continuation of the research on FKN that was initiated by Truman. The aim of this study was to broaden our understanding of FKN biology in BL with the primary focus on explaining the modification of FKN during BL cell death. The mechanism of FKN release from dying cells was also examined. In particular, the possibility that FKN is released in microparticles (MPs) during the apoptotic blebbing of BL cells was explored. Furthermore, the analysis of FKN expression in germinal centre (GC) B cells – the healthy counterparts of BL cells – was investigated.

4.2 Immunodetection of FKN in BL cells

In an attempt to repeat the results generated by Truman, BL2 cells were induced to undergo apoptosis by exposure to staurosporine and were lysed at the indicated time points. Western blot analysis of the samples was carried out using monoclonal anti-FKN chemokine domain and mucin stalk antibodies. An interesting correlation of the FKN band changes could be seen (Figure 4.1a) - as early as 2 hrs post-staurosporine exposure a 90 kDa band was replaced by a 60 kDa one. Importantly, the change took place when a large percentage of the cells started to display early apoptotic features i.e. PS exposure on cell surface (Figure 4.1b).

Next, the supernatants gathered from the cultures of staurosporine-treated BL2 cells were analysed in the same manner. Again, the 60 kDa species of FKN was detected at 2 hrs after staurosporine exposure, but the accompanying loss of the 90 kDa one was not observed this time (Figure 4.1a).

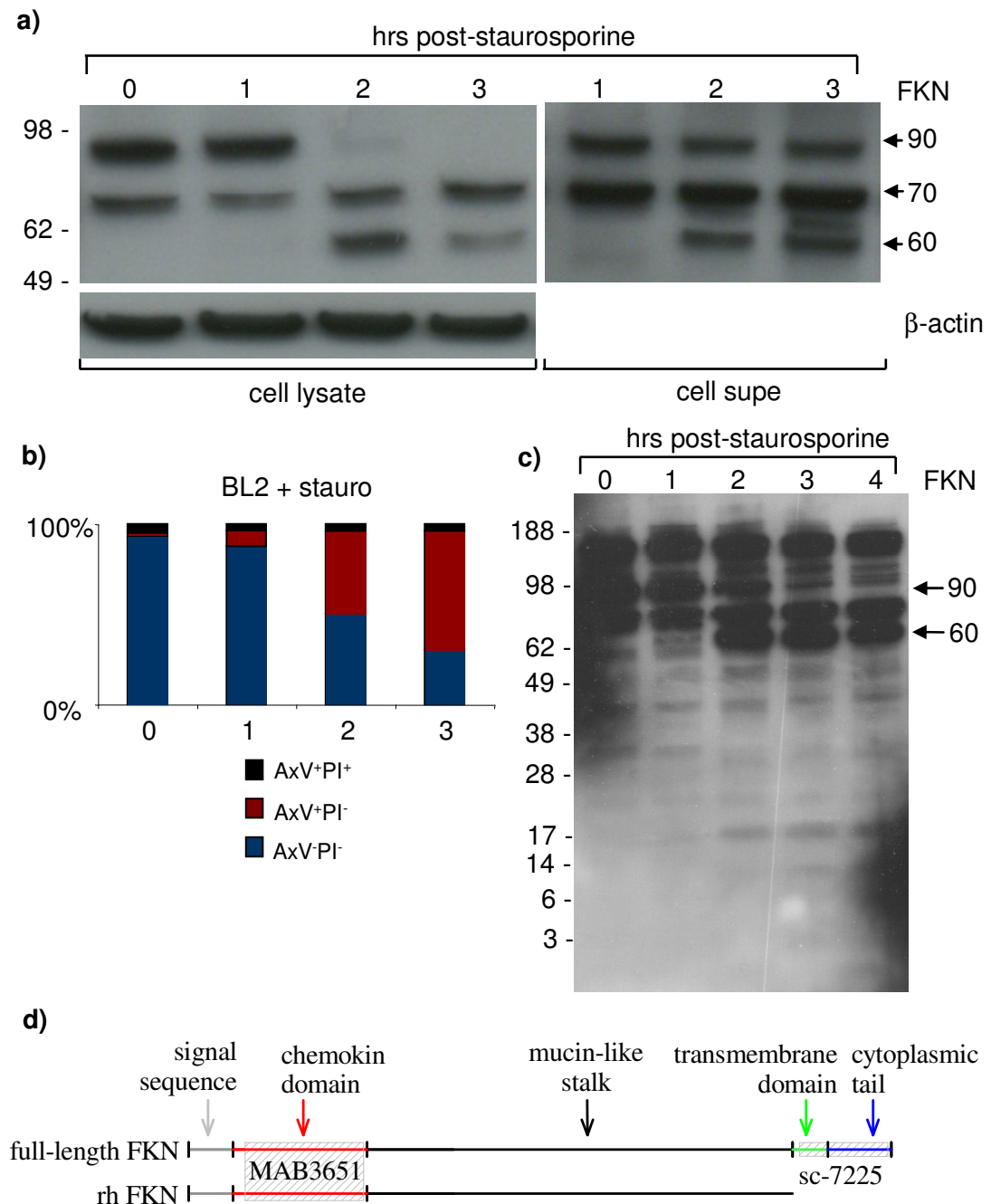


Figure 4.1 FKN detection in BL2 cells by Western blotting.

BL2 cells were induced to undergo death by staurosporine (stauro). The cells and cell-free supernatants were harvested at 1, 2 and 3 hrs after apoptosis induction. **(a)** The lysed cells and proteins precipitated from the medium by TCA were analysed by Western blotting. Blots were labelled using mouse anti-human FKN monoclonal antibodies (MAB3651). **(b)** Apoptosis was assessed by AxV/PI staining **(c)** A view of the longer exposed film. One representative result of more than twenty is shown. **(d)** Schematic picture showing fragments of wild-type full-length FKN and recombinant human FKN (R&D Systems) which are recognised by mouse monoclonal anti-human FKN chemokine domain antibodies (MAB3651) and goat anti-human FKN C-terminal end antibodies (sc-7225).

The 90 kDa-sized FKN was reported to represent either a full-length chemokine or a FKN cleaved in a membrane-proximal region - consisting of the chemokine domain and the majority of the mucin stalk (Bazan et al. 1997; Garton et al. 2001). As the 90 kDa FKN band was detected both in the lysates and as a soluble form in the supernatants (Figure 4.1a) of apoptotic BL cells it is possible that it represents the cleaved form of FKN. The 60 kDa FKN is believed to be another cleaved form of FKN, and based on its size, it is most likely cleaved further down the mucin stalk. However, due to the extensive and irregular glycosylation of FKN it is not possible to predict the exact cleavage site leading to the generation of the 60 kDa form.

The identity of the 70 kDa band detected by anti-FKN antibodies is unknown. Its size suggests that it might be an incompletely glycosylated precursor form of FKN (what could perhaps be investigated by exposing it to deglycosylating enzymes and subsequently, comparing its size with that of the deglycosylated full-length form) (Garton et al. 2001) but it might as well represent another form of cleaved FKN. However, considering that no regular changes to the intensity of this band have been detected by Western blotting of samples prepared from BL cells treated in various ways, it is equally possible that this band represents a non-specific binding of the antibodies. The nature of this band was not explored further in the studies presented in this thesis.

Although FKN bands presented in Figure 4.1 a were the most distinctive ones, within the size-range well characterised in the literature and will be in the focus of the studies presented later, it has to be noted that Western blots of apoptotic BL2 cells labelled with anti-FKN antibodies contained a wealth of differently sized bands (Figure 4.1c). The identity of those bands remains unknown and it cannot be excluded that some of them represent an unspecific antibody binding. However, their presence might also result from additional, yet unidentified, proteolytic events.

4.3 The role of apoptosis programme in FKN modulation in BL cells

In order to confirm this initial observation that the modulation of FKN seems to correlate with apoptosis of BL cells (Figure 4.1), FKN was studied in BL2 cells in which the cell death process was inhibited. Firstly, BL2 cells that were resistant to apoptosis through their stable transfection with the pro-survival gene *bcl-2* (BL2/Bcl-2 cells) were used. Despite treatment with staurosporine, these cells remained viable (Figure 4.2b). Furthermore, the 90 kDa FKN was intact in the cell lysates and the 60 kDa FKN was neither detected in the cell lysates nor in the cell-free supernatants (Figure 4.2a).

Similar results were gathered when BL2 cells were pre-treated with a poly-caspase inhibitor Z-VAD-FMK before the addition of staurosporine. Again, the 60 kDa form of FKN was not observed in any of the samples and the cells remained highly viable (Figure 4.2c, d).

Additional control reactions were also set up. Both BL2 and BL2/Bcl-2 cells were induced to death by UV light in order to exclude a possibility that the previously described (Figure 4.2a, c) modulation of FKN is a staurosporine-specific mechanism. As it is shown on Figure 4.3a, the pattern of FKN bands from UV-irradiated BL2 cells repeated that of BL2 cells induced into apoptosis by staurosporine. Also, BL2 cells cultured in RPMI 1640 with 10% FBS (as used in the experiments presented above) were compared with the cells cultured in X-Vivo media (serum-free) to ensure that the FKN previously detected by Western blotting was derived from BL cells and not from FBS. Figure 4.3b shows that the same pattern of FKN bands was detected in both samples indicating the FKN was not serum-derived.

Data presented here strongly supported the notion that the processing of 90 kDa FKN is an apoptosis-driven process in BL cells that results in the generation of 60 kDa FKN in the cell-free supernatants.

4.4 Loss of FKN from the surface of apoptotic BL cells

Given that FKN is modulated during BL cell death to generate a 60 kDa apoptosis-associated form of the molecule (Figure 4.1 and Figure 4.2) and published observations have shown that membrane-associated FKN can be released from cells as a soluble form via proteolytic cleavage (Bazan et al. 1997), it was important to monitor the expression of FKN on the surface of Burkitt's lymphoma cells.

Surface expression of FKN on BL cells has previously been assessed by both Truman and Wilkinson (Truman 2005; Wilkinson 2006). Truman demonstrated that FKN that is normally present on the surface of Mutu I cells is lost quickly when the cells enter the apoptosis programme. This indicated that the shedding of FKN might be an apoptosis-related phenomenon. However, further analysis performed by Wilkinson showed that FKN was lost not only from UV-irradiated Mutu I cells but also from Bcl-2-overexpressing (and therefore apoptosis resistant) Mutu I cells suggesting that this process is not necessarily connected to apoptosis. The studies presented here were undertaken to determine the dependence of FKN shedding on apoptosis of BL cells.

Initially, BL2 and BL2/Bcl-2 cells were exposed to staurosporine to induce apoptosis. The cells were collected up to 4 hrs post-staurosporine treatment, stained for surface FKN and subsequently analysed by flow cytometry (Figure 4.4a). The viability of the cells was also assessed by AxV/PI staining (Figure 4.4b). Although only the staurosporine-treated BL2 cells underwent apoptosis, a loss of surface FKN was observed at the 1 hr time-point in all the cases analysed. However, only the apoptotic BL2 cells showed a further decrease in surface FKN levels after 2-4 hr culture. In all the other cells the levels of FKN remained unchanged throughout the remainder of the experiment. This would suggest that only the loss of FKN observed at 2 hrs is related to apoptosis. Importantly, this correlates well with the Western blot data in which 60 kDa FKN was observed in the lysates and supernatants of apoptotic BL2 cells 2 hrs after staurosporine treatment of the cells (Figure 4.1). The loss of FKN recorded in all the samples at the 1 hr time-point might represent a constitutive

FKN shedding or be a consequence of cell handling during the early stages of the experimental procedure that could have caused damage or possibly, activation of the cells and therefore might not be significant.

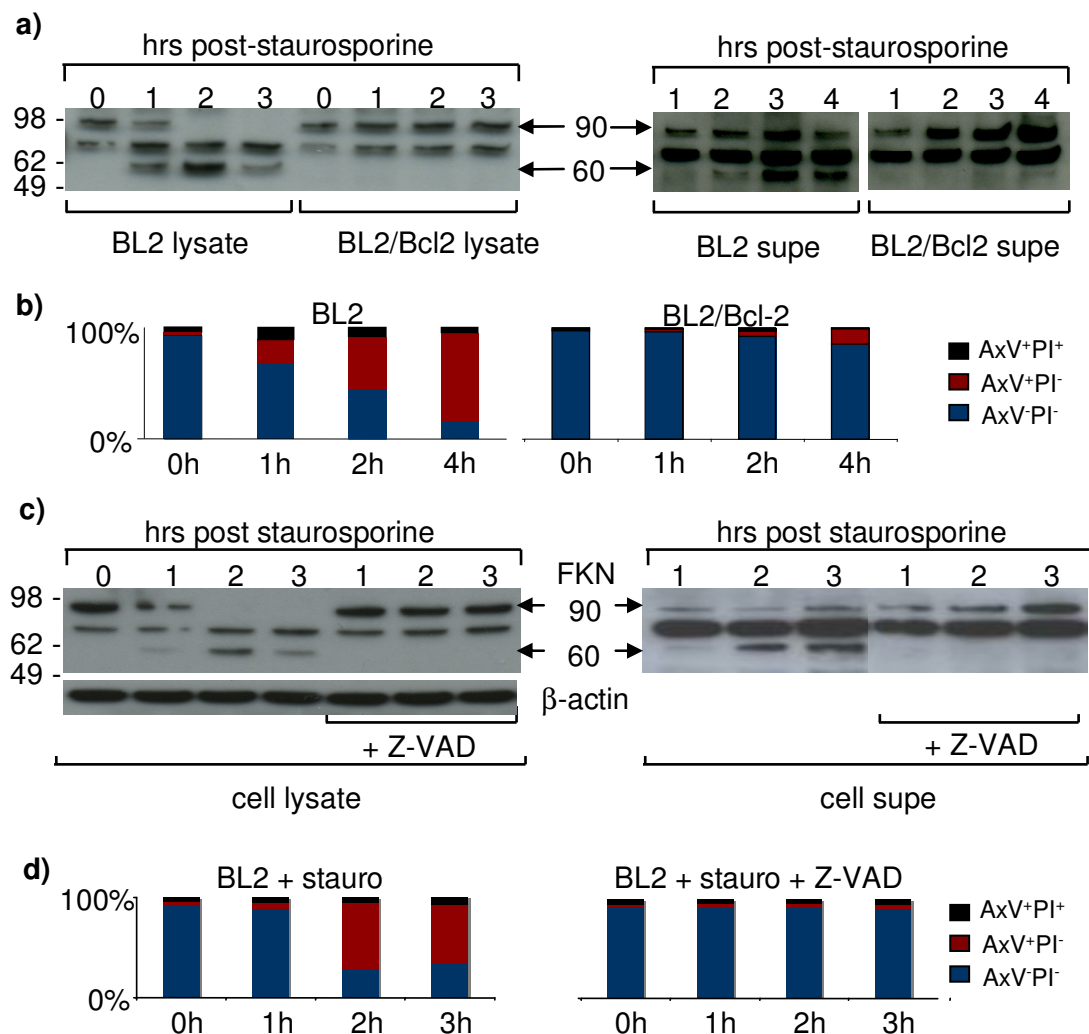


Figure 4.2 Role of apoptosis in FKN processing in BL2 cells.

(a) BL2 and BL2/Bcl-2 cells were induced to undergo death by staurosporine. The cells and cell-free supernatants were harvested at 1, 2 and 3 hrs after the apoptosis stimulus. The lysed cells and proteins precipitated from the medium by TCA were analysed by Western blotting. Blots were labelled using MAB3651 antibodies. (b) Apoptosis was assessed by AxV/PI staining. One representative result of more than five was shown. (c) BL2 cells were incubated for 30 min with caspase inhibitor Z-VAD-FMK and subsequently induced into death by staurosporine. The lysed cells and proteins precipitated from the medium by TCA were immunoblotted with MAB3651 antibodies. (d) Apoptosis was assessed by AxV/PI staining. One representative result of four is shown

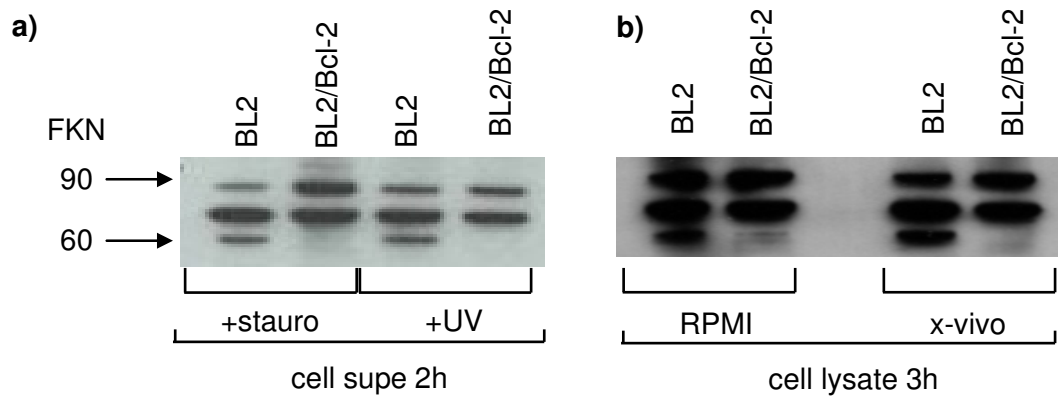


Figure 4.3 *Effect of experimental conditions on FKN detection in BL cell samples*

(a) BL2 and BL2/Bcl-2 cells were induced to death by either staurosporine or UVB light. Cell-free supernatants were immunoblotted using MAB3651 antibodies. (b) BL2 and BL2/Bcl-2 cells were placed in RPMI 1640 + 10% FBS or in X-Vivo medium, induced to undergo death by staurosporine, collected after 3 hrs, lysed and immunoblotted using MAB3651 antibodies.

A comparison of the levels of surface FKN at the 2 hr time-point of the differentially treated cells is presented in Figure 4.4c. A pre-incubation of BL2 cells with Z-VAD-FMK prior to staurosporine exposure, inhibited the loss of FKN. Similarly, BL2/Bcl-2 cells, even when incubated with staurosporine, again, showed no decrease in FKN levels. These observations led to the conclusion that substantial loss of FKN from the surface of BL cells is only observed when the cells undergo apoptosis and this loss of membrane-associated FKN occurs early in the process of apoptosis.

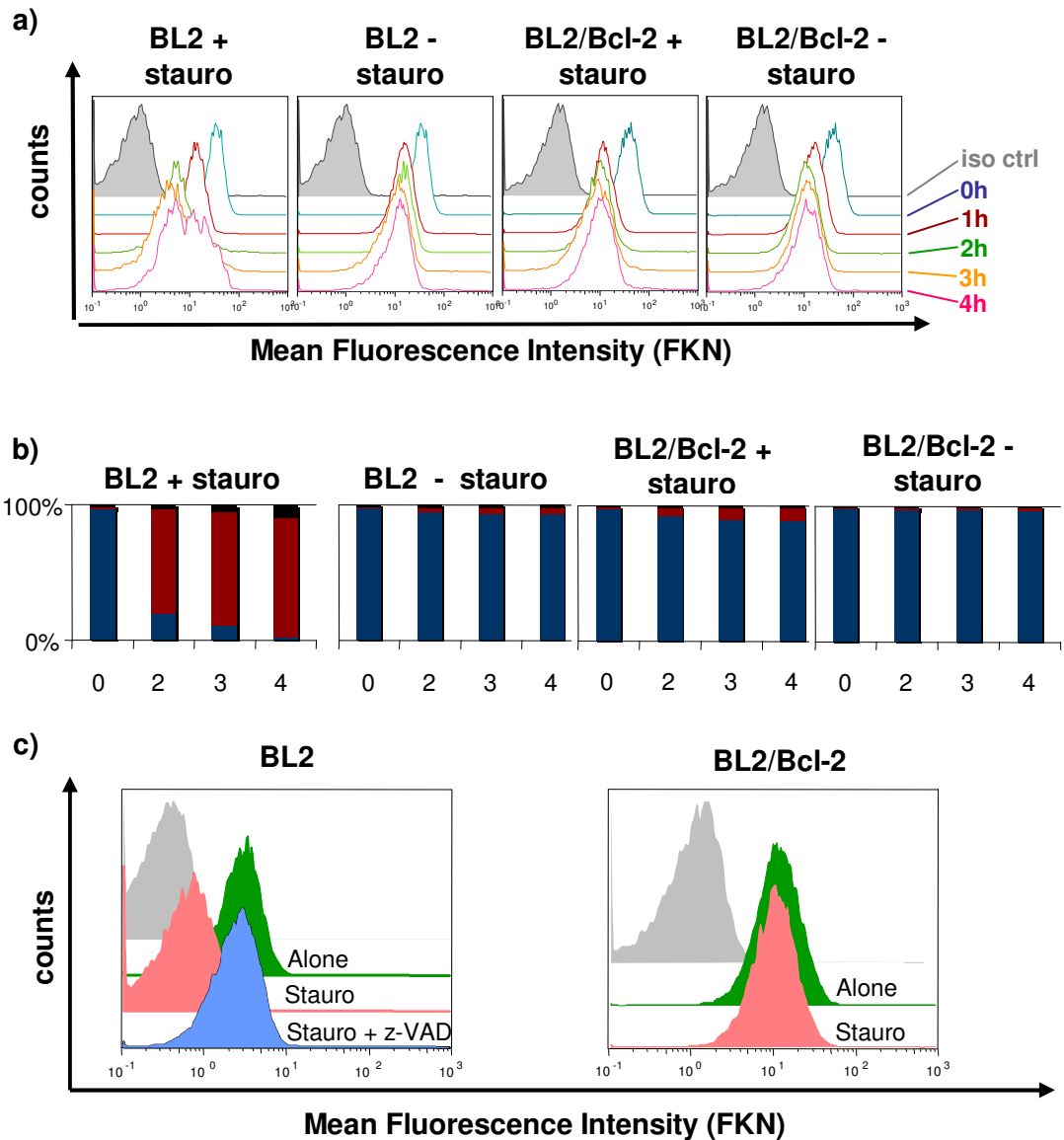


Figure 4.4 Loss of surface FKN during apoptosis of BL2 cells.

BL2 and BL2/Bcl-2 cells were either induced into death by staurosporine, pre-treated with Z-VAD-FMK for 30 min and then treated by staurosporine or left untreated. **(a)** The samples were collected after 1, 2, 3 and 4 hrs of incubation, labelled with anti-FKN antibodies (MAB3651) or IgG1 isotype control followed by FITC-conjugated anti-mouse antibodies. FKN labelling was assessed by FACS. **(b)** Apoptosis was assessed by AxV/PI staining. **(c)** FACS plots comparing the level of FKN on the cells harvested after 2 hrs of incubation and stained with anti-FKN antibodies (MAB3651) and matching secondary antibodies. One representative result from five is shown.

4.5 Searching for a protease that cleaves FKN during apoptosis of BL cells

4.5.1 The role of metalloproteinases

Members of the metalloproteinase family of proteins have been well characterised as key proteases responsible for the processing of FKN. ADAM-10 has been shown to be engaged in the constitutive shedding of membrane-bound FKN, whereas ADAM-17 (TACE) has been identified to play a role in its inducible cleavage (Garton et al. 2001; Hundhausen et al. 2003). In addition, MMP-2 has more recently emerged as a FKN-specific protease (Dean & Overall 2007). As it seemed likely that the 60 kDa form of FKN is generated from the 90 kDa form of FKN through the proteolytic cleavage (Figure 4.1), the involvement of the metalloproteinases in this process was assessed.

For this purpose, BL2 cells were pre-treated with the metalloproteinase inhibitors, GM6001, TIMP-1 and TAPI-2 (see Table 2-1 for details) prior to the induction of apoptosis. A range of inhibitors with slightly varied specificities for ADAM-10 and ADAM-17 was chosen to enable the identification of the protease involved. GM6001 is a broad-range inhibitor, known to act both on MMP and ADAM protease families. Although it is able to inhibit both ADAM-10 and ADAM-17, it has much higher effectiveness against ADAM-17 (Moss & Rasmussen 2007) and has previously been reported to inhibit ADAM-17-dependant shedding of FKN (Garton et al. 2001). TIMP-1 belongs to the family of natural tissue inhibitors of metalloproteinases. Apart from inhibiting MMPs, it is known to specifically block the activity of ADAM-10, but not ADAM-17 (Amour et al. 2000; Rapti et al. 2008). TAPI-2, although developed to block ADAM-17 activity, is now recognised as a broad-spectrum inhibitor of both MMP and ADAM family members (Ito et al. 2004; Kenny & Bissell 2007; Moss & Rasmussen 2007).

After 30 min pre-incubation with the inhibitor of choice, the cells were induced into apoptosis using staurosporine and incubated for the indicated time. FKN was detected by Western blotting in both whole cell lysates (Figure 4.5a) and supernatants (Figure 4.5b). None of the metalloproteinase inhibitors prevented

staurosporine-treated cells from losing the 90 kDa FKN (in the whole cell lysate samples) and gaining the 60 kDa FKN (in both lysate and supernatant samples), suggesting that the process of FKN cleavage in apoptotic BL cells might not be driven by metalloproteinases. Although the inhibitor activity controls were not included in these preliminary experiments, the fact that the chosen compounds are widely used and in the presented study were employed in well-established working concentrations, together with their overlapping substrate specificities, provides a high level of authenticity to the gathered data. Also, the fact that unlike FKN fragments generated by ADAM proteases, which have a size of 80-90 kDa (Garton et al. 2001; Hundhausen et al. 2003), the FKN generated during BL cell death was only of 60 kDa size may indicate that a protease different to those previously described is involved in this process.

4.5.2 Effect of broad-spectrum inhibitors

Having established that metalloproteinases are most likely not involved in cleaving FKN during BL cell death, a more general approach, involving the use of broad-spectrum inhibitors with different specificities, was taken in order to get an indication as to what type of protease might be involved in the process. Again, a set of well-characterised inhibitors with overlapping properties was selected for the study (see Table 2-1 for details). As previously, BL2 cells were pre-treated with inhibitors for 30 min prior to the addition of staurosporine. Samples were collected after 1-3 hrs of incubation and changes in FKN band pattern were analysed by Western blotting (Figure 4.6). The results obtained were surprising, as none of the used inhibitors seemed to block the generation of the 60 kDa FKN.

4.5.3 Role of caspases

4.5.3.1 Specific caspase inhibitors

From the inhibitors used thus far only the poly-caspase inhibitor Z-VAD-FMK was capable of constraining the generation of 60 kDa FKN (Figure 4.2c). Although, it is impossible to distinguish if the blockade of FKN cleavage was a direct consequence of Z-VAD-FMK inhibiting a caspase(s) responsible for cleaving FKN or it was an outcome of the blockade of apoptosis, resulting in an indirect inhibition of another

enzyme, it was decided to examine the hypothesis that the caspases are able to cleave FKN.

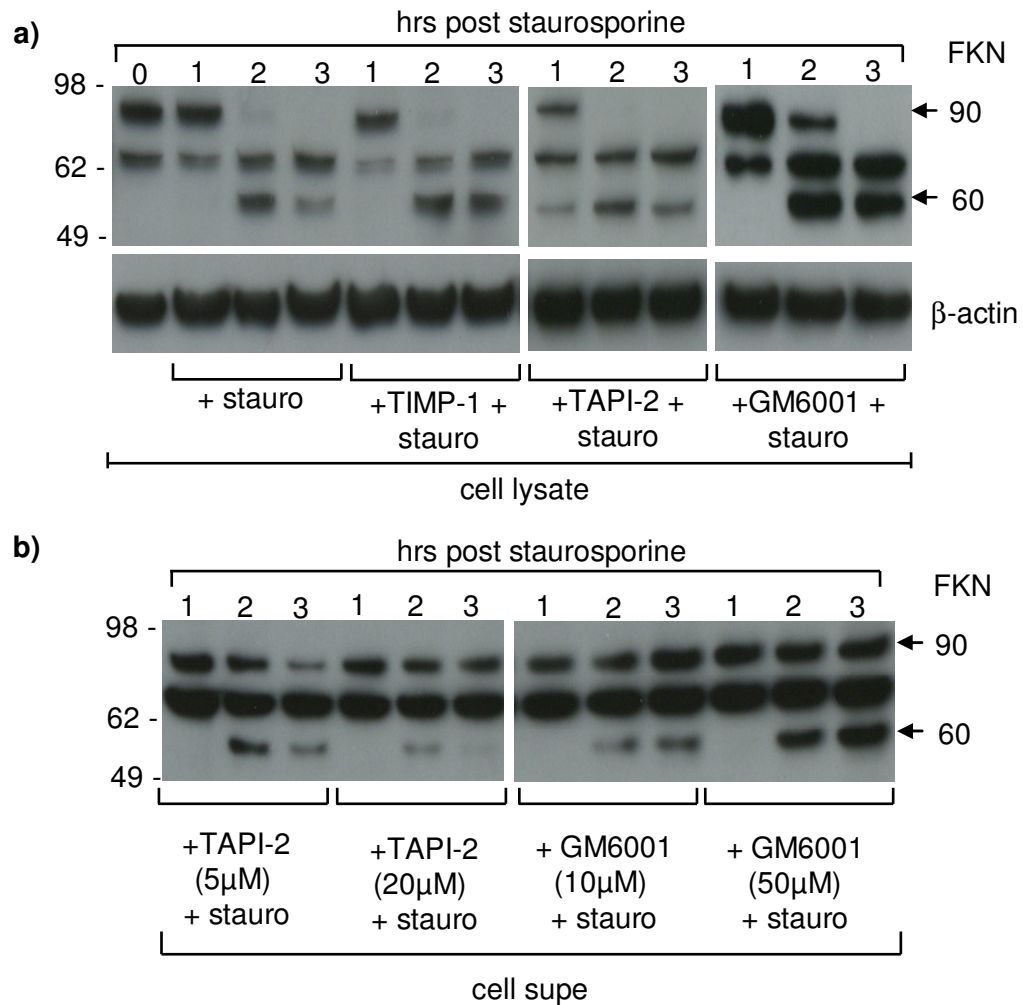


Figure 4.5 *Effect of selected inhibitors with various specificity for MMP and ADAM protease families on FKN processing in apoptotic BL2 cells.*

BL2 cells were pre-incubated for 30 min with one of the inhibitors: TIMP-1, TAPI-2 or GM6001, and subsequently induced into death by staurosporine. The samples were harvested after 1, 2 and 3 hrs of incubation and immunoblotted with anti-FKN antibodies (MAB3651). **(a)** Western blot of the whole cell lysates. **(b)** Western blot of cell-free supernatants that were concentrated using TCA. One representative result from three is shown.

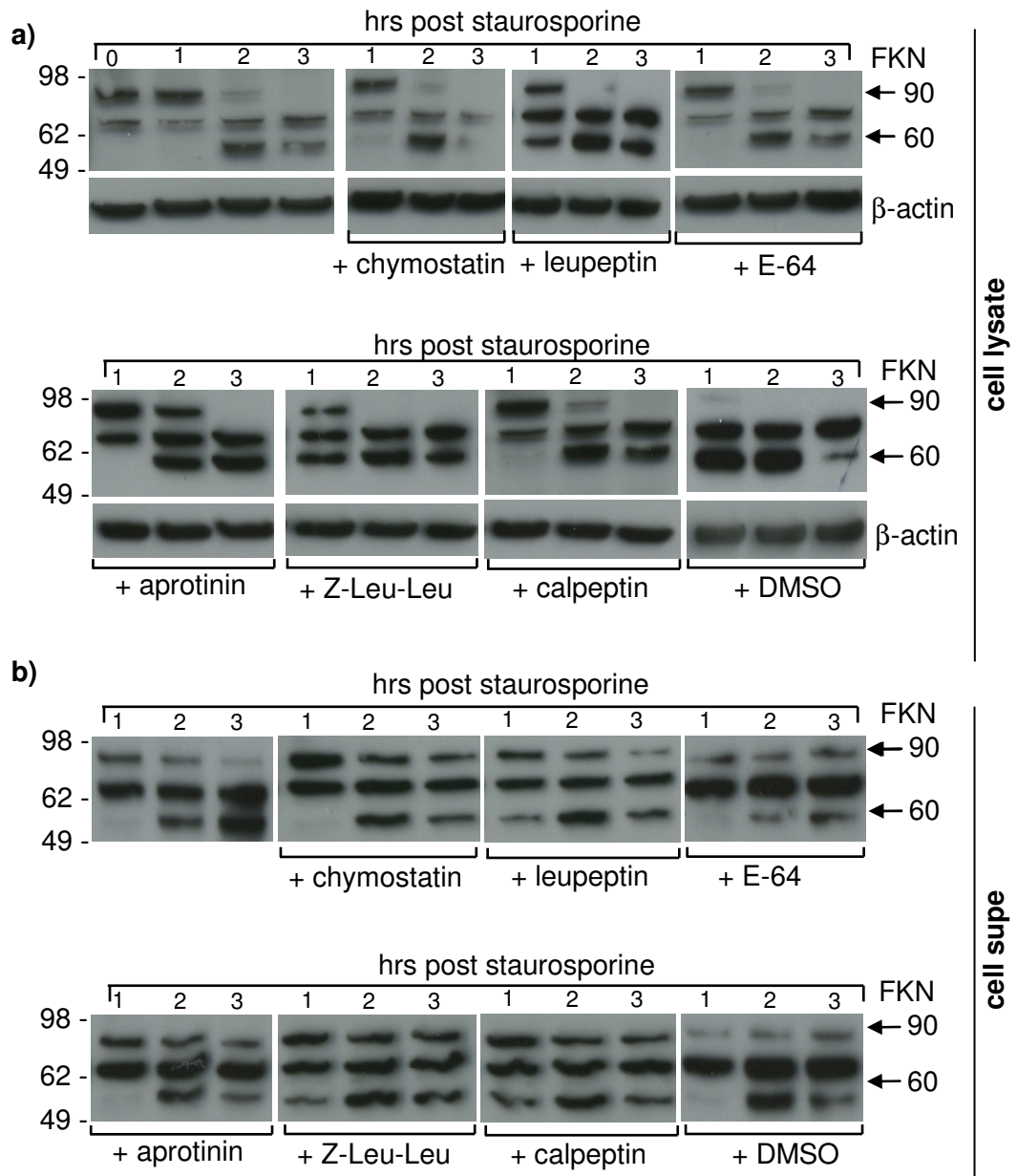


Figure 4.6 Effect of broad spectrum inhibitors on FKN processing in apoptotic BL2 cells.

BL2 cells were pre-incubated for 30 min with one of the inhibitors: chymostatin, leupeptin, E-64, aprotinin, Z-Lu-Leu-CHO, calpeptin or DMSO (ctrl), and subsequently induced into death by staurosporine. The samples were harvested after 1, 2 and 3 hrs of incubation and immunoblotted with mouse anti-FKN antibodies (MAB3651). **(a)** Western blot of the whole cell lysates. **(b)** Western blot of cell-free supernatants that were concentrated using TCA. One representative result from two is shown.

A set of more specific cell-permeable peptide inhibitors of caspases was tested at first. Although the suitability of these peptide inhibitors for identification of individual caspases involved in a studied process has been questioned (Pereira & Song 2008), it was believed that they would provide at least an indication as to if and which caspases might be involved in FKN processing. The cells were prepared as before and the obtained Western blots are presented in Figure 4.7. Two inhibitors, DEVD-FMK (inhibitor of caspase-3, -7) and AEVD-FMK (inhibitor of caspase-10 (but possibly also -6, -8, -9 – Garcia-Calvo et al. 1998) were effective in inhibiting the changes in the FKN band pattern (the result could be seen both in the whole cell lysates – Figure 4.7a and in the cell supernatants – Figure 4.7b). Importantly, the AxV/PI staining showed that the addition of specific caspase inhibitors had a less profound effect than Z-VAD-FMK on apoptosis and neither DEVD-FMK nor AEVD-FMK did inhibit the staurosporine-induced death of BL2 cells (Figure 4.8). From these results, it would seem possible that a caspase cleavage might be responsible for the occurrence of the 60 kDa fragment of FKN.

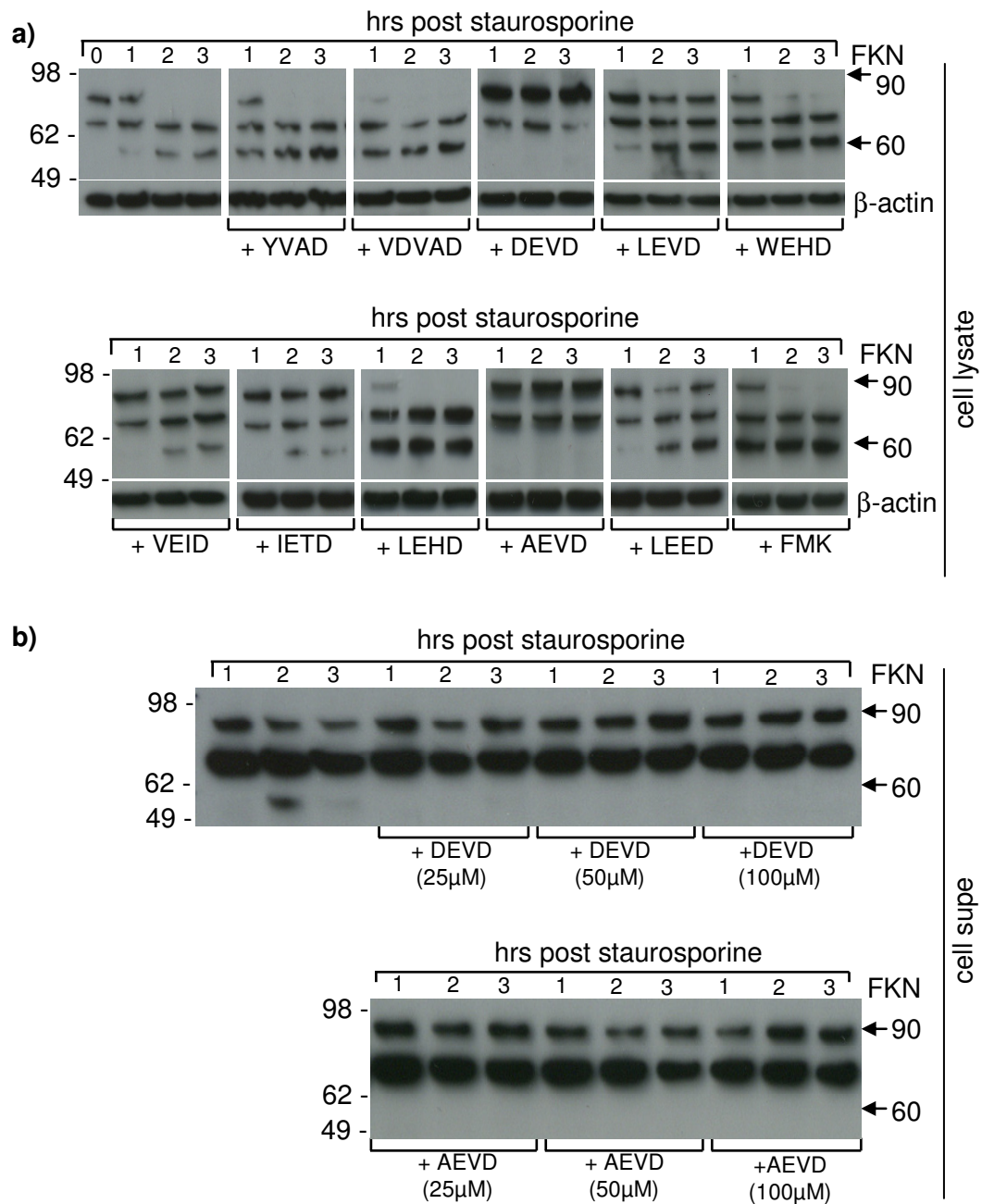


Figure 4.7 Effect of peptide caspase inhibitors on processing of FKN in apoptotic BL2 cells.

BL2 cells were pre-incubated for 30 min with one of the inhibitors: Z-YVAD-FMK, Z-VDVAD-FMK, Z-DEVD-FMK, Z-LEVD-FMK, Z-WEHD-FMK, Z-VEID-FMK, Z-IETD-FMK, Z-LEHD-FMK, Z-AEVD-FMK, Z-LEED-FMK or control compound -FMK (all at 25 μM concentration, unless stated otherwise) and subsequently induced into death by staurosporine. The samples were harvested after 1, 2 and 3 hrs of incubation and immunoblotted with anti-FKN antibodies (MAB3651). **(a)** Western blot of the whole cell lysates. **(b)** Western blot of cell-free supernatants that were concentrated using TCA.

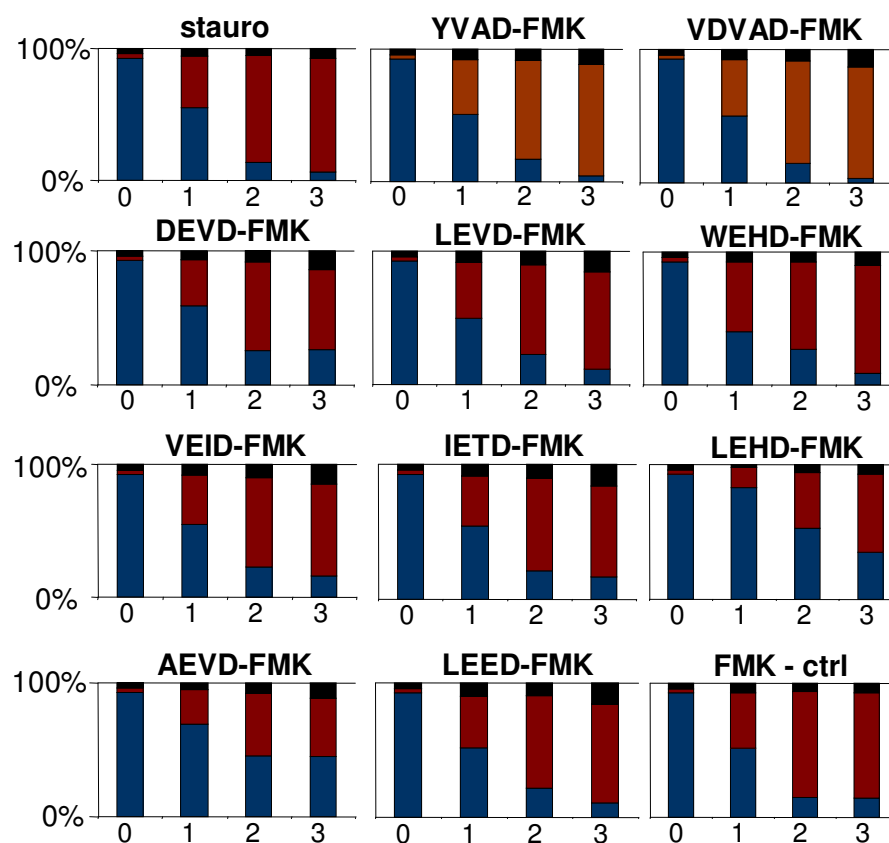


Figure 4.8 Effect of peptide caspase inhibitors on viability of BL2 cells. Apoptosis was assessed by AxV/PI staining. One representative result from three is shown.

4.5.3.2 Cleavage studies

Caspase-3 is responsible for processing the majority of caspase substrates cleaved during apoptosis (Kumar 2007; Walsh et al. 2008) and therefore was considered to be a likely candidate for a FKN-hydrolysing agent. A direct effect of caspase-3 on FKN was therefore investigated. Recombinant human caspase-3 was incubated for 2 hrs with a whole cell lysate prepared from untreated BL2 cells. Subsequently, FKN from the samples was visualised by Western blotting (Figure 4.9a). A band, of the size of the 60 kDa FKN that is observed in BL2 cells that have been treated with staurosporine for 2 hrs, was produced in the sample when recombinant caspase-3 was added. The generation of the band was completely inhibited by Z-DEVD-FMK and Z-VAD-FMK. Similarly, recombinant caspase-3 was incubated with recombinant FKN (Figure 4.9b). A lower molecular weight, most likely a hydrolysis product – as it was sensitive to Z-DEVD-FMK, was detected after the addition of recombinant

caspase-3. Taken together, this *in vitro* data suggests that caspase-3 is capable of cleaving FKN.

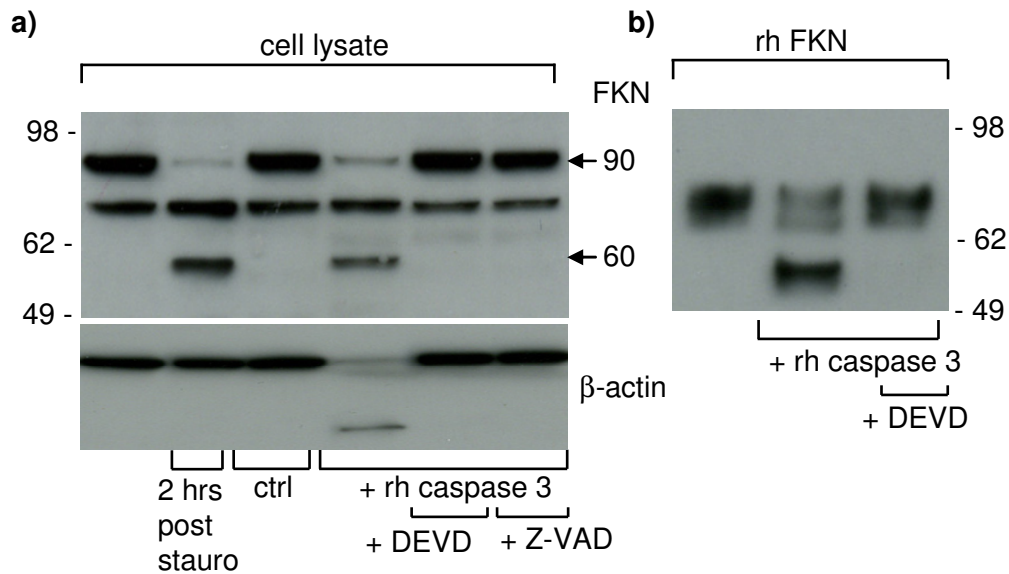


Figure 4.9 Recombinant human caspase-3 cleaves FKN to generate 60 kDa form.

(a) Whole cell lysate of viable BL2 cells was incubated for 2 hrs with recombinant human caspase-3 alone or with addition of either Z-DEVD-FMK or Z-VAD-FMK. Whole cell lysate of 2 hrs post-staurosporine BL2 cells was included for a comparison. (b) Recombinant human FKN was incubated for 2 hrs with recombinant human caspase-3 alone or with addition of either Z-DEVD-FMK or Z-VAD-FMK. Samples were immunoblotted using anti-FKN antibodies (MAB3651). One representative result from four is shown.

The studies were later extended to caspase-10, as its inhibitor had also showed a potency to block generation of 60 kDa FKN (Figure 4.7), and to caspase-7 – another executioner caspase, believed to share a number of substrates with caspase-3 (Jang et al. 2007; Walsh et al. 2008). The activity of caspase-7 towards FKN was tested both in BL2 cell lysates and in an assay with recombinant FKN. However, the low molecular weight FKN was not detected this time (Figure 4.10a). Recombinant caspase-10, when incubated with recombinant FKN also did not seem to be able to cause hydrolysis of FKN under these conditions (Figure 4.10b). This data does not exclude the possibility that under different conditions e.g. physiological conditions or in a higher concentration of caspase, caspase-7 and caspase-10 (but also caspases-6, -8, or -9) may be capable of processing FKN. In fact, it seems to be consistent with the notion that caspase-3 is generally more potent than other caspases in cleaving most of their shared substrates (McStay et al. 2007; Walsh et al. 2008). However, in

order to confirm the validity of these results it would be important to perform additional control analysis of the activity of recombinant caspase-7 and caspase-10. An assay monitoring the ability of these caspases to process some of their well known substrates (like p23 for caspase-7 or Bid for caspase-10) in parallel to assessing their capacity for cleaving FKN would be of use here (Walsh et al. 2008; Wachmann et al. 2010).

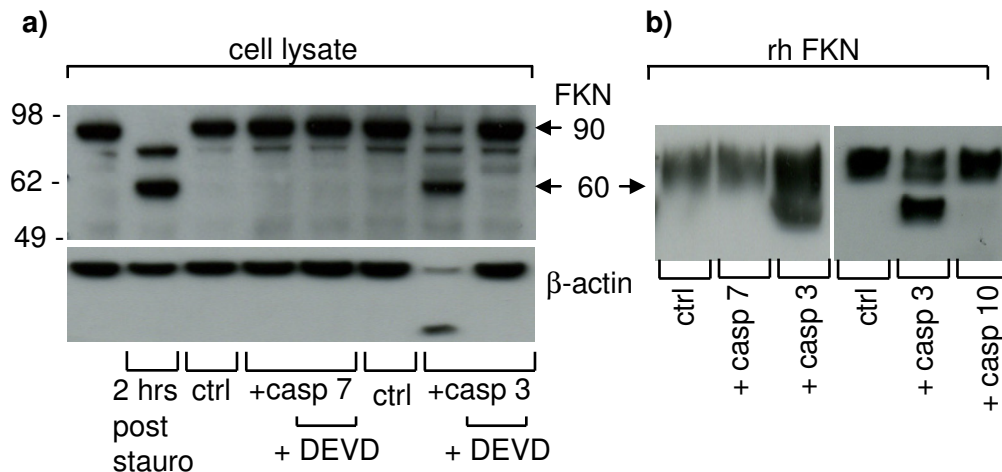


Figure 4.10 Examining capability of caspase-7 and caspase-10 to cleave FKN
(a) Whole cell lysate of viable BL2 cells was incubated for 2 hrs with rh caspase-7 or rh caspase-3, alone or with addition of Z-DEVD-FMK. One representative result from two is shown. **(b)** Rh FKN was incubated for 2 hrs with rh caspase-7, caspase-3 or caspase-10. One representative result from two is shown. Samples were immunoblotted using anti-FKN antibodies (MAB3651).

4.5.3.3 Down-regulation of caspase-3 in BL2 cells

To attempt to confirm the involvement of caspase-3 in FKN processing in the course of apoptosis of BL2 cells, the decision was made to silence the caspase-3 gene in BL2 cells. The addition of caspase-3 inhibitor – Z-DEVD-FMK did not previously block apoptosis of BL2 cells (Figure 4.8), indicating that other proteases activated during the staurosporine-induced death can compensate for caspase-3. Therefore, the down-regulation of caspase-3 in BL2 seemed to be an appropriate measure for discriminating between the role of caspase-3 as an apoptosis executioner and FKN-cleaving protease.

The caspase-3 gene was knocked-down using a lentiviral shRNA system and stably transduced cells were selected using puromycin. The non-targeting shRNA vector was used as a control. Figure 4.11a shows the efficiency of the caspase-3 down-

regulation, as assessed by reverse transcription PCR with caspase-3 specific primers. The presence of a very faint band representing the caspase-3 product proved the success of the gene knock-down.

The successful generation of caspase-3-depleted BL2 cells enabled the effect of caspase-3 knock-down on FKN processing during cell death to be evaluated. Caspase-3 knocked-down BL2 cells (BL2/casp3kd), control BL2 cells (infected with control vector-expressing lentivirus) (BL2/ctrl) and parental BL2 cells were incubated with staurosporine and analysed after 2 hrs. Both cell lysates and conditioned media were immunoblotted using anti-FKN and anti-caspase-3 antibodies (Figure 4.11b). Although the substantial reduction in caspase-3 protein level was clearly visible in the BL2/casp3kd samples, it did not seem to have any effect on the level of the 60 kDa FKN, which remained similar to that in control samples. Again, this was not due to blockade of apoptosis in BL2/casp3kd cells as the amount of AxV+/PI- cells was comparable among all the analysed cell types (Figure 4.11c). As the expression of caspase-3 was not completely abrogated and some portion of the protein was still present in the BL2/casp3kd cells, it is, unfortunately, impossible to state if the complete processing of the 90 kDa FKN was possible because the remaining amount of caspase-3 was sufficient to cleave it or the cleavage was carried out by another protease. The possibility that the remaining caspase-3 can still cause the complete degradation of its substrates might be further examined by analysing the rate of cleavage of another known substrate of caspase-3 (e.g. gelsolin or p23) in the knocked-down cells (Walsh et al. 2008).

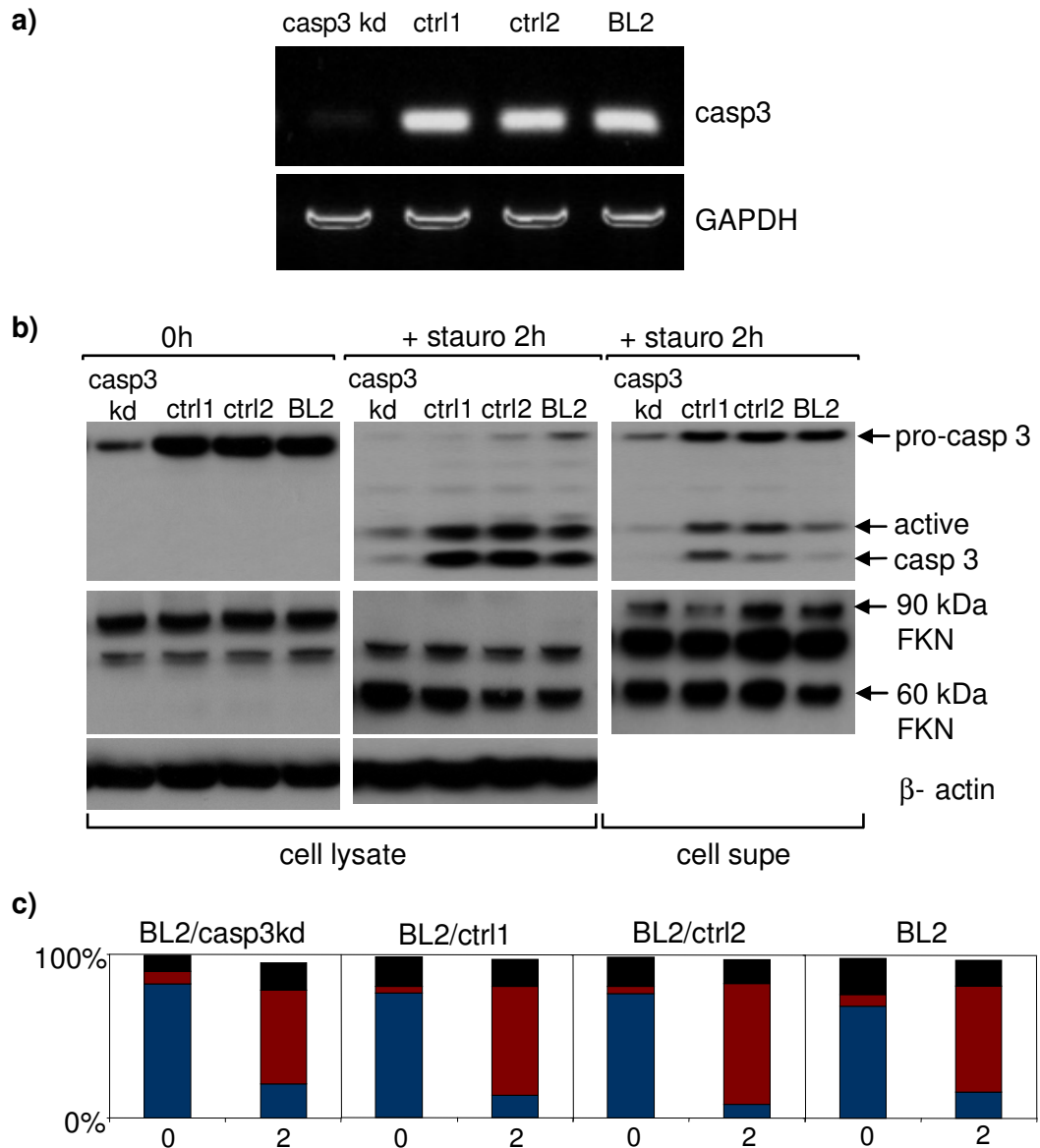


Figure 4.11 Down-regulation of caspase-3 in BL2 cells.

(a) Total RNA was extracted from BL2/casp3kd (casp3kd), BL2/ctrl (ctrl1, ctrl2) and parental BL2 (BL2) cells and reverse-transcription-PCR for caspase-3 was performed. Control reactions for GAPDH were also included. One representative result from five is shown. (b) Caspase-3 and FKN expression in BL2/casp3kd, BL2/ctrl and parental BL2 cells was analysed by Western blotting. Cell lysate samples from untreated cells as well as cell lysate and supernatant samples from 2 hrs post-staurosporine cells were immunoblotted using anti-caspase-3 antibodies (9662) or anti-FKN antibodies (MAB3651). One representative result from six is shown. (c) Apoptosis was assessed by AxV/PI staining.

4.5.3.4 Introducing point mutation into the FKN gene sequence

The work presented thus far did not give the final confirmation for the involvement of caspases in FKN cleavage in BL cells. As caspases are highly conserved and are only able to cleave their substrates after aspartic acid residues (Asp; D) (Timmer & Salvesen 2007), it should be possible to determine their role in processing of FKN by introducing point mutations in the Asp residues of the FKN sequence. The sequence of FKN mucin stalk, accommodating putative cleavage sites for proteases, contains seven aspartic acid residues (see Appendix A). Computational tools were used to predict which of them might be involved in caspase-mediated cleavage. No consensus cleavage site was detected, but one of the used applications identified sequence STQD (D223) as a possible site of cleavage. Nonetheless, it was decided to introduce the mutations not only in D223 but also in the remaining mucin stalk Asp residues. Plasmid constructs carrying the FKN gene sequence were used for site-directed mutagenesis and all the Asp residues were successfully replaced with glutamic acid (Glu; E) (see Appendix A for details). Glutamic acid was chosen for the substitution as it should be able to impede caspase-3-driven cleavage (caspases were shown to have 10^4 lower specificity for Glu than Asp (Stennicke et al. 2000) but at the same time glutamic acid has similar physical and chemical properties to aspartic acid so is not believed to cause a change to the structural conformation of the protein. As the conformation is known to play a role in substrate - caspase interactions (Timmer & Salvesen 2007) it seemed important to ensure that this aspect of the protein structure remained unchanged. Unfortunately, due to the technical problems encountered during the attempts to express the mutated-FKN plasmids in human cells, it was not possible to determine the effect of the mutations on the cleavage of FKN before the end of period prescribed for the completion of this thesis.

4.6 Role of apoptosis-derived microparticles (MPs) in the release of FKN from BL cells

Generation of membrane blebs carrying portions of dying cell content is one of the most characteristic features of apoptosis (Kerr et al. 1972). There is a growing recognition of the importance of MP-carried signals in various physiological conditions. In particular, apoptotic cell-derived MPs have been reported to have a potency to induce death of macrophages (Distler et al. 2005). In another study Segundo et al. demonstrated that blebs derived from germinal centre B cells were able to cause macrophage chemoattraction (Segundo et al. 1999). Studies undertaken previously in this group have revealed that FKN was also released in association with MPs (Truman et al. 2008). Very intriguingly, it was demonstrated that 60 kDa FKN is present exclusively in MPs but not as a soluble form in MP-free supernatants. 90 kDa FKN, on the other hand, was found both in MPs and MP-free supernatants. The possibility that 90 kDa FKN may be processed to 60 kDa FKN while in MPs seemed plausible.

Following on from the observation that various proteases were found to be secreted in MPs (Taraboletti et al. 2002), it was decided to investigate caspase-3 presence in MPs released from BL cells.

4.6.1 FKN and caspase-3 co-release in BL2-derived MPs

At first, the presence of caspase-3 in BL2-derived MPs was examined by Western blotting. The MP fraction was acquired by ultracentrifugation of cell-free conditioned media from staurosporine-treated BL2 cultures collected after 2 hrs. The MP fraction was lysed using mild detergent prior to electrophoretic separation. Two different anti-caspase-3 antibodies were used to label the blots. The first one was able to recognise only the active form of caspase-3 whereas the second one was able to detect both the active form and pro-caspase-3 (Figure 4.12a). Both antibodies visualised a substantial amount of caspase-3. Interestingly, the MP fraction contained both intact pro-caspase-3 as well as its active form, again suggesting that the

apoptosis-driven processing of cell content may well be extended into MPs. Similarly, MMP-2 and MMP-9 that were reported to be released in vesicles, were present in MPs both in their inactive (pro-enzyme) and active forms (Taraboletti et al. 2002). Subsequently, the particular ability of MP-derived caspase-3 to hydrolyse fluorescently-labelled specific peptide substrate ((Z-DEVD)₂-Rh110) was tested. The intensity of fluorescent light emitted by the cleaved substrate was measured (Figure 4.12b). Lysed MP samples and apoptotic BL2 cell lysate samples were equally potent in generating fluorescent cleavage product, thus, supporting the possibility that proteolytic cleavage of caspase substrates might be continued in vesicles that detached from the cells. The main requirement for this is that both protease and its substrate are released in the same vesicles. It was therefore decided to examine if FKN and caspase-3 are co-released in the same MPs. For this reason MPs obtained from the supernatants of 2 hrs post-staurosporine-treated BL2 cells that were labelled with DAPI (DNA stain) as well as with anti-FKN and anti-active caspase-3 antibodies and matching fluorescently-labelled secondary antibodies, were analysed by confocal microscopy. Figure 4.12c shows a representative picture of stained MPs. Although these very basic experiments do not allow the percentage of MPs that were positively stained with both of the used antibodies in the whole MP population to be calculated, they clearly demonstrated that at least some of the MPs contain both FKN and caspase-3. Taken together, the studies presented in this section revealed that FKN and proteolytically active caspase-3 can be co-released from dying BL2 cells in the process of membrane-blebbing. This mechanism can possibly enable a further regulation of FKN action, e.g. prolong FKN presence in the environment.

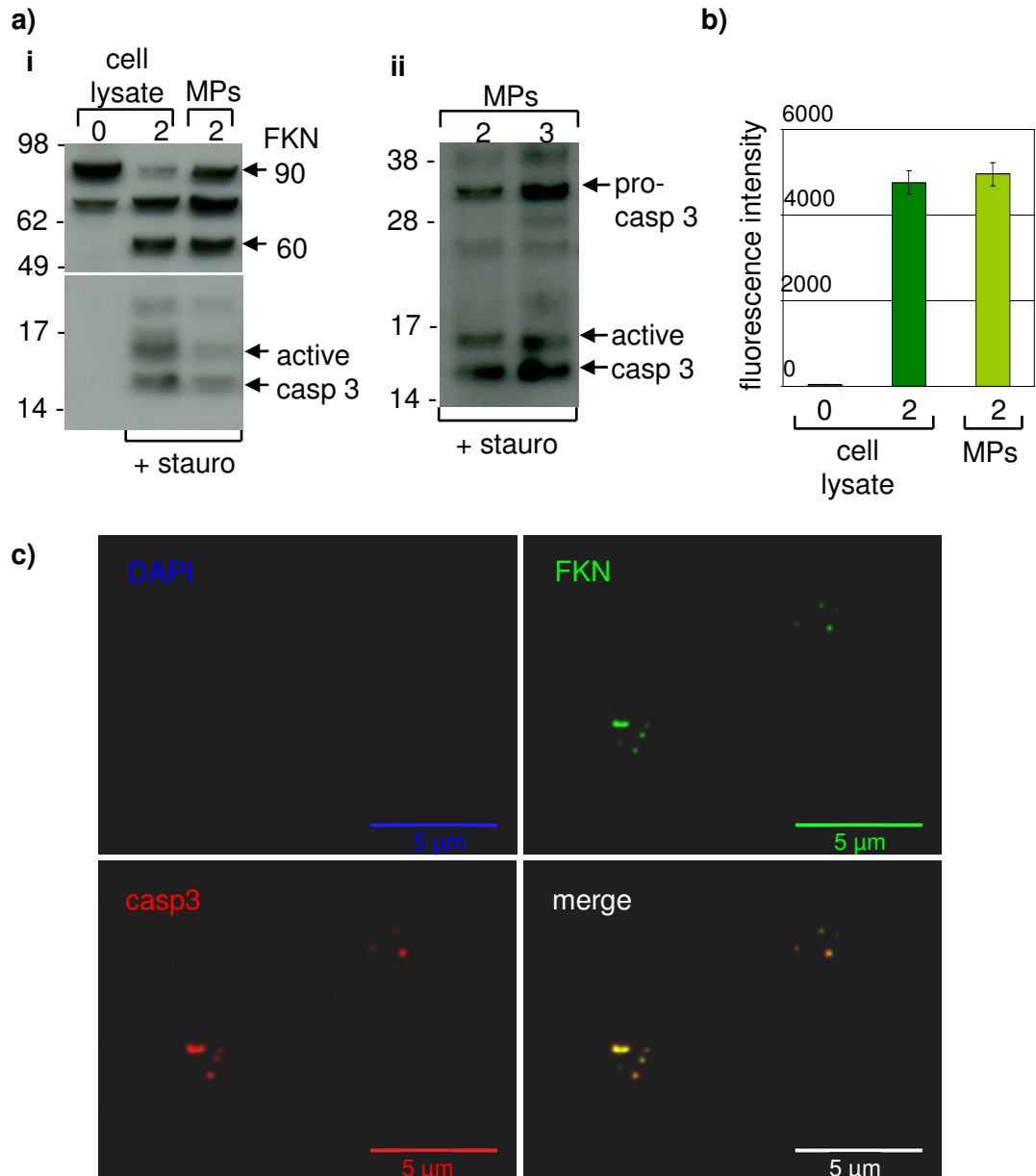


Figure 4.12 Presence of FKN and active caspase-3 in MPs from BL2 cells

(a) Cell lysates from untreated or staurosporine-treated BL2 cells and MPs acquired by ultracentrifugation of supernatants from BL2 cells induced into death with staurosporine for 2 or 3 hrs were immunoblotted using anti-FKN antibodies (MAB3651) or anti-active caspase-3 antibodies (PC679-upper panel or 9662-lower panel). One representative result from four similar experiments is shown. (b) Detection of active caspase-3 in the assay with caspase 3-specific fluorogenic peptide substrate (Z-(DEVD)₂-Rh110). Cell lysates from untreated or 2 hrs staurosporine-treated BL2 cells and lysed MPs acquired from supernatants of staurosporine-treated BL2 cells were incubated for 1 hr with the substrate. Samples were analysed by measurement of fluorescence. One representative result from two is shown. The error bars represent the standard error of the mean (n=3). (c) MPs acquired by ultracentrifugation of supernatants from staurosporine-treated BL2 cells were labelled using anti-FKN antibodies (MAB3651) and anti-active caspase-3 antibodies (9661) as well as with nuclear stain - DAPI, mounted on the microscope slide and analysed by confocal microscopy. One representative result from two is shown.

4.6.2 Characterisation of BL cell-derived MPs

To increase our understanding of the mechanisms of FKN release, an attempt was made to further characterise BL cell-derived MPs.

4.6.2.1 Time lapse imaging of MP release from apoptotic BL cells

The formation and subsequent release of MPs from staurosporine-induced BL2 cells were monitored using phase contrast widefield microscopy and a time lapse imaging system. Figure 4.13 contains the representative stills from the picture sequence, showing the morphology of the cells from the beginning of the experiment (~ 40 min post-staurosporine) and when the apoptotic features were clearly visible (~120 min post-staurosporine). The whole picture sequence can be found in Appendix B. Analysis of the gathered material revealed that the intensity of vesicle budding and detachment increased dramatically around 90 min after the induction of apoptosis. The observed time of MP release seemed to correlate very well with previously described changes in FKN expression e.g. the loss of surface expression detected in apoptotic BL2 cells (Figure 4.4).

4.6.2.2 Morphological characteristics of vesicles produced during BL cell death

It was also decided to examine the morphological characteristic of MPs produced by BL cells. For this purpose, BL2 cells were induced into death by 2 hrs of staurosporine treatment. The MP fraction was acquired by ultracentrifugation of conditioned media and the MPs were analysed by flow cytometry. Two control samples containing 50 nm and 1 μ m beads (Figure 4.14 a and b, respectively) were also measured using the same protocol to allow an approximate size-estimation.

From the dot plots of isolated MPs two major populations could be distinguished: population of small vesicles (possibly around 50 nm size), which accounted for less than 30% of events and a larger population of bigger vesicles (probably below 1 μ m size) (Figure 4.14). Vesicles with a diameter of around 1 μ m are generally believed to be cell membrane-derived MPs whereas a size of 30-100 nm is usually assigned to exosomes - vesicles of endocytic origin (Denzer et al. 2000; Aharon et al. 2008; Lakkaraju & Rodriguez-Boulan 2008). This study revealed that the vesicle fraction acquired by ultracentrifugation of apoptotic BL2 cell-conditioned media, not only

contained the characteristic apoptotic cell-derived MPs but also had a population of smaller vesicles, possibly exosomes.

In order to understand the cellular origin of the BL cell-derived vesicles, examination of markers present on the surface of the vesicles would be required. MPs are known to express cell surface antigens whereas exosomes are characterised by expression of proteins like MHC molecules or members of the heat shock protein family (Aharon et al. 2008). The analysis of apoptotic BL2 cell-derived vesicles (Figure 4.12c) using confocal microscopy demonstrated that FKN and caspase-3 expression was associated with MPs (~ 1 μm). It would however be interesting to try to determine if any FKN is present in smaller vesicles or if there is any difference in the distribution of different forms of FKN between these two populations of vesicles.

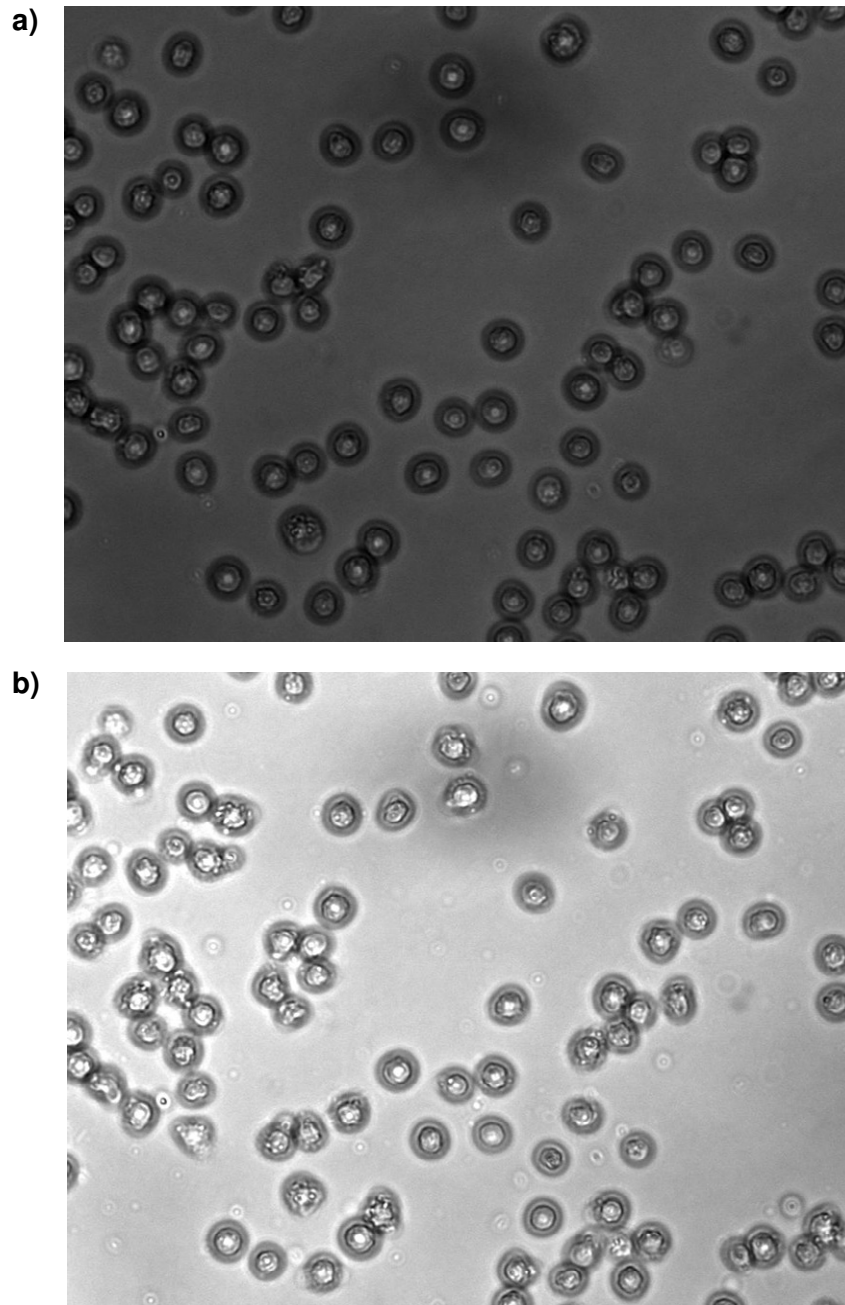


Figure 4.13 Time lapse imaging of MP release from apoptotic BL cells
BL2 cells in 25 mM HEPES-buffered RPMI 1640 + 10% FBS medium that was saturated with 5 % CO₂ were placed in POC mini chamber on a heated microscope stage (37°C) of an inverted microscope. A low-magnification image (10x objective) of the cells was collected every 15 sec, for 150 min, starting 30 min after the cells were exposed to staurosporine. **(a)** BL2 cells ~ 40 min post-staurosporine **(b)** BL2 cells ~ 120 min post-staurosporine. The full sequence of pictures can be found in Appendix B.

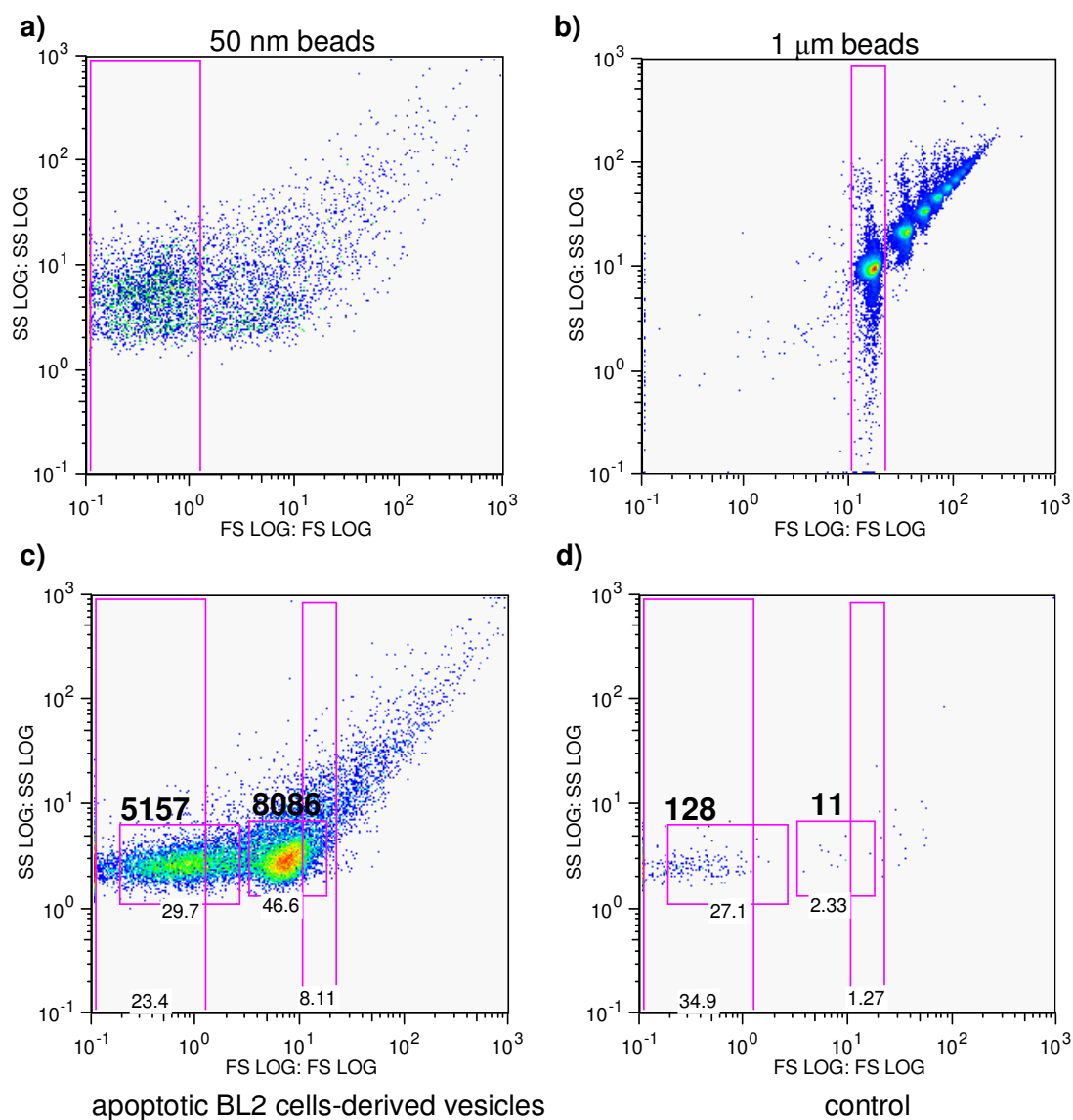


Figure 4.14 Flow cytometric analysis of vesicles derived from apoptotic BL cells.

Dot plots of logarithmic forward versus side scatters of **(a)** 50 nm diameter beads **(b)** 1 μm diameter beads **(c)** MPs acquired by ultracentrifugation of supernatants from BL2 cells induced into death with staurosporine for 2 hrs **(d)** control: filtered PBS – MPs diluent. Values on top of gates represent the number of gated events, whereas values at the bottom of gates show the percentage of gated events in total number of events. One representative result of five similar is shown.

4.7 FKN localisation in apoptotic BL cells

Surface expression of FKN has been studied extensively whereas intracellular localisation of FKN remains poorly characterised. Liu et al. have found that in human endothelial cells stably transfected with FKN, the chemokine is dynamically distributed between two cellular compartments: cell surface and juxtannuclear endomembrane vesicles (Liu et al. 2005). The results presented thus far indicated that most of the FKN processing in BL2 cells takes place intracellularly (Figure 4.1). The subcellular localisation of FKN in BL cells was thus investigated. The localisation of caspase-3 was also examined in BL2 cells. Two hours after the induction of apoptosis by staurosporine, the BL2 cells were fixed, permeabilised and stained with DAPI as well as anti-FKN, anti-active caspase-3 and matching fluorescently-labelled secondary antibodies. The samples were analysed by confocal microscopy (Figure 4.15). FKN, although distributed throughout the cytoplasm, seemed to be more abundant in the membrane-proximal area. Furthermore, active caspase-3 appeared to co-localise with FKN in the proximity of the cell membrane. To determine whether the visualised FKN was localised inside the cell or extracellularly, the intact BL2 cells were stained with anti-FKN antibodies. This showed that a substantially lower level of clustered FKN was detected on the cell surface (Figure 4.16), indicating that the membrane-proximal FKN was mainly present intracellularly (Figure 4.15).

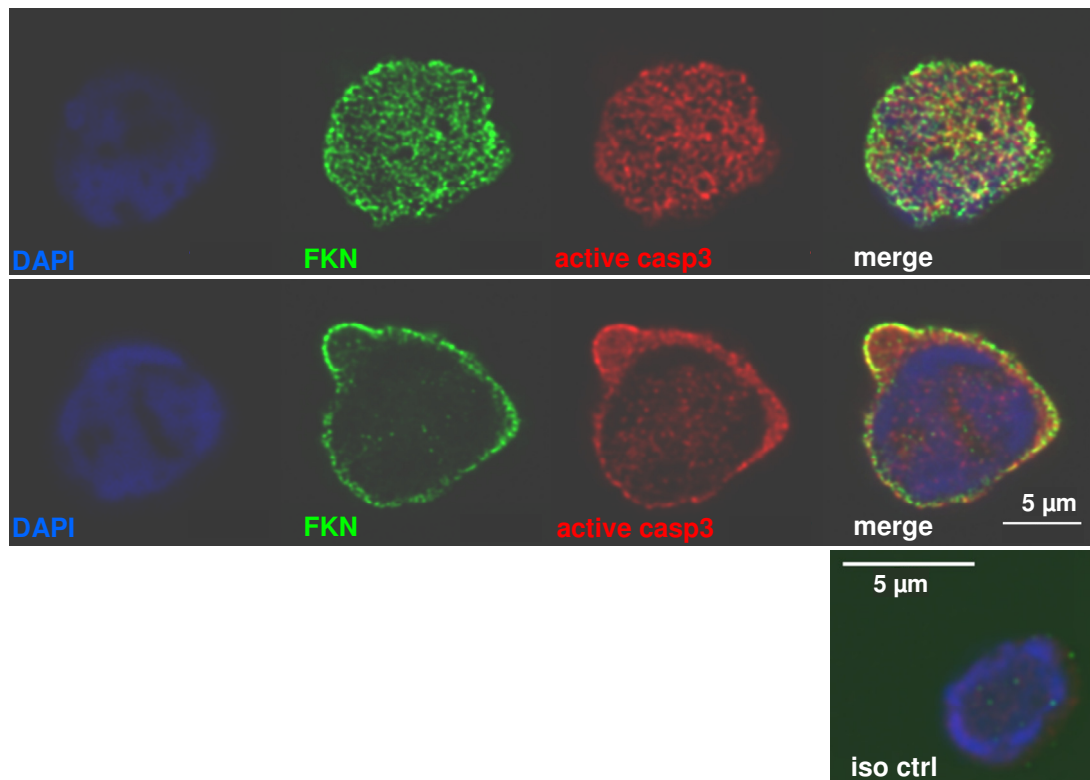


Figure 4.15 FKN and active caspase-3 localisation in apoptotic BL cells

BL2 cells were induced into death with staurosporine for 2 hrs, fixed, permeabilised and labelled using mouse anti-FKN antibodies (MAB3651) and rabbit anti-active caspase-3 antibodies (9661) and matching fluorescently-labelled secondary antibodies. Nuclear stain – DAPI (blue), was also used. Labelled cells were mounted on a microscope slide and analysed by confocal microscopy. The two rows of pictures represent different optical sections of the cell. Control staining with DAPI and the secondary antibodies was included. One representative experiment of two is shown.

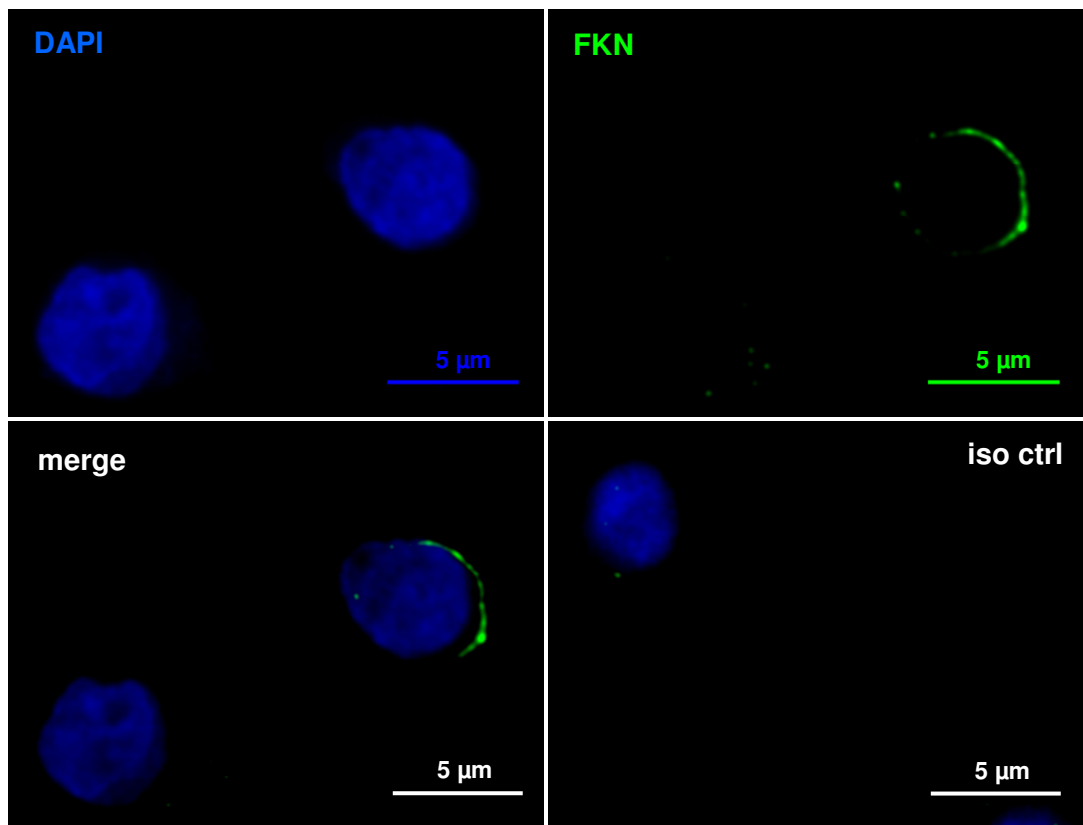


Figure 4.16 FKN staining of non-permeabilised BL cells

BL2 cells were fixed and labelled using DAPI (blue), mouse anti-human FKN monoclonal antibodies (MAB3651) and isotype matched AF488-conjugated goat anti-mouse IgG antibodies (green). Labelled cells were mounted on a microscope slide and analysed by confocal microscopy. Control staining with DAPI and IgG1 isotype control was included. One representative result from two is shown.

4.8 Release of FKN by germinal centre B cells

The occurrence and release of the 60 kDa form of FKN observed in Burkitt's lymphoma cells has been shown to be of prime importance for the attraction of macrophages to the site of apoptosis and hence for clearance of apoptotic debris (Truman et al. 2008). The potential role of FKN during the cell death of other cell types remains unknown. The studies presented here aimed at extending our knowledge to germinal centre (GC) B cells – the healthy counterparts of Burkitt's lymphoma cells (Gregory et al. 1987). During affinity maturation in the germinal centre apoptosis of B cells occurs at a very high rate therefore it seemed likely that FKN may play a key role in attracting tingible body macrophages and ensuring effective clearance of apoptotic cells.

Murine germinal centre B cells were isolated from the spleens of animals immunised with sheep red blood cells. Germinal centre and follicular (resting) B cells were separated based on their IgD expression. As GC B cells are known to undergo spontaneous apoptosis also in the culture conditions (Liu et al. 1989), IgD⁻ germinal centre B cells and IgD⁺ follicular B cells were placed in fresh media, cultured for a specified time and subsequently, their supernatants were immunoblotted using anti-mouse FKN antibodies (Figure 4.17a). The 60 kDa form of FKN was detected in all the samples. However, its level was considerably higher in GC B cells, which are known to undergo spontaneous apoptosis (Figure 4.17b).

Next, germinal centre and follicular B cells were separated from human tonsils excised from healthy individuals. Similarly, the cells were cultured for the indicated time and conditioned media was analysed by Western blotting using anti-human FKN antibodies (Figure 4.17c). The results resembled those obtained from murine germinal centres, that is a substantial amount of the 60 kDa FKN was detected in GC B cells whereas the follicular B cells showed significantly lower levels of this form of FKN.

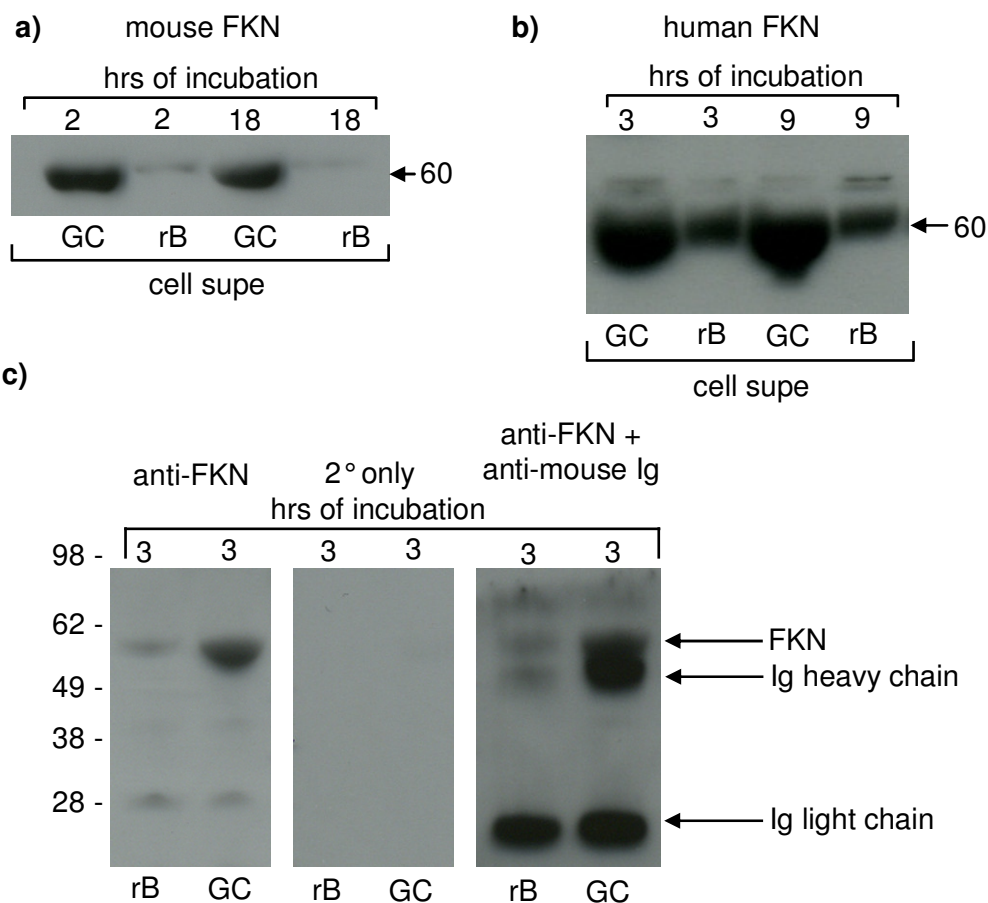


Figure 4.17 FKN release from GC B cells

(a) Cell-free supernatants were collected from murine germinal centre (GC) and follicular (resting) B cells (rB) after 2 and 18 hrs of culture. Proteins were precipitated from the medium by TCA and immunoblotted using rabbit anti-mouse FKN antibodies (TP-213). One representative result from three similar experiments is shown (b) Supernatants were collected from human germinal centre and resting B cells after 3 and 9 hrs of incubation and immunoblotted using mouse anti-human FKN monoclonal antibodies (MAB3651). One representative result from three is shown (c) Control Western blots of murine germinal centre and resting B cells labelled using rabbit anti-mouse FKN antibodies (TP-233), followed with goat anti-rabbit Ig antibodies (left); goat anti-rabbit Ig antibodies only (middle); rabbit anti-mouse FKN antibodies (TP-233) followed with goat anti-rabbit Ig antibodies together with sheep anti-mouse IgG antibodies (right).

Out of concern over the specificity of the anti-mouse FKN antibody binding and the possibility that what the antibodies detected on the Western Blot was not 60 kDa FKN but rather was an immunoglobulin heavy chain, which is of a similar size, some additional controls were performed for these experiments. The blots that were previously labelled with rabbit anti-mouse FKN antibodies were stripped and re-labelled. Initially, to highlight the real position of the mouse IgG on the blots and to compare its size with the size of FKN, rabbit anti-mouse FKN and goat anti-rabbit

IgG were used together with goat anti-mouse IgG antibodies. The antibodies clearly detected two separate bands (Figure 4.17c). This result was additionally supported by the fact that goat anti-rabbit antibodies alone were unable to label any proteins on the blots.

This subsection demonstrates that the apoptosis-dependant release of FKN is not just restricted to Burkitt's lymphoma cells, but is also characteristic of normal B cells undergoing selection in germinal centres. Further analysis carried out in the group, involving comparing the numbers of macrophages in the active germinal centres of wt and CX3CR1^{-/-} mice, proved that FKN is, indeed, involved in attracting tingible body macrophages to germinal centres (Truman et al. 2008).

4.9 Conclusions

In summary, the studies described in this chapter:

- showed that, in BL cells, the hydrolysis of 90 kDa FKN resulting in the generation of a 60 kDa fragment of FKN as well as the loss of surface FKN are apoptosis-dependent processes
- identified FKN as a potential substrate for caspases
- demonstrated that caspase-3 is capable of cleaving FKN *in vitro*
- showed that FKN and fully functionally active caspase-3 are released together in apoptotic BL cell-derived MPs
- illustrated that FKN and active caspase-3 co-localise together in the membrane-proximal region of BL2 cells
- demonstrated the release of the 60 kDa form of FKN from germinal centre B cells.

Chapter 5

Role of calpain in FKN cleavage in dying Burkitt's lymphoma cells

5.1 Introduction

The previous chapter has demonstrated that the 60 kDa form of FKN, previously shown to be generated in BL2 cells during apoptosis, is a likely product of caspase-dependent proteolysis. Here, the appearance of another cell-death-related form of FKN in BL cell lines is reported and further studies aiming at explaining its origin are presented.

5.2 Expression of FKN in group I BL lines

The studies on FKN expression and processing in Burkitt's lymphoma, presented in Chapter 4, were performed using the BL2 cell line. Here, FKN expression analysis was extended to other group I BL cell lines. This included another EBV-negative line – L3055 as well as two EBV-positive lines: Mutu I and SAV (Gordon et al. 2000). The cells were induced to undergo apoptosis by exposure to staurosporine. At indicated time points whole cell lysate and cell-free supernatant samples were

prepared. Cell viability was assessed by AxV/PI staining. FKN was detected by immunoblotting with mouse monoclonal anti-FKN antibodies (Figure 5.1a). FKN band pattern in apoptotic L3055 cells resembled that observed in BL2 cells (Figure 4.1a). However, in SAV cells (which remained mostly viable) the 60 kDa form of FKN was not observed. Instead, a band of approximately 55 kDa was present in all the SAV samples. In addition, the intensity of the 90 kDa FKN was reduced. Interestingly, in apoptotic Mutu I cells 60 kDa FKN was detected in cell lysates but in the supernatants the 55 kDa band was present instead. This analysis suggested the existence of a possible alternative to the caspase-dependent mode of processing FKN. The results obtained from Mutu I cells were further confirmed when UVB light exposure was used to induce these cells into apoptosis (Figure 5.2). Again, an unusual FKN band pattern was observed with the 60 kDa form present in the cell lysates and 55 kDa form in cell-free supernatants, indicating that this mechanism of FKN processing is independent of the type of apoptosis stimulus.

60 kDa FKN has previously been reported to be released from apoptotic BL2 cells in association with MPs (Truman et al. 2008). It therefore seemed interesting to determine whether FKN released from Mutu I cells is also released in MPs. Also, establishing which of the two processed forms (60 or 55 kDa) is present in MPs would shed some light on the mechanism of FKN release. MP lysate samples prepared from supernatants of 2 hrs post-staurosporine BL2 and Mutu I cells were analysed by Western blotting. Figure 5.1b shows that apoptotic Mutu I-derived MPs contained 90 and 55 kDa species of FKN. As none of the 55 kDa FKN was present in the Mutu I cell lysate and none of the 60 kDa FKN was detected in MPs it seemed likely that intracellular hydrolysis of FKN, and its cleavage in MPs might be two independent processes.

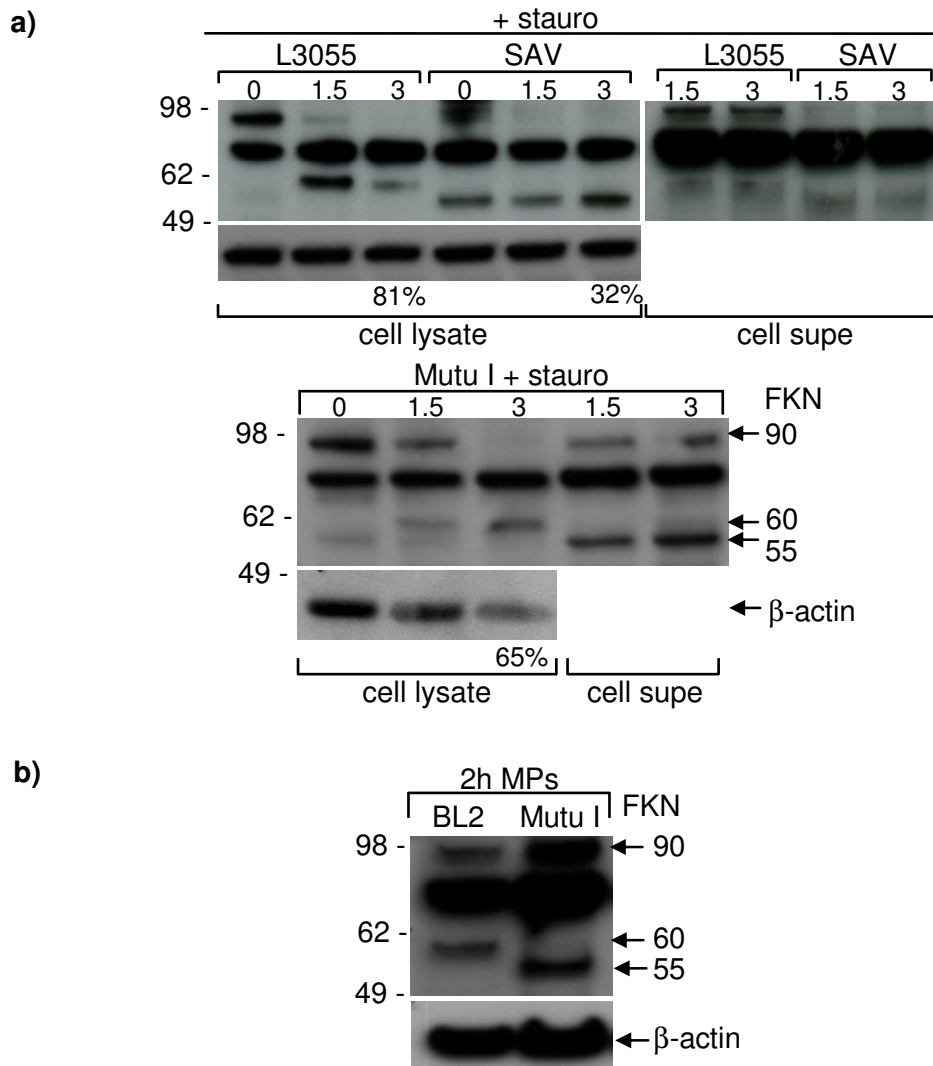


Figure 5.1 Expression of FKN in group I BL lines

(a) Type I BL cell lines L3055, SAV and Mutu I were induced to undergo apoptosis by staurosporine. Cells and cell supernatants were collected at the indicated times after apoptosis induction. Lysed cells and proteins precipitated from the medium by TCA were immunoblotted using anti-FKN antibodies (MAB3651). Cell death was assessed by AxV/PI staining and the percentage of AxV/PI- cells at the 3 hrs time point is shown underneath the blots. One representative result of more than five is shown. **(b)** MPs collected from the supernatants of BL2 and Mutu I cells 2 hrs post-staurosporine treatment, were immunoblotted with MAB3651 antibodies. One representative result of two is shown.

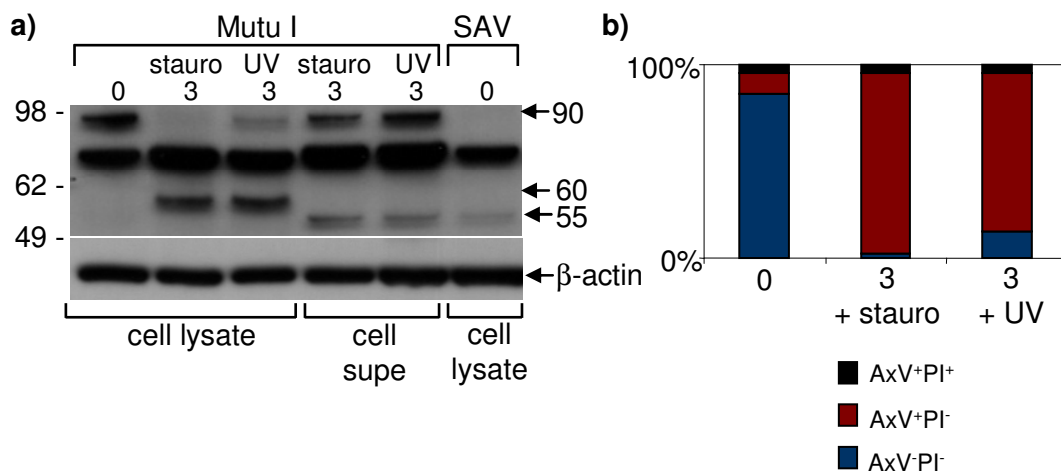


Figure 5.2 Effect of different apoptosis induction methods on the FKN processing in Mutu I cells

Mutu I were induced to undergo death by either staurosporine or UV treatment. (a) Cell lysates and proteins precipitated from the supernatants were immunoblotted with MAB3651 antibodies. (b) Apoptosis was assessed by AxV/PI staining.

5.3 Involvement of calpain in cleavage of FKN

The analysis of FKN release from Mutu I cells (Figure 5.1 and 5.2) showed that, similarly to the generation of 60 kDa FKN, cleavage of FKN to 55 kDa might be a cell-death-driven process. Calpain, a ubiquitous, Ca²⁺-activated protease, has a well-recognised role in various types of cell death (e.g. Blomgren et al. 2001; Gil-Parrado et al. 2002; Mandic et al. 2002; Fettucciari et al. 2006; Golstein & Kroemer 2007). While interplaying with caspases in inducing cell death under different conditions, it is also known to share with them a number of substrates (Wang 2000). Therefore, it was hypothesised that it is calpain that is involved in proteolytic cleavage of FKN and generation of a 55 kDa cleavage product. In order to test this hypothesis, the *in vitro* cleavage assay was set up: recombinant human FKN was incubated with active human calpain I. This resulted in generation of two different product bands and suggested that calpain might not only be capable of cleaving FKN but also might have more than one cleavage site within the FKN sequence (Figure 5.3a). Subsequently, the sizes of the products of calpain and caspase cleavages of

recombinant FKN were compared (Figure 5.3a). The largest product from the calpain cleavage-derived bands appeared to be smaller than that generated by recombinant caspase-3, reminiscent of the size difference between 60 and 55 kDa FKN bands (Figure 5.1).

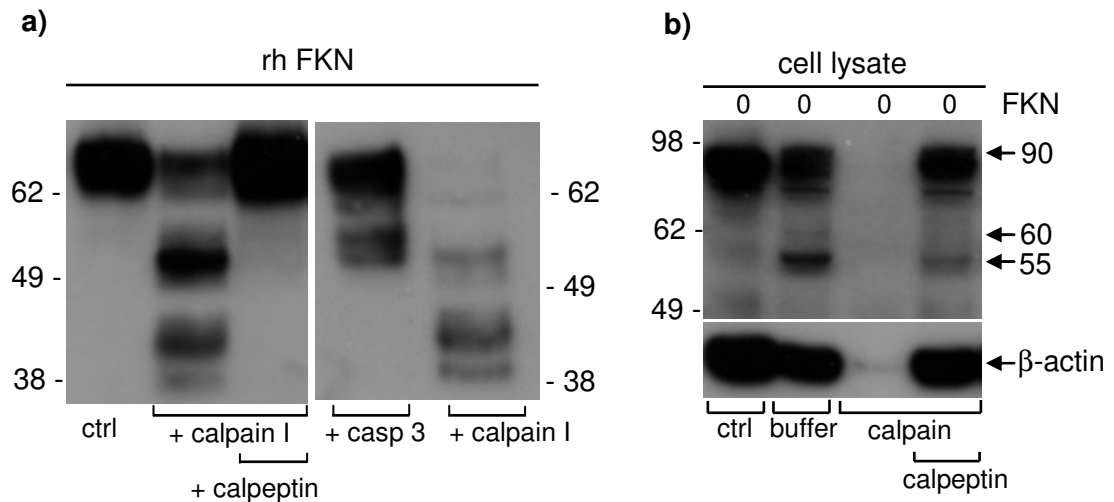


Figure 5.3 Involvement of calpain in FKN cleavage

(a) Recombinant human FKN was incubated for 2 hrs with human calpain I alone or with addition of specific calpain inhibitor - calpeptin. The sizes of recombinant FKN processed by recombinant caspase-3 or calpain I were compared. Samples were immunoblotted using anti-FKN monoclonal antibodies (MAB3651). One representative result from 3 is shown. (b) Whole cell lysate of viable BL2 was incubated for 2 hrs with calpain reaction buffer only, human calpain I or calpain I with addition of calpeptin. Samples were analysed by Western blotting using MAB3651 antibodies. One representative result of three similar is shown.

The addition of recombinant caspase-3 to the BL2 whole cell lysate samples has been proven effective in mimicking the process of generation of 60 kDa FKN in the apoptotic cells (Figure 4.9). Consequently, human calpain I was added to BL2 cell lysates in order to confirm its involvement in the production of 55 kDa species of FKN. This approach, however, proved to be ineffective, as the excess of calpain in the sample caused the complete degradation of proteins (Figure 5.3b). Interestingly, incubation of the cell lysate in the calpain reaction buffer alone (containing Ca^{2+} of a concentration within the calpain activation range) caused the generation of approximately 55 kDa-size form of FKN. The specific calpain inhibitor, calpeptin, managed to inhibit the degradation. Of note, the amount of 55 kDa band in the sample containing both calpain and calpeptin seemed to be reduced when compared

with the sample containing buffer alone. This could have been due to the incomplete inhibition of degradation as well as to calpeptin inhibiting calpain-dependent cleavage of FKN and production of 55 kDa species. These observations were compared with the results obtained from incubation of BL2 lysates in caspase reaction buffer (Figure 5.4a). Here as well, the generation of 55 kDa FKN was observed in the samples incubated in the buffer only. However, further analysis using various protease inhibitors revealed that the cleavage of FKN can be blocked by chymostatin (known to inhibit calpain activity – Nilsson & Karlsson 1986), calpeptin and Z-Leu-Leu-CHO (two calpain inhibitors). On the other hand, metalloproteinase inhibitors – GM6001 and TIMP-2 – were not able to inhibit this process. Taken together, these studies implied that cell-derived calpain might, under certain conditions, be activated in the samples and drive FKN proteolysis. An interesting question arose here as to which component of the reaction buffers used was responsible for the calpain activation. Two buffers presented here were compared with the ADAM reaction buffer that was previously demonstrated not to trigger uncontrolled protein degradation (Figure 4.9) (see section 2.9 for buffer compositions). As only one of the buffers causing FKN degradation contained Ca^{2+} it seemed that increased Ca^{2+} concentration in the samples was not a requirement for calpain activity. The only compound common to the degrading buffers but not present in the non-degrading one was dithiothreitol (DTT). DTT is used for its strong reducing capability. The role of DTT in FKN degradation is in accord with the work of Guttman et al., reporting that oxidation causes inhibition of substrate hydrolysis by calpain (Guttman et al. 1997). Therefore, it seems possible that DTT facilitates calpain-driven proteolysis by changing redox conditions in the sample.

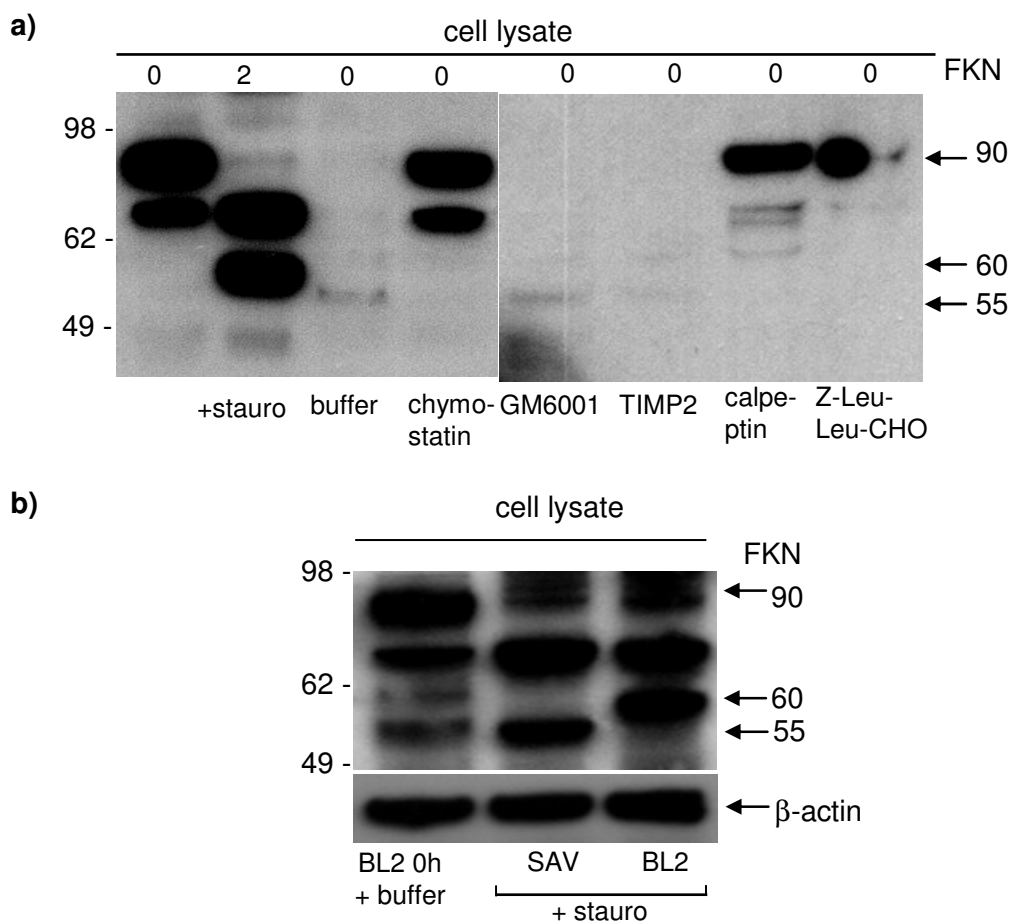


Figure 5.4 Effect of protease inhibitors on FKN cleavage in BL2 cell lysates
(a) Whole cell lysate of viable BL2 was incubated for 2 hrs with caspase reaction buffer only or with reaction buffer with addition of inhibitors: chymostatin, GM6001, TIMP2, calpeptin or Z-Leu-Leu-CHO. Samples were analysed by Western blotting using MAB3651 antibodies. One representative result from 2 similar is shown. **(b)** Sizes of the FKN bands generated in BL2 cell lysates incubated in caspase reaction buffer and staurosporine-treated SAV and BL2 cells were compared.

Figure 5.4b shows the comparison of the size of FKN detected in SAV BL cell line with FKN generated in the calpain reaction buffer by uncontrolled proteolysis of BL2 cell lysates as well as with lysates of BL2 cells exposed to staurosporine for 2 hrs. Demonstration that the 55 kDa FKN band from SAV cells (and also from Mutu I cells – Figure 5.1) was the same size as buffer-generated FKN seemed to support the hypothesis that calpain may be involved in proteolytic cleavage of FKN during BL cell death.

5.4 Ionomycin-induced death of BL2 cells

Further work focused on examining the role of calpain involvement in the proteolytic processing of FKN in the cellular system. For this purpose, BL2 cells were incubated with calcium ionophore – ionomycin, known to activate calpain and to induce BL cell death (Gil-Parrado et al. 2002; Truman 2005). Figure 5.5a shows that although in the lysates of ionomycin-treated cells only 60 kDa FKN was present, in the supernatants 55 kDa was detected as early as 30 min after the addition of ionomycin, much before the 60 kDa band was also visible. Interestingly, 55 kDa FKN had started to be visible before any active caspase-3 was detected in the samples and even after 3 hrs of incubation with ionomycin not all the pro-caspase-3 was transformed to its active form. In addition, unlike in the staurosporine-treated BL2 cells, not all the 90 kDa FKN disappeared from the lysate at the time when 60 kDa form emerged. The appearance of 55 kDa FKN in the ionomycin-treated BL2 cell supernatants gave further evidence for a possible calpain activity in hydrolysis of FKN. Intriguingly, both for BL2 cells incubated with ionomycin and for Mutu I cells that were UVB-irradiated or incubated with staurosporine (Figure 5.1 and Figure 5.2), 55 kDa FKN was only observed in cell supernatants. This indicated that this form might be shed from the cell surface.

The AxV and PI labelling of ionomycin-treated BL2 cells (Figure 5.5b) revealed that the effect of ionomycin on BL2 cells differs considerably from what was previously observed with staurosporine (Figure 4.1). Unlike staurosporine, ionomycin caused a rapid increase in the number of PI-positive, and thus presumably leaky, cells. The population of AxV-positive (PS-displaying) cells, however, only counted for approximately 30 per cent of cells after 3 hrs (compared with around 75% of staurosporine-treated cells). Early loss of plasma membrane integrity, lack of the characteristic features of apoptosis (such as PS exposure and caspase activation) and frequently an increase of Ca^{2+} concentration are considered to be characteristic for necrosis (Golstein & Kroemer 2007). Thus, it was concluded that ionomycin-induced death of BL2 cells is more similar to necrotic rather than apoptotic cell death.

Importantly, calpain activity is also often associated with necrotic cell death (Harriman et al. 2002; Liu et al. 2004; Moubarak et al. 2007).

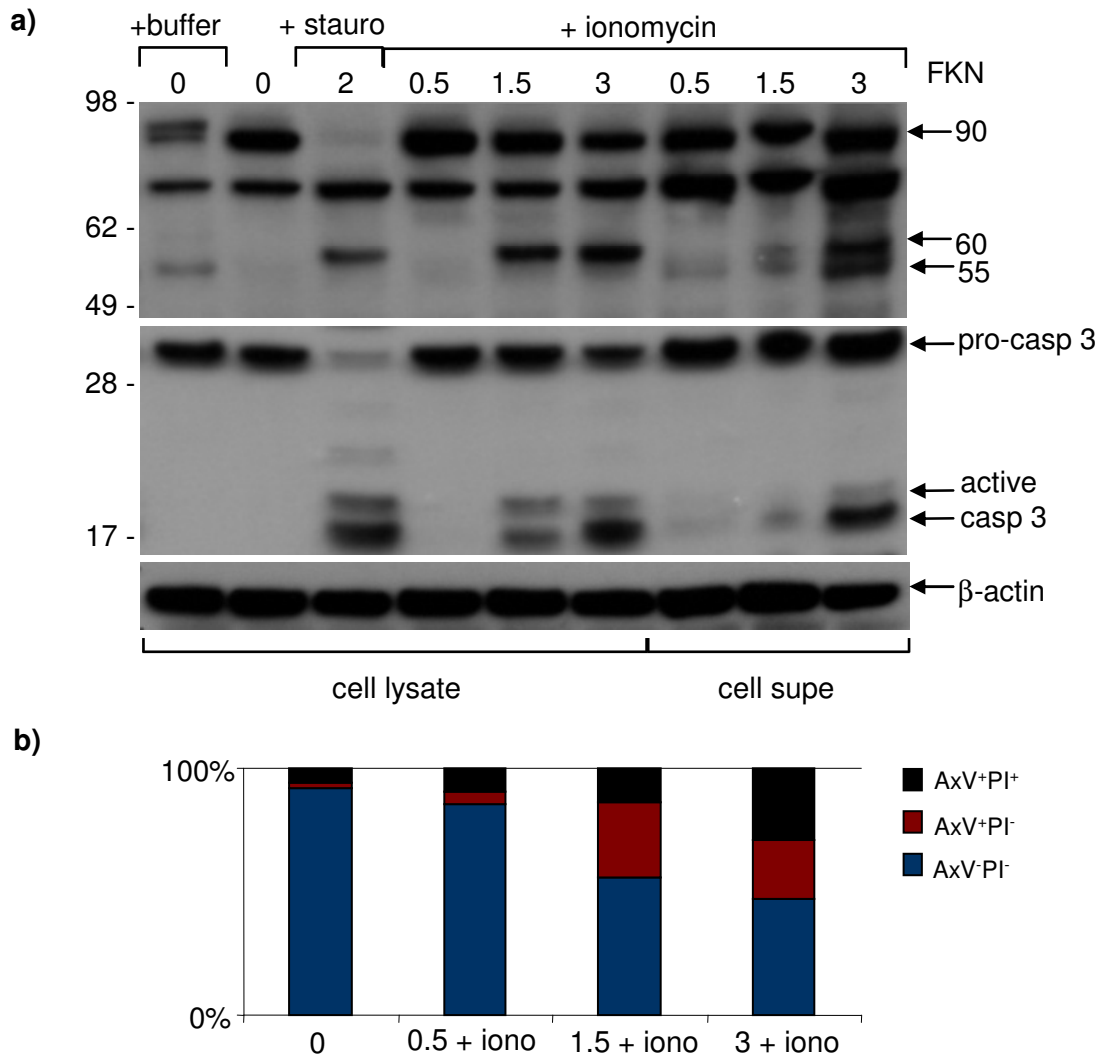


Figure 5.5 Ionomycin-induced death of BL2 cells

BL2 cells were induced to undergo death by ionomycin. **(a)** Cells and cell supernatants were collected at the indicated times (hrs) after death induction. Lysed cells and proteins precipitated from the medium by TCA were analysed by Western blotting. Blots were labelled using mouse anti-FKN antibodies (MAB3651) or rabbit anti-caspase-3 antibodies (9662). **(b)** Cell death was assessed by AxV/PI staining. One representative result of more than five similar is shown.

5.5 Ionomycin-induced death of Mutu I cells

The effect of ionomycin on FKN cleavage in Mutu I cells was also assessed. These cells have already been reported to release 55 kDa FKN in response to staurosporine (Figure 5.1), however, after exposure to ionomycin, 55 kDa FKN was detected not only in the supernatants but also in the cell lysates (Figure 5.6a). At the same time, no 60 kDa form of FKN was detected in these cells. In Mutu I, similar to BL2, ionomycin induced only partial activation of caspase-3 and a low percentage of cells became AxV-positive (Figure 5.6a, b). These results suggested that the ionomycin-driven increase in the intracellular Ca^{2+} level in Mutu I cells may lead to altered processing of FKN and generation of products other than the previously described form of FKN. Dependence of this process on ionomycin stimulation further supported a role for calpain involvement. In addition, the differences in BL2 and Mutu I cell response to various stimuli became more clearly visible here.

5.6 Effect of calpeptin and Z-VAD-FMK on the FKN processing in ionomycin-treated BL cells

Having established that ionomycin induces release of 55 kDa FKN from both BL2 and Mutu I cells it was next decided to examine if the specific calpain inhibitor – calpeptin is capable of inhibiting this release. Poly-caspase inhibitor Z-VAD-FMK was also included in the experiment in order to investigate the level of caspase involvement in the supposed calpain-induced cleavage of FKN. BL2, Mutu I and L3055 cell-free supernatants were collected 2 hrs after addition of ionomycin. The

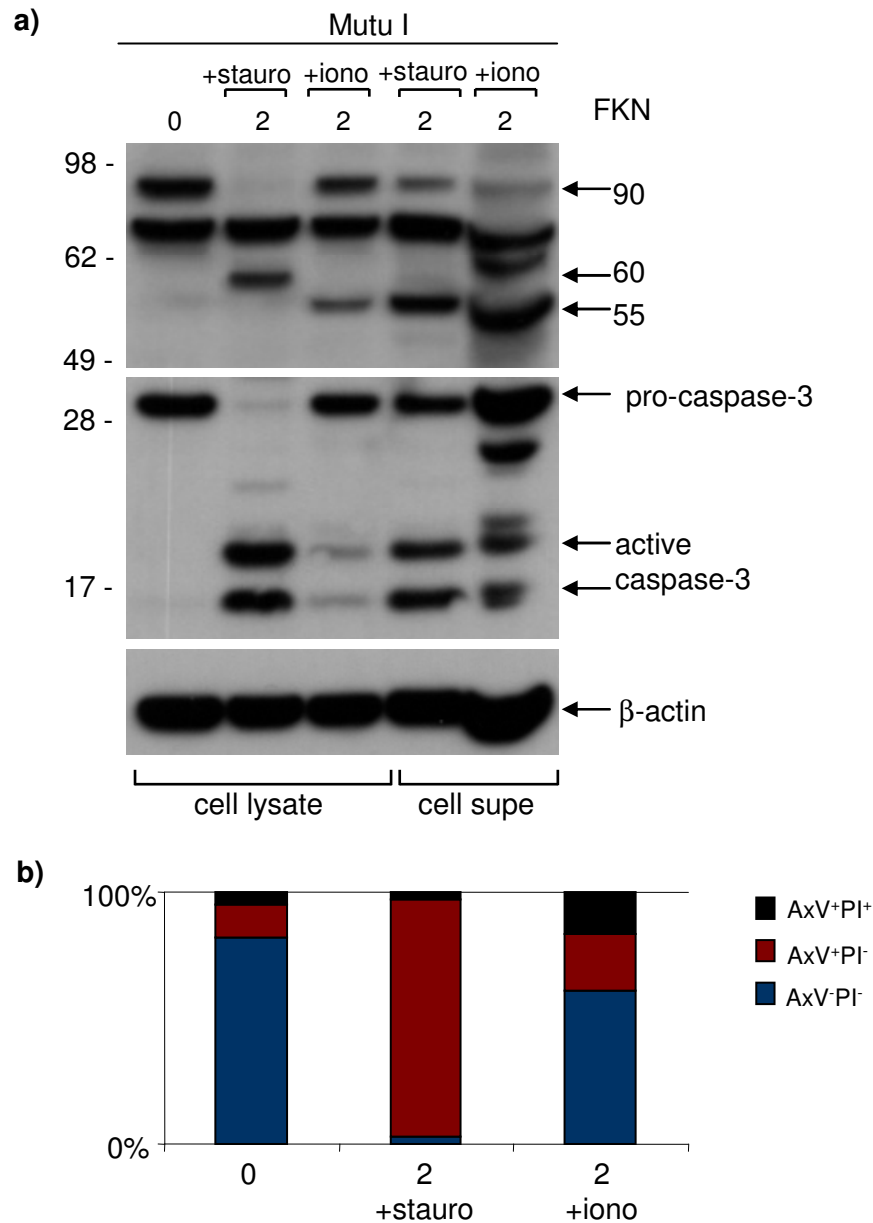


Figure 5.6 Ionomycin-induced death of Mutu I cells

Mutu I cells were induced to undergo death by ionomycin or staurosporine. **(a)** Cells and cell supernatants were collected at the indicated times (hrs) after death induction. Lysed cells and proteins precipitated from the medium by TCA were analysed by Western blotting. Blots were labelled using anti-FKN antibodies (MAB3651) or anti-caspase-3 antibodies (9662). **(b)** Cell death was assessed by AxV/PI staining. One representative result of four similar is shown.

resulting pattern of FKN bands (Figure 5.7a) revealed that calpain- and caspase-mediated proteolysis seemed to be two mostly independent processes. In BL2 and L3055 ionomycin-treated cells, which contained both the 60 and 55 kDa forms of FKN in the supernatants, the addition of calpeptin caused complete inhibition of the release of the 55 kDa FKN. When Z-VAD-FMK was added to these cells, the

production of 60 kDa was completely blocked but the amount of 55 kDa band was inhibited only partially. When calpeptin was added to ionomycin-treated Mutu I cells the generation of the 55 kDa band was significantly reduced. The addition of Z-VAD-FMK did not have such a profound effect, although some degree of cleavage inhibition was also observed. The high efficiency of calpeptin in abolishing the release of 55 kDa FKN seemed to further advocate calpain engagement in FKN hydrolysis. At the same time, calpeptin appeared not to affect the production of 60 kDa FKN, thus suggesting that although in ionomycin-treated cells activation of caspases is a Ca^{2+} -dependent process, the direct involvement of calpain is not needed for caspase activation. Similarly, as was demonstrated by addition of Z-VAD-FMK, the activity of caspases appeared to be only partly required for calpain activation. Although calpeptin had a strong effect on specific FKN cleavage inhibition, it did not seem to be as powerful in blocking other cell death-related events (Figure 5.7b). Strikingly, Z-VAD-FMK was able to inhibit to a certain extent loss of membrane integrity, which normally is not thought to be caused by caspases. As calpain appeared not to have a leading role in driving cell death induced by ionomycin, it also seemed less likely that the calpeptin-mediated blockade of FKN cleavage was a result of indirect inhibition of other proteases. Additionally, AxV and PI staining revealed that Mutu I cells are less susceptible than BL2 or L3055 to ionomycin-induced death.

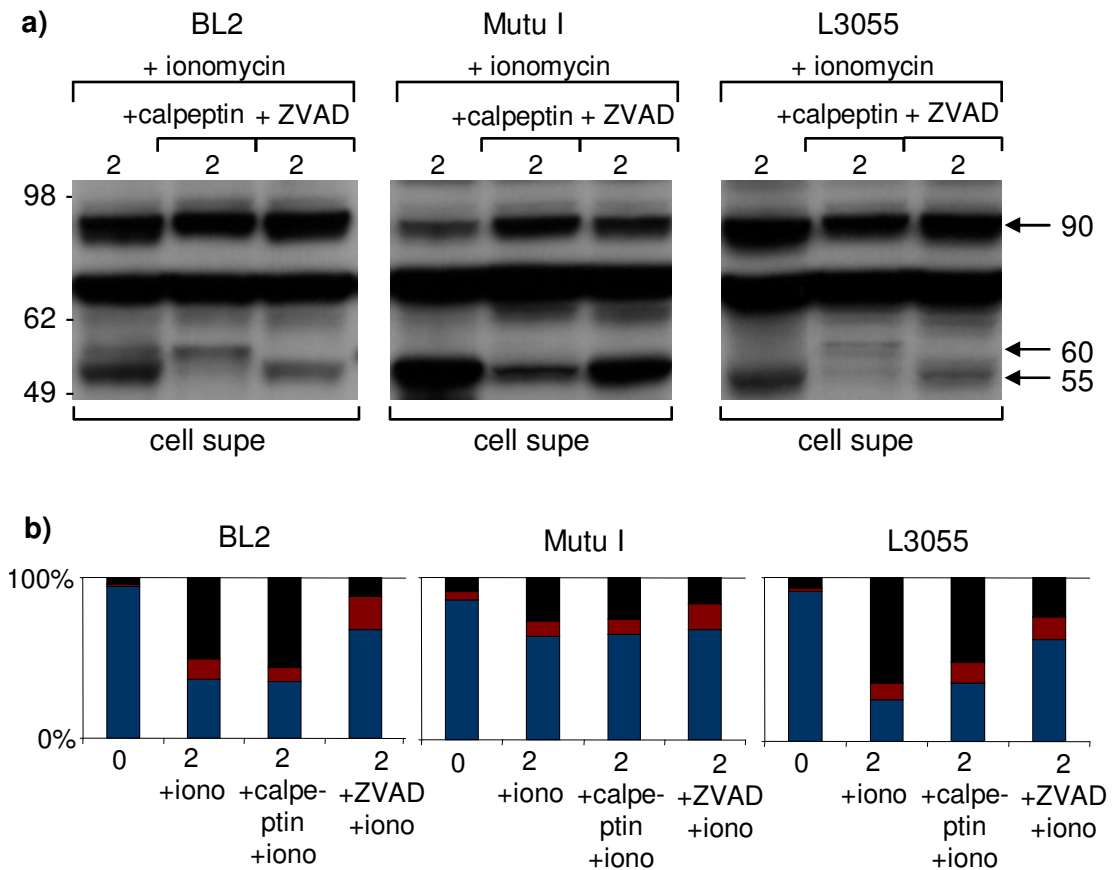


Figure 5.7 Effect of calpeptin and Z-VAD-FMK on the FKN processing in ionomycin-treated BL cells

BL2, Mutu I and L3055 cells were incubated with ionomycin alone or in the presence of inhibitors: calpeptin or Z-VAD-FMK. **(a)** Cell-free supernatants were collected after 2 hrs. Proteins were precipitated from the medium by TCA and were analysed by Western blotting using MAB3651 antibodies. **(b)** Cell death was assessed by AxV/PI staining. One representative result of two similar is shown.

5.7 Calpain expression in BL cells

Previous experiments have revealed the differences between BL cell lines as to the way in which FKN is processed after cell death stimulus. As the 55 kDa form of FKN was likely to be generated by calpain cleavage, it seemed possible that variation in FKN cleavage in different lines resulted from altered expression of calpain. Thus, FACS analysis of calpain I level was performed in the previously used cell lines. The effect of both ionomycin and staurosporine on calpain expression was compared as

these two compounds had differently affected FKN fate in these lines. Figure 5.8a shows the labelling of total calpain, performed in previously permeabilised cells. In the untreated BL2, Mutu I and L3055 cells the detected calpain levels were similar. Also, neither ionomycin nor staurosporine seemed to affect calpain expression as after 2 hrs incubation its levels remained unchanged. The appearance of the small calpain-low populations of ionomycin-treated BL2 and L3055 cells most probably results from the increase in the number of leaky cells (as showed in Figure 5.7b). Although presented results were plotted from the cells that fell into a “viable” gate according to their forward versus side scatter characteristic, it cannot be excluded that a small percentage of these cells were already leaky. Intracellular changes in calpain expression seemed therefore to be an unlikely cause of differences in FKN processing. The surface expression of calpain was also measured. Anti-calpain antibodies labelled surface of BL cells. Moreover, the staining revealed differences between analysed cell lines (Figure 5.8b). Untreated Mutu I cells, unlike untreated BL2 or L3055 cells, were shown to display convincing levels of calpain I on their surface. Interestingly, preliminary results of labelling of SAV cells also revealed calpain I presence on the surface. The addition of either staurosporine or ionomycin to Mutu I did not seem to dramatically change the level of calpain I on the cell surface. Moreover, none of the cell death-inducing compounds was able to induce calpain I expression on the surface of BL2 or L3055 cells. The small population of calpain-positive cells visible in the plot of ionomycin-treated L3055 cells is, again, believed to represent cells that have lost membrane integrity. The two isoforms of calpain (calpain I and calpain II) seem to have the same substrate specificity and therefore it was impossible to conclude from the results presented thus far which of them is responsible for FKN hydrolysis.

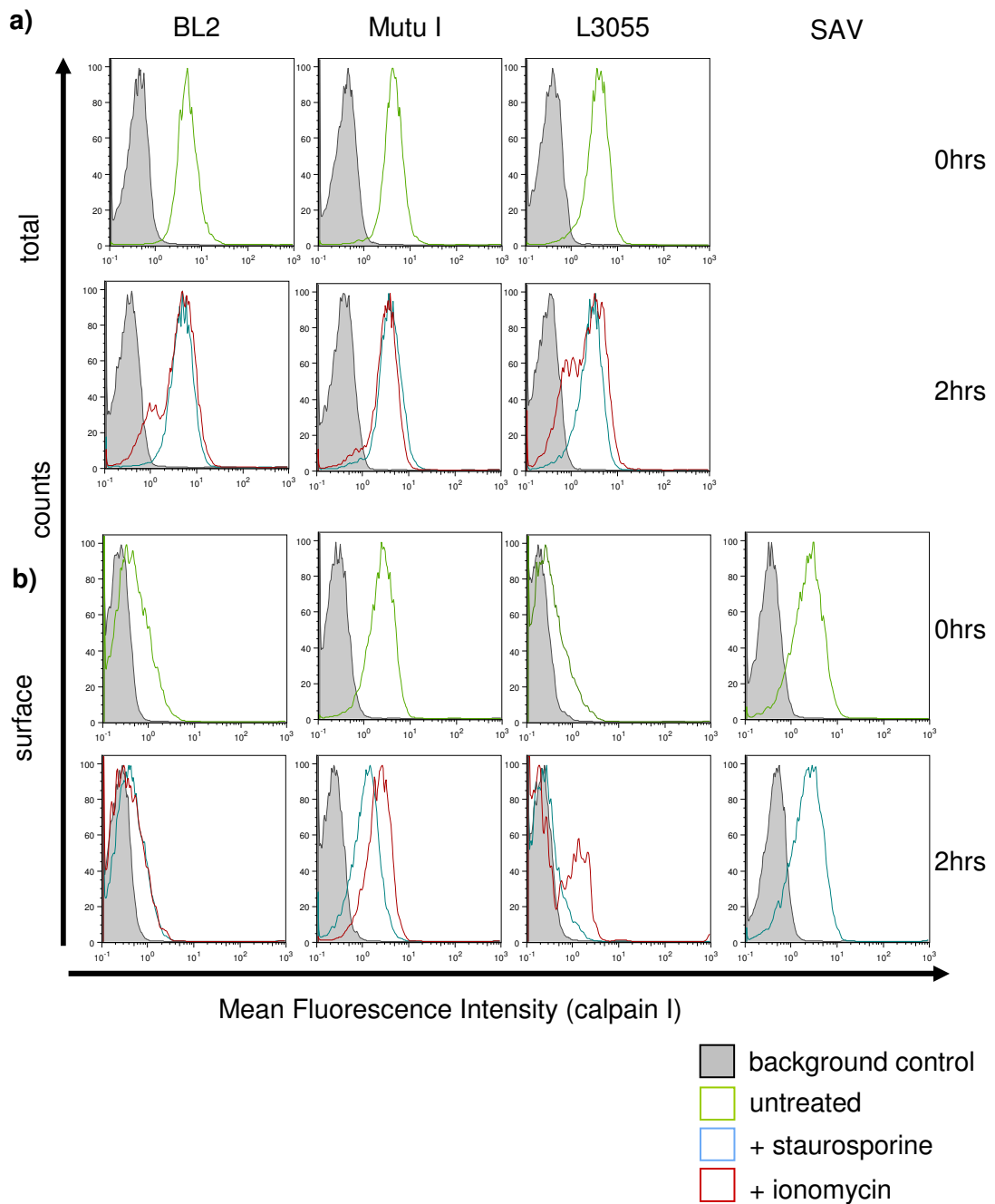


Figure 5.8 Calpain I expression in BL cells

FACS plots showing (a) permeabilised BL2, Mutu I and L3055 cells or (b) intact BL2, Mutu I, L3055 and SAV cells, treated with staurosporine or ionomycin, which were stained for calpain I using rabbit anti-calpain I large subunit antibodies (2556), and FITC-conjugated goat anti-rabbit secondary antibodies. One representative result of four is shown. For SAV cells the only result is shown.

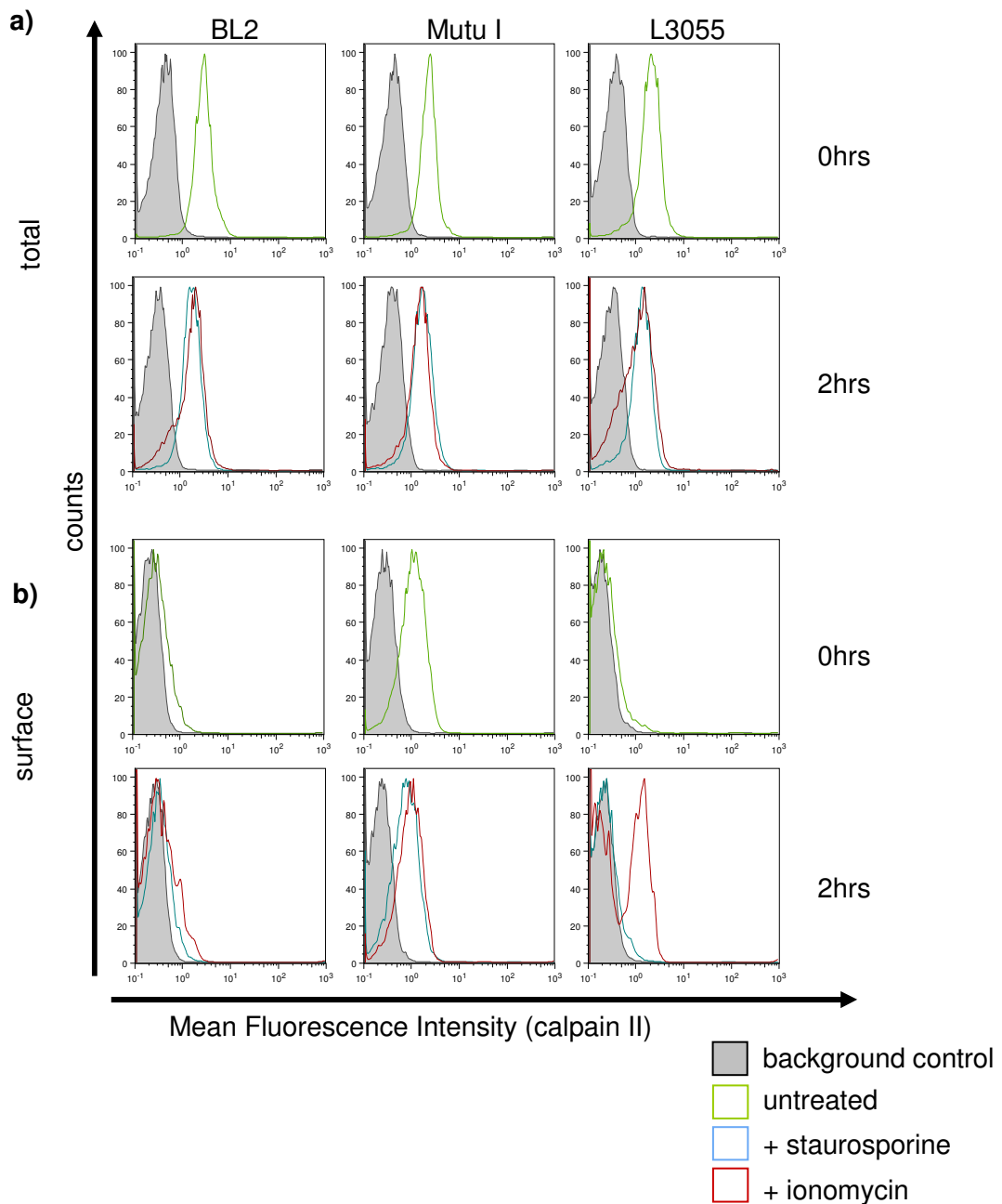


Figure 5.9 Calpain II expression in BL cells

FACS plots showing (a) permeabilised or (b) intact BL2, Mutu I, L3055 cells which were, treated with staurosporine and ionomycin, stained for calpain II using rabbit anti-human calpain II large subunit antibodies (2539), and FITC-conjugated goat anti-rabbit secondary antibodies. Control staining with secondary antibodies only was included. The only result is shown.

For this reason, the staining was later repeated using anti-calpain II antibodies (Figure 5.9a, b). The preliminary results showed the pattern of calpain II expression to closely resemble that of calpain I. Although the detection of calpain on the cell surface seemed rather unusual, it was quite striking that the pattern of differences in

the surface levels of calpain closely resembled the pattern of the differences between the cell lines ability to produce 55 kDa FKN in response to staurosporine.

5.8 Calpain activation in BL cells

The variation in calpain expression between BL cell lines did not fully explain the discrepancy in their ability to generate 55 kDa FKN. Thus, the possibility that calpain activity is diversely regulated between the cell lines under different conditions was explored. In the event of an increase in the Ca^{2+} concentration, calpain is known to undergo autolytic proteolysis. In this two-step process, the 80 kDa large subunit of calpain is initially cleaved to a 78 kDa form of calpain, which is subsequently trimmed to a 76 kDa form (sizes given are for calpain I) (Zimmerman & Schlaepfer 1991; Michetti et al. 1996). The second of these two proteolytic events is responsible for reducing the Ca^{2+} concentration required for calpain activity (Li et al. 2004). Calpain I was detected by immunoblotting in ionomycin and staurosporine-treated cells (Figure 5.10). As predicted, the degradation of calpain was observed under increased Ca^{2+} concentration in all the ionomycin-induced cells. The cleavage seemed to be inhibited by addition of both calpeptin and Z-VAD-FMK. Also, identical cleaved forms of calpain I were detected in the supernatants of ionomycin-treated cells regardless of cell type. The effects of staurosporine on the cells differed from that of ionomycin. The cleaved form of calpain I appeared not to be present in these cells. Intriguingly, soluble calpain I detected in the supernatants of all the staurosporine-treated cells was of the size of the active enzyme. Importantly, no differences in the way calpain I was activated under different conditions were detected between the studied cell lines although they have been shown to differentially process FKN (Figure 5.5 and Figure 5.7). This suggested that some other regulatory mechanism inhibiting calpain-driven cleavage of FKN must be in operation in these cells. Intriguingly, the presence of active calpain I was detected also in the cytosol of ionomycin-treated BL2 and L3055 cells, where 55 kDa of FKN has not been seen (Figure 5.7). Similarly, active calpain was detected in all the three cell lines, in the supernatants collected from staurosporine-treated cells, but 55 kDa FKN has been shown to be present only in Mutu I cells (Figure 5.1). An intriguing

effect of Z-VAD-FMK was also observed. Its addition to the cells that were subsequently exposed to ionomycin seemed to inhibit calpain I cleavage. At the same time, however, Z-VAD-FMK did not block the generation of 55 kDa FKN. Further studies need to be conducted in order to broaden our understanding of the complex interplay between availability of active hydrolases (caspases and calpain) and proteolytic processing of FKN. Calpastatin, a ubiquitously expressed natural calpain inhibitor, involved in regulating calpain activation and its proteolytic activity (Wendt et al. 2004; Melloni et al. 2006) is an obvious target for future examination. However, as it has been reported that calpain activity can be finely regulated by specific phosphorylation (Shiraha et al. 2002; Smith 2003; Chen et al. 2010) it also seems probable that phosphorylation of either calpain or FKN might be responsible for the differences in the FKN processing.

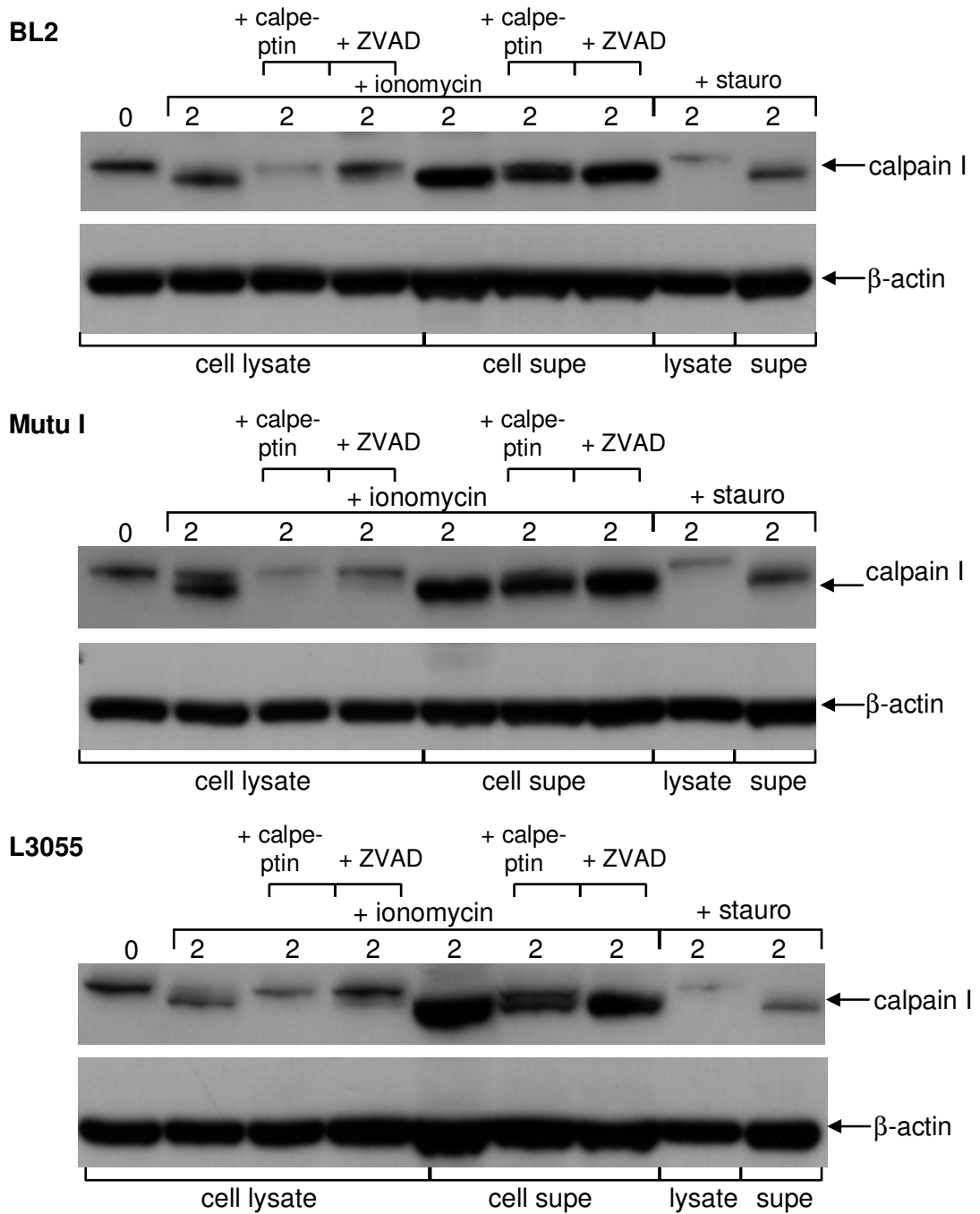


Figure 5.10 Calpain I activation in BL cells

BL2, Mutu I and L3055 cells were incubated with stauro or with iono alone or in the presence of calpeptin or Z-VAD-FMK. Cells and cell-free supernatants were collected after 2 hrs. Cell lysates and proteins precipitated from the medium by TCA were immunoblotted using anti-calpain I antibodies (2556). One representative result of three similar is shown.

5.9 Monitoring FKN release from late apoptotic BL cells

The initial studies presented in this thesis have demonstrated that the 60 kDa cleaved form of FKN is generated very early in the cell death process and thus, further analysis also focused at these early periods of BL cell death. Here, however, the pattern of FKN release was monitored for extended period of time, up to 24 hrs after BL2 cell death induction with staurosporine. The stability of 60 kDa FKN was of main interest here. As it can be seen in Figure 5.11, 60 kDa form of FKN, first detected 2 hrs post-staurosporine, seemed to disappear from the supernatants by 6 hrs. In contrast the 90 kDa form of FKN remained stable in the supernatants until the end of the assay. Remarkably, as late as 12 hrs 55 kDa FKN started to be visible in the samples. The striking discrepancy in the lifespan of 60 kDa and 90 kDa FKN indicated that these two forms might have different functions. It appeared likely that 60 kDa form serves only as a transient chemotactic signal for phagocytes sent by dying cells. Moreover, the observation that BL2 cells were able to produce 55 kDa FKN as late as 12 hrs post-staurosporine, presumably, when they already turned secondary necrotic, additionally confirmed calpain involvement in FKN processing under necrotic conditions.

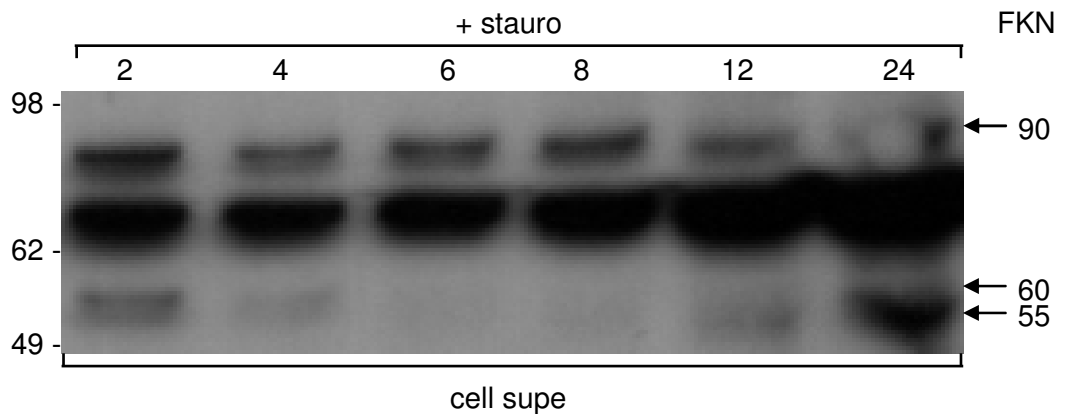


Figure 5.11 Monitoring FKN release from late apoptotic BL2 cells

BL2 cells were induced to undergo death by staurosporine. Cell supernatants were collected at the indicated times (hrs) after death induction. The proteins precipitated from the medium by TCA were analysed by Western blotting using anti-FKN (MAB3651) antibodies. One representative result of three is shown.

5.10 Searching for FKN C-terminal cleavage products

Having assumed that 60 kDa and probably 55 kDa FKN is generated by proteolytic cleavage of 90 kDa, it was next decided to look for the C-terminal products of this reaction. This was designed to clarify whether 90 kDa FKN detected in BL cells constitutes full-length or already cleaved FKN, as well as finding confirmation for the 55 kDa FKN origin from the 90 kDa form. Some previously performed Western blots were selected for this purpose and relabelled with antibodies raised against the C-terminal part of FKN (transmembrane and intracellular domains). The predicted size of the C-terminal fragments generated from the 90 kDa form FKN by caspase or calpain cleavage would be approximately 30 kDa and 35 kDa, respectively. However, bands of these sizes were not detected by the antibodies used (Figure 5.12). Instead, a very intense band of around 25 kDa was visible in all the samples. The 25 kDa band remained unchanged throughout the time course of the experiment and was detected in all the assayed cell lines. Moreover, its presence was not correlated with the appearance of neither 60 kDa nor 55 kDa band. Altogether, these results suggested that the 25 kDa band is a product of different proteolytic process

than that presented here. Lack of both the 30 kDa and 35 kDa forms suggested that the 90 kDa form of FKN from which 60 and 55 kDa forms are generated might represent an already cleaved form of FKN. This, however, should result in production of small C-terminal fragment, probably around 6 kDa size (Hundhausen et al. 2007) which was not clearly visible on any of the labelled blots.

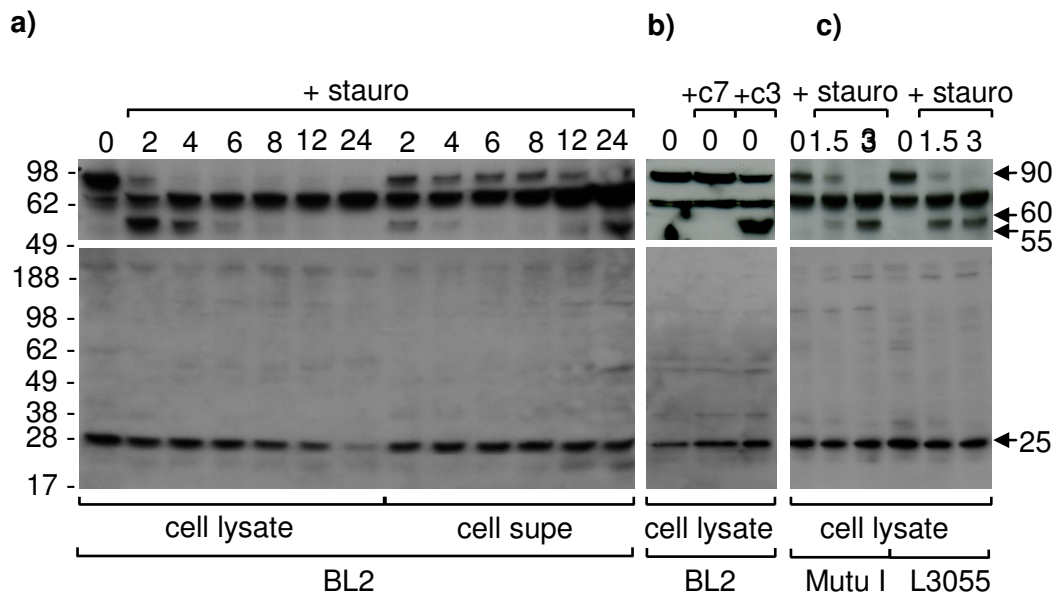


Figure 5.12 Searching for FKN C-terminal cleavage products

(a) Cell lysates and cell-free supernatants from BL2 cells incubated for up to 24 hrs with staurosporine, (b) BL2 cell lysates incubated for 2 hrs with recombinant human caspase-7 (c7) or caspase-3 (c3), (c) and cell lysates from staurosporine-treated Mutu I and L3055 cells were immunoblotted with anti-FKN antibodies (MAB3651) and anti-FKN C-terminus antibodies (sc-7225).

5.11 Conclusions

In summary, this chapter demonstrates that:

- various BL cell lines may differently process FKN in response to the same death stimulus and that an “alternative” 55 kDa form of FKN might be released from dying cells along with 60 kDa FKN
- calpain is capable of cleaving FKN and is likely to be responsible for generation of 55 kDa FKN

- differences in the way FKN was cleaved in different BL cell lines did not seem to be caused by altered levels of total calpain expression or its changed activation
- a 25 kDa C-terminal fragment of FKN is present in the BL cell lines independently of the occurrence of caspase-mediated cleavage, suggesting an existence of cleavage event other than those previously reported

Chapter 6

Discussion of the results

6.1 Introduction

The work presented in this thesis was aimed at understanding the mechanisms responsible for attraction of macrophages to Burkitt's lymphoma (BL) tumours. The initial, preliminary studies showed that monocyte chemoattractant protein 1 (MCP-1) is unlikely to play a role in this process. Fractalkine (FKN), which has previously been reported to be partly responsible for macrophage infiltration of BL (Truman 2005), was shown here to be processed in dying BL cells via mechanisms that have not previously been described. Two new death-associated forms of FKN, of 60 and 55 kDa, which are most likely responsible for attraction of macrophages to BL cells were identified. Moreover, caspase-3 and calpain I were demonstrated to be capable of cleaving FKN and to be likely to be responsible for generation of the 60 and 55 kDa forms, respectively. Thus, it was proposed that apart from executing cell death the two proteases might at the same time be able to send 'find me' signals to macrophages and thus, ensure effective clearance of dying cells. The potential participation of two distinct families of cell death-related proteases, caspases and calpains, in FKN cleavage suggests that the modulation of this molecule is important during cell death.

Furthermore, the research presented here revealed the complexity of the mechanisms modulating the functions of FKN in the cells that naturally express FKN. It should be further investigated, however, whether the observed complexity is a consequence of aberrant processing of FKN in the BL tumour cells or is it also typical for untransformed cells.

6.2 FKN processing and role of differently cleaved fragments

The processing of FKN by various proteases results in the generation of FKN fragments of different lengths and therefore, presumably different functions. Numerous studies on the signalling cascades triggered by FKN binding to its only known receptor – CX3CR1, showed differential responses to soluble and cell surface-bound FKN. Cell surface-exposed FKN has been shown to be able to mediate firm adhesion independently of signalling events mediated by CX3CR1 and this cell adhesion can persist in the presence of pertussis toxin (PTX) which is known to uncouple the receptor from G proteins responsible for transferring the signals downstream of the receptor (Imai et al., 1997; Fong et al., 1998). Other researchers have observed that FKN/CX3CR1 binding was able to induce Ca^{2+} flux in the adherent cells but the response was weaker than that stimulated by the soluble form of FKN (Harrison et al. 1998). Unlike membrane-bound FKN, its extracellular form has the ability to induce signalling cascades and chemotaxis (Haskell et al. 1999; Mizoue et al. 2001). Additionally, soluble FKN has been shown to increase cell adhesion by activating integrins (Goda et al., 2000; Cambien et al., 2001). Different roles of the various forms of FKN have also been observed in a series of *in vivo* experiments conducted by Vitale et al. As FKN has been reported to have the anti-tumour properties, the authors investigated the contribution of three forms of FKN: wild type, uncleavable membrane-bound and constitutively soluble, to the anti-tumour effects of FKN. The cell surface-exposed FKN showed virtually no effect on the tumour while both wild type and constitutively soluble forms seemed to inhibit its growth. Interestingly, the wild type FKN was shown to be more effective than the constitutively released form (Vitale et al. 2007). Unfortunately, limited information is available on the effect of differential cleavage on the function of FKN. The existence of conflicting reports on the capability of soluble FKN to induce the migration of mononuclear phagocytes, however, indicates the existence of some variability in FKN-mediated chemotaxis (i.e. good chemoattractant for mononuclear phagocytes: Bazan et al., 1997; Harrison et al., 1998; Chapman et al., 2000; Lucas et al., 2003; poor chemoattractant: Imai et al. 1997; Pan et al. 1997; Umehara et al.

2001; Ludwig et al. 2002; Vitale et al. 2004). The observed discrepancy in the response of phagocytes to FKN might be due to the use of phagocytes from different sources that could have different CX3CR1 expression levels or differential mechanisms of signal transduction. It could also be due to different origins and glycosylation of FKN, differences in other experimental conditions or from the differential cleavage of FKN. Although Harrison et al., who performed an extensive analysis of the role of the chemokine domain and the mucin stalk in the interaction of FKN with its receptor, did not find any correlation between the length of the mucin stalk and the ability of the chemokine to effectively bind to CX3CR1 or to induce the calcium flux (Harrison et al. 2001), there are some examples in the literature of differently cleaved FKN having different effects on the migration of monocytic phagocytes. Bazan and collaborators have demonstrated that the 95 kDa soluble FKN (containing probably the whole extracellular part of the chemokine) was very potent in mediating the migration of monocytes whereas a truncated form of soluble FKN (47-55 kDa) had significantly weaker chemoattractant properties (Bazan et al. 1997). In contrast, Pan et al. have demonstrated that neither the whole extracellular part of FKN nor the chemokine domain alone were capable of attracting monocytic phagocytes in the *in vitro* chemotactic assay but they also showed that the injection of the chemokine domain only but not the whole extracellular fragment of FKN to the mouse peritoneal cavity was able to regulate monocyte infiltration (Pan et al. 1997). Similarly, the work presented by Imai and colleagues has revealed that the full extracellular FKN was less potent in attracting monocytes when compared with MCP-1 (Imai et al. 1997). These observations were further confirmed in another paper from this group (Umehara et al. 2001). In addition, Ludwig and colleagues have reported that 80 kDa soluble FKN (containing the majority of the extracellular part of FKN) did not induce migration of monocytes (Ludwig et al. 2002). By contrast, a recombinant fragment of rat FKN, containing the chemokine domain only, has been shown to be chemotactic for rat microglial cells (Harrison et al. 1998). Unfortunately, in the majority of the published studies on the chemotactic properties of FKN the information on the size of the chemokine was omitted (presumably due to the lack of recognition of possible importance of the soluble chemokine size for its function) and thus only a restricted number of papers contain more detailed analysis

of the roles of various sizes of soluble FKN. However, the work performed previously in our group seems to be in accordance with most of the results discussed above. It has been demonstrated that only the FKN released from dying BL cells was able to attract macrophages (Truman 2005; Truman et al. 2008). It has been shown in this thesis that while the 90 kDa soluble form of FKN was present in all the examined samples, both from viable and dying BL cells, FKN fragments of 60 and 55 kDa were detected only in the supernatants from dying cells (Figures 4.2 and 5.1). Therefore, it was assumed that these two shorter forms of FKN, but not the constitutively released 90 kDa form, are inducing macrophage chemotaxis. Altogether, these results suggested that the specific proteolytic cleavage resulting in the generation of the shortened form of FKN is required to enable FKN to chemoattract mononuclear phagocytes. This hypothesis might be further supported by the fact that FKN has been reported to be cleaved by ADAM-10 and ADAM-17 proteases (Garton et al. 2001; Tsou et al. 2001; Hundhausen et al. 2003). ADAM-10 has been shown to induce constitutive shedding of surface FKN whereas ADAM-17 is responsible for inducible cleavage of FKN. Both proteases are believed to play a role in regulating FKN-dependent cell adhesion (Hundhausen et al., 2003). Since the cleavage mediated by ADAM proteases takes place either constitutively or under non-inflammatory conditions it seems reasonable that the produced 90-95 kDa form of FKN has no chemoattracting properties and does not attract phagocytes in a constitutive manner. It is therefore imaginable that a regulatory step, such as another specific proteolytic cleavage, is required for FKN to gain new function. Furthermore, it has already been suggested that ADAM-cleaved FKN might act as a CX3CR1 antagonist and in this way further regulate the level of cell adhesion (Hundhausen et al. 2007). As a form that was generated as a result of the negative regulation of FKN function, 90 kDa FKN seems likely to be unable to induce signal transduction and has an antagonistic role. Additionally, it is possible that 90-95 kDa soluble FKN, which contains almost all the extracellular part of FKN, retains the properties of the membrane-bound form of FKN and similarly does not induce signal transduction through CX3CR1. An interesting question to be addressed in the future is whether and how the change in the length of the soluble form of FKN can affect the way it interacts with its receptor.

6.2.1 Origin and function of 90 kDa form of FKN

The origin of the 90 kDa form of FKN was not fully explained in this work. Based on the available literature, the 90 kDa form of FKN might represent either the full-length protein or a form of the chemokine that is cleaved in a membrane-proximal region (Bazan et al., 1997; Garton et al., 2001). Although the 90 kDa FKN band was present in the BL cell lysates its detection as a soluble form also in the supernatants of BL cells would indicate that it may represent a cleaved form of FKN (Figure 4.1). On the other hand, no difference in size was detected between the 90 kDa FKN in the cell lysate and that in the cell-free supernatants, which would indicate that the 90 kDa form observed in the supernatant was the full-length, uncleaved chemokine. Assuming that the 90 kDa FKN band visible in the lysate is representing cleaved FKN would mean that another, slightly bigger band of full-length FKN should be present in these cells since the presence of the full-length form of FKN in BL cells has been confirmed by FACS and confocal microscopy analysis, where membrane-bound FKN was detected (Figures 4.4 and 4.16). However, bands of the correct size were not observed on the normally exposed Western blots. Some 95-100 kDa-sized bands became visible only on the overexposed films (Figure 4.1c). It is difficult to prove that these bands were representing FKN and were not a result of unspecific binding of the antibodies, it is, however, imaginable that the 95-105 kDa full-length FKN is an unstable form and its majority is proteolytically converted to 90 kDa form, which is abundant in BL cells (Figure 4.1). There is only one clear high molecular weight FKN band (~150 kDa) detected in the BL2 cell lysates (Figure 4.1c) and, despite the fact that it is approximately 40-50 kDa bigger than the reported size of full-length FKN (Bazan et al. 1997; Garton et al. 2001), it cannot be excluded that this band represents the full-length chemokine. However, the fact that neither the 150 kDa form itself nor an expected C-terminal cleavage product of approximately 40-60 kDa were detected by the anti-C-terminal FKN antibodies (Figure 5.12) would state against this possibility.

Analysis of the FKN association with microparticles, which was previously performed by this group, suggested that the 90 kDa FKN is a soluble and therefore, most likely cleaved form of the chemokine. It was demonstrated that whereas 60 kDa FKN was found exclusively in MPs, the 90 kDa form was present both in MPs and in

the supernatants remaining after the MPs were collected by ultracentrifugation (Truman et al. 2008).

Further support for the hypothesis that the 90 kDa FKN found in the lysates of BL cells a cleaved form of the chemokine comes from Western blot analysis using anti-FKN C-terminus antibodies (Figure 5.12). The generation of a 60 kDa form of FKN from the 90 kDa fragment is expected to also produce a 30 kDa fragment. As no such fragment was found, it could perhaps mean that the 90 kDa form was already cleaved when the caspase-3-mediated generation of 60 kDa FKN took place. On the other hand, it would be expected that in the process of proteolytical generation of the 90 kDa form of FKN from its bigger full-length precursor a small (6-9 kDa) fragment should be generated (Hundhausen et al. 2007). A fragment of this size, however, was not visualised by the antibodies recognising the C-terminal part of FKN. In future, immunoprecipitation and sequence analysis of the 90 kDa fragment of FKN will perhaps help explain the origin of this form.

6.2.2 Origin of 25 (and 70) kDa FKN

Intriguingly, the only distinctive band that was visible on the blots labelled with anti-FKN C-terminus antibodies was 25 kDa in size (Figure 5.12). This band is most likely a product of the proteolysis of full-length FKN. Its most characteristic feature is a stable expression in all the analysed cell lines (BL2, Mutu I, L3055) and independently of the cell death programme or caspase-3-dependent proteolysis of 90 kDa FKN. The size of this band indicates that it is unlikely to be a product of ADAM-mediated proteolysis. FKN C-terminal products generated by ADAMs have been shown to have a size of only 6 - 9 kDa (Hundhausen et al. 2007). Therefore, it appears likely that this band was a product of proteolytic events other than those that have been described thus far. As full-length FKN has been reported to have a size of around 90-105 kDa (Bazan et al., 1997; Garton et al., 2001; Hermand et al., 2008) a form of around 65-75 kDa should be produced alongside the 25 kDa one. It is therefore possible that the strong 70 kDa band detected in all BL cell samples (e.g. Figures 4.1, 5.1 and 5.12) might not reflect, as it was previously assumed (Truman et al., 2008 and earlier in this thesis), the unspecific binding of antibodies nor an unglycosylated FKN precursor but may in fact be another product of the proteolytic

cleavage of FKN. A similar, strong and constant expression of both bands seems to support this possibility.

Importantly, Truman has also detected a 25 kDa band in the Mutu I cell lysates using other (than described in this thesis) types of anti-FKN C-terminus antibodies (Truman 2005). Although there does not seem to be FKN bands of these sizes mentioned elsewhere in the literature, it has to be remembered that the overwhelming majority of publications on the release of FKN and its role in various medical conditions is limited to the ELISA-based detection of FKN and therefore the presence of the 70 and 25 kDa bands in some of the studied conditions cannot be excluded. Furthermore, much of the literature has used cells transfected with FKN rather than studying endogenous FKN and due to the extensive post-translational modification of FKN this is likely to affect the size of this protein.

In order to test this hypothesis, it should first be confirmed that the 70 kDa form of FKN is not an unglycosylated precursor. This could be achieved by comparing sizes of the various forms of FKN treated with deglycosylation enzymes. To try to identify the protease responsible for a potential cleavage, experiments involving protease inhibitors could be performed. The incubation times, however, would need to be longer than the ones used in the assays described in Chapter 4 and viable cells would need to be used. In addition, the function of these hypothetical forms of FKN would be an interesting subject of future studies.

6.2.3 Role of caspases in FKN proteolysis

The work presented in this thesis showed that caspase-3, the main executioner protease of apoptotic cell death, may be responsible for cleavage of FKN in apoptotic BL cells. The released 60 kDa product of this cleavage has been suggested to have chemotactic activity towards macrophages (Truman et al. 2008). This may be the first demonstration of the direct involvement of caspase-3 in the attraction of macrophages and warranting the efficient removal of dying cells. However, previous studies by Knies et al. have demonstrated that macrophage chemoattractant EMAP II is also processed and released from apoptotic cells in a caspase-dependant manner and Behrendorf and colleagues have identified EMAP II to be a substrate of caspase-7 (Knies et al. 1998; Behrendorf et al. 2000). However, the ability of this

caspase to cleave EMAP II has later been challenged (Zhang & Schwarz 2002). The report on the release from apoptotic cells of another chemotactic factor for mononuclear phagocytes - lysophosphatidylcholine (LPC) has proposed the indirect role of caspase-3 in these processes (Lauber et al. 2003). The release of this phospholipid has been shown to depend on caspase-3-driven activation of phospholipase A₂β (iPLA₂β) (Lauber et al. 2003).

Several lines of evidence presented in this thesis seemed to suggest that caspase-3 might be responsible for the generation of the 60 kDa chemotactic form of FKN. However, this work did not address the important issue of spatial availability of FKN for caspases. It would be expected that FKN, a protein produced in the endoplasmic reticulum and destined for the plasma membrane, has its mucin stalk well hidden from enzymes operating in the cytosol, including caspases. The process of generating 60 kDa FKN was shown here to be highly sensitive to caspase inhibitors (Figure 4.2 and Figure 4.7). Some of the used inhibitors were able to effectively block cleavage of FKN even when they were not capable of inhibiting the cell death process itself (as assessed by AxV/PI staining – Figure 4.8). Presumably, the activity of the other cell death-related proteases was unaffected as they were still able to drive the process of death. It has to be noted, however, that the peptide caspase inhibitors, including Z-VAD-FMK and Z-DEVD-FMK, which were used here, have also been shown to effectively block other cysteine proteases, particularly cathepsins (Rozman-Pungercar et al. 2003). For this reason, it cannot be excluded that these are not caspases but cathepsins that are responsible for the production of the 60 kDa form of FKN. Particularly as cathepsins have already been shown to be involved in the processing of FKN (Clark et al. 2007; Clark et al. 2009) and are known to actively participate in the process of cell death (Conus & Simon 2008). The possibility that cathepsins participate in the hydrolysis of FKN in dying BL cells is also supported by the fact that these proteases are normally involved in the lysosomal degradation of membrane proteins internalised by endocytosis.

On the other hand, the fact that the incubation of the lysates from untreated BL2 cells with rh caspase-3 resulted in the generation of one clear band of the size of the 60 kDa FKN that is observed in BL2 cells after staurosporine treatment (Figure 4.9), would indicate that caspase-3 may be directly involved in this process. This

appeared particularly striking as another protease(s) (calpain?) that seemed to take part in the hydrolysis of FKN during cell death was responsible for the production of a band of a clearly different size (55 kDa) (e.g. Figure 5.1, Figure 5.5 and Figure 5.6). Therefore, it seems rather unlikely that there is one particular position in the FKN mucin-like stalk that would serve as a cleavage hotspot, resulting in different proteases producing cleavage products of the same size.

Further work would be required to assess how/if FKN cleavage by caspases might be achieved in BL cells. An attempt was described here (section 4.5.3.4, Appendix A) to confirm the position of the caspase cleavage site in the FKN sequence by replacing all the aspartic acid residues in the FKN mucin stalk sequence with glutamic acid residues using site directed mutagenesis. Due to technical difficulties the transfection of the mutated vectors turned out to be impossible within the period prescribed for submission of this thesis, however, the effect of the mutations on the FKN expression and cleavage in BL cells is currently being analysed in our laboratory. These studies are likely to tell us if it is possible that caspases are responsible for the production of the 60 kDa FKN in apoptotic BL cells.

If these analyses confirm the involvement of caspases in the FKN processing, it will be important to understand the mechanisms that allow caspases to directly interact with FKN. Searching for an alternative, cytosolic form of FKN would be perhaps a good starting point.

By contrast, if the role of caspases in the process of cleaving FKN is not confirmed, other factors (perhaps from among those known to operate within membrane compartments) will need to be considered. The possibility that FKN is hydrolysed by cathepsins would be the first to explore. In addition, although, some experiments presented in this thesis seemed to exclude the possibility that metalloproteinases may be responsible for the cleavage (Figure 4.5), in order to confirm those initial observations it would be sensible to repeat the experiments using more powerful inhibitors of metalloproteinases (e.g. 1,10-phenanthroline, captopril). Moreover, other proteases, which are known to operate in the lumen of the ER/Golgi system (e.g. cysteine protease ER-60), could be considered here. This would be particularly interesting as the activity of these enzymes is not normally associated with cell death.

Assuming that the role of caspases in the processing of FKN will be confirmed it still should be remembered that although caspase-3 was shown here to be capable of hydrolysing FKN (Figure 4.9) the involvement of other caspases in this process *in vivo* cannot be excluded. Caspase-3 is generally considered to be the most potent of all the caspases since it has the highest affinity to its substrates and the largest number of identified substrates. However, the efficient knock-down of caspase-3 in BL2 cells did not block the generation of the 60 kDa form of FKN when the cells were induced into apoptosis, indicating that other caspases might compensate for caspase-3 in this circumstances (Figure 4.11). Therefore, it seems possible that caspase-7 and caspase-10, which were also tested for their FKN-cleavage properties (Figure 4.10) may (for example at higher concentration) be able to hydrolyse FKN. Although the caspase inhibitor studies (Figure 4.7) indicated that other caspase family members are not engaged in FKN cleavage during BL cell death, the specificity of the peptide inhibitors that were used in these assays has been questioned (Pereira & Song 2008) therefore it is feasible that they might play a role in this process.

The work aiming at expressing vectors carrying the mutated FKN sequences (Appendix A) might reveal where in the FKN sequence is the location of the caspase cleavage place. However, even now, although the experimental evidence is not available, there are indications that the aspartic acid residue in position 223 (D223) might be recognised by caspases. In the paper of Bazan and colleagues it has been demonstrated that the 180 amino acid long fragment of human FKN sequence gives rise to a peptide of 47-55 kDa in size (Bazan et al. 1997). From that it can be estimated that the remaining 161 (the sequence of FKN chemokine and mucin stalk is 341 amino acid-long) amino acid of the mucin stalk sequence would correspond with approximately 40 kDa of the molecular weight of the protein. Therefore, every 10 kDa of mucin stalk would correspond to a 40 amino acid-long fragment of the nucleotide sequence and a 60 kDa fragment of FKN would be assumed to correlate to around 220 amino acids of the nucleotide sequence. This rough estimation was later confirmed by the search for putative caspase cleavage sites performed by CASVM prediction software. STQD (D223) was the only site identified in the human FKN sequence. Intriguingly, in mouse and rat FKN sequences the software

predicted caspase cleavage in sites distant from the one identified in the human sequence. For mouse FKN the sequence is placed in the N-terminal part of the mucin stalk (TLED, D136). This raises question as to the origin of the 60 kDa FKN that was detected in the supernatants collected from mouse germinal centre cell cultures (Figure 4.17). A proteolysis at D136 would be expected to result in the generation of a band with a molecular weight smaller than 60 kDa. Is it therefore possible that a protease other than caspase is responsible for FKN cleavage in dying mouse germinal centre cells? The form of FKN found in the supernatants of human germinal centre cells appears to be of the same size as the one produced by apoptotic BL2 cells suggesting similar processes take place in these two types of cells. Although it seems less likely, uneven distribution of glycans resulting in an increase of the molecular weight of the N-terminal part of the mucin stalk may be another explanation of the discrepancy between the predicted cleavage site and the unexpectedly high weight of the cleaved form of FKN released by mouse germinal centre cells. In addition, caspase cleavage in sites distinct from the predicted location of cleavage could result in the production of a larger than anticipated form of FKN. This is conceivable as caspase-3 has been shown to be hydrolysing its substrates in non-canonical sites (Fuentes-Prior & Salvesen 2004).

Caspase-mediated proteolysis is widely believed to cause a modification of substrate function rather than its degradation. While some proteins might be inactivated and quickly degraded as a consequence of caspase cleavage (Ditzel et al. 2003), others can gain new function and become stabilised (Dix et al. 2008). Caspase-driven cleavage of FKN is likely to activate a new function as the apoptosis-derived FKN has been shown to mediate macrophage migration (Truman 2005). Interestingly, this form was present in the apoptotic BL2 cell supernatants very early after the cell death induction and only for approximately 2-3 hrs after the release. Thus, it is possible that it may serve as a transient signal, ensuring quick but limited phagocyte action. In the context of FKN gaining an ability to attract macrophages after the caspase-mediated hydrolysis it is, however, difficult to explain the role of the 60 kDa form of FKN that was produced in the apoptotic Mutu I cells since this form did not seem to be liberated from the cells (neither as a soluble form nor in association with MPs) but rather these cells released a 55 kDa form of FKN (Figure 5.1).

6.2.4 Role of calpain in FKN proteolysis

The results presented in Chapter 5 indicate the involvement of another type of cell death-related protease, calpain, in the cleavage of FKN in dying BL cells. The generation of the 55 kDa form of FKN in BL cells was shown to be induced by the calcium ionophore, ionomycin (Figures 5.5 and 5.6) as well as to be sensitive to specific calpain inhibitors, calpeptin and Z-Leu-Leu-CHO (Figures 5.3 and 5.7). Furthermore, it was demonstrated that human calpain I is capable of cleaving recombinant human FKN (Figure 5.3).

However, these results do not rule out the possibility that *in vivo* the generation of 55 kDa form of FKN is mediated by other proteases. As cathepsins have a well recognised role in the effector phase of cell death (Conus & Simon 2008) and cathepsin S has already been demonstrated to be involved in FKN cleavage (Clark et al. 2007; Clark et al. 2009) it appears possible that these proteases might also be involved in the cleavage of FKN in dying cells. It is conceivable that the observed inhibition of the generation of the 55 kDa form of FKN (Figure 5.4 Figure 5.7) by calpeptin and Z-Leu-Leu-CHO might be a result of these compounds inhibiting action of calpain and thus, of calpain-dependent release of lysosomal cathepsins (Yamashima 2000). In addition, it is possible that these inhibitors had a direct effect on the activity of cathepsins (Barnoy et al. 1997). Evidence exists also for the role of ionomycin in directing the release of the lysosomal enzymes (Rodríguez et al. 1997) and a similar effect of ionomycin on BL cells has not been excluded by this work. Therefore, an investigation into the role of cathepsins in the FKN processing during BL cell death seems to be an important task for the future.

In the previous section, an important issue concerning chances for spatial and temporal co-localisation of FKN, a protein produced in the ER and destined for the plasma membrane, and cytosolic caspases was discussed. Here, the same problem has to be addressed in the context of a potential calpain-driven cleavage of FKN. However, unlike in the case of caspases some evidence exists for calpain localisation in the lumen of the ER and Golgi apparatus. Calpain II, but not calpain I nor small regulatory subunit, has been detected in the ER lumen of pulmonary smooth muscle cells as well as in the lumen of the ER and Golgi complex of lung adenocarcinoma

(A-549) cells (Hood et al. 2004; Samanta et al. 2007; Samanta et al. 2010). The mechanism allowing for calpain II transport to this location, however, has not yet been provided. Nevertheless, these reports bring a potential explanation for the results presented in Chapter 5. However, the potential of this mechanism to be in operation in BL cells would need to be further investigated.

It is impossible to establish based on the data presented in Chapter 5 which of the two ubiquitous form of calpain, calpain I (μ) or calpain II (m) might be responsible for the FKN cleavage in BL cells. Although only calpain I was tested here for its ability to cleave FKN, it is very likely that calpain II would be just as efficient since these proteases are known to have a very similar specificity for substrates (Goll et al. 2003; Satish et al. 2005). For the same reason the experiments involving calpain inhibitors were unable to indicate which of the calpains were responsible for the cleavage. The two enzymes vary in the level of calcium required for their activation, with calpain I being activated by calcium concentrations within the low micromolar range and calpain II requiring near-millimolar Ca^{2+} concentrations (Friedrich 2004). Therefore, intracellular calcium levels as well as the availability of the enzymes are the two most likely factors affecting the FKN cleavage by either calpain I or calpain II.

Although calpain has been shown to mediate very limited proteolysis it does not seem to require any specific amino acid sequence for its substrate recognition. Rather secondary structural features and the substrate conformation are responsible for the identification of its target (Tompa et al. 2004; Cuerrier et al. 2005). Thus, it was impossible to predict the calpain cleavage site from the FKN sequence. However, based on the calculations made for the putative caspase cleavage site it is possible to suggest that calpain cleavage may take place in the region between amino acids 190 and 200 of the human FKN sequence. Assuming that caspases cleave after the Asp residue at the position 223 to generate the 60 kDa fragment of FKN, to produce a 5 kDa smaller form of FKN the cleavage should take place around 20 amino acids toward the N-terminus of FKN (on the assumption that in the case of FKN 10 kDa = 40 amino acids; see the previous section for details). There are some arguments in the literature in favour of these estimations. Hundhausen et al. have also shown the generation of a 55 kDa form of FKN in response to ionomycin

stimulation. Importantly, their observations were made using a hybrid protein consisting of the N-terminal fragment of FKN (amino acids 1-206) that was placed on top of the transmembrane fragment of the gp130 signal transducer (Hundhausen et al. 2007). This indicated that the cleavage responsible for the production of the 55 kDa fragment of FKN takes place before residue 206. The authors have demonstrated not only that ionomycin drove the release of the 55 kDa FKN but also that ionomycin-induced release of FKN cannot be fully inhibited by the addition of specific inhibitors of ADAM-10 and ADAM-17. They therefore concluded that ADAM proteases are not the only proteases to process FKN during incubation with ionomycin, the other protease, however, was not identified.

6.2.4.1 Regulation of calpain activity in BL cells

The discrepancies in the way that FKN is processed in different BL cell lines continue to be an intriguing issue. BL2 and L3055 appeared to respond differently to Mutu I or SAV cells upon staurosporine and ionomycin exposure. In Mutu I and SAV cells staurosporine induced the release of 55 kDa FKN whereas in BL2 and L3055 a 60 kDa form of FKN was liberated in response to this stimulus (Figure 5.1). On the other hand, intracellularly, in all but one (SAV) of the examined cell lines staurosporine induced the generation of 60 kDa FKN only. Moreover, the cell lines responded differently when treated with ionomycin. In BL2 (and probably L3055) cells ionomycin caused the release of 55 kDa FKN but only the 60 kDa form was found intracellularly (Figures 5.5 and 5.7). Mutu I cells in response to ionomycin started to generate 55 kDa also intracellularly (Figure 5.6). Intriguingly, the differences in the staurosporine-driven release of FKN seemed to correlate with the exclusive presence of calpain on the surface of the cell lines (Mutu I and SAV) that released the 55 kDa form of FKN (Figures 5.8 and 5.9). Although the detection of calpain on the outer leaflet of the plasma membrane appeared at first rather unusual, careful analysis of the available literature revealed that calpain (particularly calpain II) has previously been observed in this location. It has been found to be exteriorised from activated platelets and to be responsible for the processing of surface glycoproteins (McGowan 1983, Schmaier 1990). Moreover, Dourdin and colleagues have shown that calpain is present on the outer surface of myoblasts and that the calpain-mediated proteolysis of fibronectin is indispensable for myoblast

fusion to take place (Brustis et al. 1994; Dourdin et al. 1997). A role of extracellular calpain in arthritis has also been described. Active calpain II has been found in the synovial fluid from arthritic joints as well as in the cartilage affected by arthritis, where it has been shown to process proteoglycans (Suzuki et al. 1990; Suzuki et al. 1992; Yamamoto et al. 1992; Szomor et al. 1999). In addition, Shimizu and colleagues have demonstrated that extracellular calpain II has a role during the process of cartilage calcification and Nakagawa and co-workers have observed that it is also involved in the healing of fractured bones (Shimizu et al. 1991; Nakagawa et al. 1994). More recent advances in this field, demonstrating that calpain II can be associated with the ER/Golgi apparatus structures and with the Golgi microdomain-derived vesicles as well as that calpain II can be secreted from various cell lines in vesicles, may help to understand how the action of calpain in the extracellular space can be achieved (Nishihara et al. 2001; Hood 2003; Hood et al. 2004). In the light of these observations, it seems that it is not impossible that the 55 kDa FKN detected in the supernatants of the staurosporine-treated Mutu I and SAV cells (Figure 5.1) might derive from the calpain-mediated cleavage of surface FKN. In support of the results showing calpain presence on the outer side of the plasma membrane, it should be remarked that, when the antibodies used for the FACS-based detection of surface calpain were used for immunoblotting, no unspecific (other than 78-80 kDa bands predicted for calpain) bands could be observed (Figure 5.10). Therefore, it does not appear very likely that the labelling of the surface of BL cells resulted from the unspecific binding of the anti-calpain antibodies to another protein. However, it still cannot be excluded that these are other proteases that are responsible for the cleavage. As was discussed above, cathepsins, having a well established role in cell death and cleavage of extracellular substrates, are possible candidates (Briozzo et al. 1988; Buck et al. 1992; Conus & Simon 2008). Moreover, there is a growing recognition of the involvement of MMPs in cell death (Mannello et al. 2005). As it was proposed before, in order to exclude the possibility that MMPs are responsible for the production of 55 kDa form of FKN, it might be reasonable to repeat some of the experiments presented in Chapter 4 (Figure 4.5) using more powerful metalloproteinase inhibitors (e.g. captopril or 1,10-phenanthroline).

An explanation for the differences in the forms of FKN that were detected intracellularly is more difficult to identify. The total level of calpain expression in BL2, L3055 and Mutu I cells seemed to be the same (Figures 5.8 and 5.9). Furthermore, calpain I activation status (assessed by the level of its autoproteolysis) was the same (Figure 5.10). It should be noted, however, that calpain is known to have a certain level of activity even prior to its Ca^{2+} -driven autoproteolysis (Johnson & Guttman 1997; Li et al. 2004). These results were in line with expectations for ionomycin-treated cells in which increased calcium concentration would undoubtedly cause calpain activation and autolysis. In addition, the fact that calpain did not seem to become activated in the staurosporine-treated cells was understandable. It was, however, intriguing that the calpain forms detected in the supernatants of both ionomycin- and staurosporine-treated cells were different (more extensively processed) than the forms that were found in the cell lysates. This was particularly striking in case of the cells that were incubated with staurosporine. Moreover, although calpain inhibitors have previously been reported to be able to inhibit the conversion of the 78 kDa form of calpain into the 76 kDa one (Melloni et al. 1996; Michetti et al. 1996), the mechanism by which Z-VAD-FMK blocks this process is worth further investigation (inhibition of caspase-mediated cleavage of calpastatin (Wang et al. 1998; Shi et al. 2000) might be one possible explanation). These experiments were only performed for calpain I but it seems likely that similar results would be obtained for calpain II (as the dramatically elevated Ca^{2+} concentrations in ionomycin-treated cells should be sufficient for calpain II activation). Under physiological conditions, however, these two proteases might be both differentially activated and differentially localised. As neither the level of calpain expression nor the efficiency of its activation seemed to be responsible for the variance in FKN cleavage in different BL cell lines it seems possible that the different distribution of the two main calpain isoforms in the cell is responsible for the observed effect. As calpain II, unlike calpain I, might be found in the lumen of the ER/Golgi network, it is possible that it has better access than calpain I to the substrate. It would need to be further investigated if these are the differences in the subcellular localisation of calpain II that make the studied BL cell lines produce different forms of truncated FKN (Figure 5.1). Other mechanisms known to regulate

calpain function may also be responsible for the observed discrepancies. Among them the expression of the natural calpain inhibitor – calpastatin, known to regulate the action of the active form of calpain (Melloni et al. 2006), is potentially responsible for the observed discrepancies. Yet, the variance may also be caused by differences in the pattern of either calpain or FKN phosphorylation. Specific phosphorylation has been reported to cause either an increase in calpain activity (McClelland et al. 1994; Glading et al. 2001) or its inhibition (Shiraha et al. 2002; Smith et al. 2003). Moreover, also here, it might be the different expression or activation of proteases other than calpain that results in the observed discrepancies.

Calpain-mediated proteolysis, like that of caspases, is believed to modify the function of its substrate rather than cause its degradation (Johnson & Guttman 1997; Goll et al. 2003). This might be true also in case of FKN since FKN derived from staurosporine- and ionomycin-treated Mutu I cells (which released 55 kDa FKN) has been shown to be responsible for attraction of macrophages (Truman 2005).

6.2.5 Possible cooperation of caspases and calpain in FKN cleavage

Caspases and calpains are the major protease families with a recognised role in cell death. Caspases are the key mediators of apoptosis. Calpain, initially linked with acute necrotic death, has more recently gained recognition also for its function during apoptotic events (Wang 2000; Liu et al. 2004).

While these two types of proteases are generally believed to cooperate during cell death, they have also been shown to influence each other's activity. Calpain has been demonstrated to activate caspases under some death conditions (Ruiz-Vela et al. 1999; Blomgren et al. 2001; Altnauer et al. 2004) but also to mediate caspase degradation and inactivation (Chua et al. 2000). On the other hand, caspases have been shown to regulate calpain activity (Wood & Newcomb 1999). The endogenous calpain inhibitor – calpastatin, has been reported to be hydrolysed by both caspases and calpain (Wang et al. 1998; De Tullio et al. 2000; Shi et al. 2000). Remarkably, calpastatin is not an exception as a considerable number of substrates that can be cleaved by both proteases have been identified (Chan & Mattson 1999; Wang 2000;

Liu et al. 2006). Among them many cytoskeletal, plasma membrane and signal-transducing proteins have been found. In the experimental pathological conditions, like transient brain injury, where both the proteases are activated, they have been shown to be cleaving their substrates simultaneously (Liu et al. 2006). Interestingly, in many of the dually cleaved proteins caspases- and calpain-driven cleavage leads to the production of similarly-sized products; the differences between most of them are within a range of 0-10 kDa (Wang 2000). For instance, in the anti-apoptotic protein Bcl-XL the cleavage sites for caspases and calpain adjoin (Clem et al. 1998; Nakagawa & Yuan 2000). In α -fodrin the two cleavage sites are separated by only nine residues (Wang 2000). Similarly, the difference between the two cell death-related forms of FKN amounted to 5 kDa and, according to the estimations presented above, the potential cleavage sites may be situated approximately 20-30 amino acids apart. The fact that the products potentially generated by calpain- and caspase-mediated cleavage can have identical or comparable molecular weights might suggest that they can also have similar functions. Therefore, it is possible that the 60 and 55 kDa forms of FKN are also playing similar roles. This, however, has to be further investigated. The chemotaxis experiments performed by Truman have revealed that only the cell death-derived FKN had the property of attracting macrophages. Unfortunately, the differences in FKN processing in different BL cell lines had not yet been perceived and Truman's studies were limited to Mutu I cells induced into death by various compounds (Truman 2005). As Mutu I cells were shown here to exclusively release 55 kDa FKN (Figures 5.1 and 5.6), the chemotactic properties of 60 kDa FKN, although likely, have still to be additionally characterised.

Interestingly, it was observed here that FKN as well can be simultaneously hydrolysed by two different enzymes, as it was visible in the staurosporine-treated Mutu I cells (Figure. 5.1).

Moreover, the same cells were shown to produce both forms, one after another. In the 24 hrs time-course of staurosporine-treated BL2 cells, where 60 kDa form of FKN was detected in the supernatant at the 2 hrs time-point, the 55 kDa form became visible after 12 hrs (Figure 5.11). Interestingly, that pattern of FKN cleavage in the staurosporine-treated BL2 cells matched the pattern of cell-death related changes

observed by Wood and Newcomb in apoptotic HL-60 cells (Wood & Newcomb 1999). They have reported that in drug (9-AC)-treated cells, the effects of caspase activity could be visible as early as 2 hrs after the treatment, while calpain action could be detected 10-12 hrs after the start of the experiment. This might be a confirmation of both caspase and calpain involvement in FKN cleavage. Also, the results from BL2 cell studies imply that early apoptotic (2 hrs post-treatment) cells send (caspase-mediated) “find-me” signals to macrophages to ensure they are cleared rapidly and that second (calpain-mediated) signal is sent when the still uncleared cells are becoming secondary necrotic (12 hrs post-treatment).

Altogether the observations presented here suggest that these complex mechanisms serve to ensure that, independently of nature of death stimulus and pathway involved in the cell death execution, the “find-me” signal will be sent by dying cells.

6.3 Mechanism of FKN release

The mechanism by which FKN is released from dying BL cells remains incompletely understood. The shedding of membrane-bound FKN by proteases ADAM-10 and ADAM-17 has long been seen to be a major method of generating soluble FKN. However, the experiments performed previously in this laboratory have revealed that a 60 kDa form of FKN, derived from apoptotic BL cells, is exclusively associated with MPs. The easiest explanation for the presence of 60 kDa FKN in MPs is that intracellularly cleaved FKN would subsequently be directed to MPs. Yet, it is also possible that the processing of FKN takes place in MPs themselves. The support for this hypothesis came from the experiments with staurosporine-treated Mutu I cells. Whilst the lysates of these cells only produced the 60 kDa FKN, the supernatants/MPs contained the 55 kDa form instead (Figure 5.1). Hence, it seems that intracellular cleavage of FKN and its hydrolysis in MPs are two independent processes. The fact that no 60 kDa FKN was found in supernatants from Mutu I cells also implies that two separate pools of FKN exist in these cells: one, which would be directed to MPs and another, which would not be released. This would be in accordance with the report by Liu *et al.* in which FKN was only detected as a

membrane-bound form and in a unique endomembrane compartment (Liu et al. 2005). Likewise, Hermand and colleagues have demonstrated that only around 50% of cellular FKN is exposed on the plasma membrane (Hermand et al. 2008). The analysis of FKN localisation in apoptotic BL2 cells showed that although some portion of it was present on the cell surface, most seemed to be concentrated intracellularly, in the membrane-proximal area (Figures 4.16 and 4.15). FKN appeared to be equally distributed in other parts of the cells and no special cytoplasmic storage compartment could be distinguished. It was, however, difficult to assess the location of compartments without using additional markers in cells like lymphocytes since they contain only scarce amount of cytoplasm.

In addition, the loss of FKN from the surface of BL2 cells that was observed 2 hrs after staurosporine treatment (Figure 4.4) is likely to be due to the release of MPs. Using time lapse microscopy it was revealed that the intensity of MPs formation and liberation from BL2 cells dramatically increased around 1.5 – 2 hrs after the induction of apoptosis (Figure 4.13, Appendix B). Segundo and colleagues have also observed a loss of cell surface proteins resulting from the release of MPs from apoptotic cells (Segundo et al. 1999). On the other hand, the decrease in the levels of surface FKN observed at the 1 hr time point was clearly independent of the apoptosis programme since the control cells were affected in the same way as the staurosporine-treated BL2 cells (Figure 4.4). At the same time, the release of 90 kDa FKN also appeared to be independent of apoptosis and was detected in the supernatants as early as 1 hr after the treatment. As it was discussed before (section 6.2.1), the results presented in this thesis do not allow for distinguishing if 90 kDa form of FKN represents a full-length transmembrane molecule or a truncated form. However, as it was detected in the supernatants in a soluble form it seems possible that the observed, apoptosis-independent, loss of surface FKN expression results from an ADAM-mediated, constitutive or cell handling-induced, proteolysis of surface FKN.

Furthermore, the role of two separate pools of microvesicles that were produced by staurosporine-treated BL2 cells (Figure 4.14) in the release of FKN could be investigated. FKN was shown here to be present in the bigger vesicles, which according to their size represent MPs (Figures 4.12 and 4.14) (Charras 2008). The

other population of vesicles were of around 50-100 nm in diameter (Figure 4.14) and probably represent exosomes. Unlike MPs, which are generated as protrusions from the plasma membrane, exosomes derive from the endosomal compartment (Lakkaraju & Rodriguez-Boulan 2008). Having different origins to MPs, exosomes are likely to carry distinct sets of proteins. Therefore, it would be interesting to assess whether exosomes also contain FKN and to determine the differences in the forms of FKN contained within these two populations of vesicles.

6.4 Role of FKN in cell death

The mechanisms responsible for migration of specialised phagocytic cells to the sites where cell death occurs are not well characterised. So far, only a few chemotactic factors released from dying cells have been described. First, endothelial monocyte-activating polypeptide II (EMAP II), a cytokine with attractant properties for macrophages, has been found to be processed in apoptotic cells in developing mouse embryo. Its subsequent release has correlated with macrophage infiltration (Knies et al. 1998). In addition, ribosomal protein S19 has been identified as a factor mediating monocyte chemotaxis to heat-treated HL-60 cells (Horino et al. 1998). Moreover, Lauber et al. have demonstrated that a phospholipid lysophosphatidylcholine (LPC) drives the migration of THP-1 monocytic cells to UV-irradiated MCF-7 cells (Lauber et al. 2003). While some groups have also reported that LPC has chemotactic properties (Radu et al. 2004; Yang et al. 2005), its involvement in macrophage attraction to apoptotic cells has not been observed in other systems, e.g. studied by this group and others (Truman, unpublished; Elliott et al. 2009). In addition, another lipid, sphingosine-1-phosphate (SP1) has been reported to mediate both chemoattraction of macrophages to apoptotic cells as well as their survival and shift towards M2-like phenotype (Weigert et al. 2006; Gude et al. 2008; Johann et al. 2008). More recently, Elliott and colleagues have shown that the release of the nucleotides ATP and UTP from apoptotic T cells induced migration of mononuclear phagocytes both *in vitro* and *in vivo* (Elliott et al. 2009). The identification of the role of the cell death-associated form of FKN in attraction of macrophages added the first

chemokine to this group (Truman et al. 2008). Here it was demonstrated that it might be caspases that are directly involved in the production of chemotactic signals for macrophages. Moreover, calpain – another protease that plays a crucial role in the execution of cell death, was also proposed here to potentially have a role in cleaving FKN during cell death. The susceptibility of FKN to hydrolysis by two proteases, known to complement one another during various types of death, suggests that this chemokine may have a crucial role during the process of cell death.

The role of FKN in attracting macrophages has also been confirmed in systems other than BL environment. Germinal centre B cells (healthy counterparts of BL cells) which undergo spontaneous apoptosis during the process of specificity selection, have been shown to attract tingible body macrophages in a FKN-dependent manner (Truman et al. 2008). Here, it was established that dying GC B cells, unlike control follicular B cells, released a form of approximately 60 kDa molecular weight (Figure 4.17). It is therefore tempting to hypothesise that other FKN-expressing cells can also use it to signal their death.

There are some indications that FKN can play a similar role during neuronal cell injury. Calpain activation is well established under various cell injury-causing conditions i.e. toxicants, hypoxia and ischemia, (Rami 2003; Liu et al. 2004). Moreover, it has been shown that during experimental excitotoxic injury, FKN, which is widely expressed by neurons, is released at an early stage of the death programme and that it has chemoattractant properties for monocytes (Chapman et al. 2000). The authors have further demonstrated that the release can be inhibited by metalloproteinase inhibitors; this part of their study, however, was not performed under cell death conditions and the size of the chemotactic form of FKN was not provided. Finally, FKN-deficient mice have been shown to be partly protected from ischemia-mediated damage (Soriano et al. 2002). Based on the results presented in this thesis it can be speculated that injury-activated calpain would cleave FKN and that it would be the cleaved, chemotactic form of FKN that would drive an accumulation of microglial cells and monocytes. These cells could in turn mediate the damage observed following neuronal cell injury (Amantea et al. 2009). FKN is constitutively expressed in the central nervous system and is known to play various, often contradictory roles (e.g. Boehme et al. 2000; Meucci et al. 2000; Zujovic et al.

2000; Soriano et al. 2002; Cardona et al. 2006; Clark et al. 2007). Its specific cleavage by calpain might be indispensable to modulate its function and ensure specificity of its action.

The final evidence for FKN having an important role in cell death comes from the fact that it has been found to be directly regulated by the transcription factor p53 (Shiraishi et al. 2000). It might mean that FKN expression can be up-regulated by p53 (along with pro-apoptotic proteins, for example: Bak and Bax) as a part of the cells reaction to stress e.g. DNA damage or hypoxia (Levine 1997).

6.5 Role of FKN in BL

FKN has been shown to play an important role in the process of attracting macrophages to BL tumours (Truman et al. 2008). As tumour-associated macrophages (TAMs) are known to support tumour growth in many ways (Mantovani et al. 1992), FKN can be seen as a factor that exerts an indirect effect on tumour growth by shifting the immune response to act in favour of the tumour.

It has, however, been shown that FKN is not the only chemokine released by dying BL cells to attract macrophages (Truman et al. 2008). Studies gathered in Chapter 3 were focused on determining the role of MCP-1 in this process. As it was established that MCP-1 probably is not produced by BL cells, the chemokine(s) responsible for this process have yet to be identified. From the chemokines with known ability to attract mononuclear phagocytes, CXCL12 stands out as a potential candidate. This chemokine has been found to participate in the mediation of an immunosuppressive environment, neovascularisation and metastasis in various types of tumours, including non-Hodgkin's lymphoma (Rempel et al. 2000; Kryczek et al. 2007; de Oliveira et al. 2009). Intriguingly, a strong expression of CXCL12 has been found in malignant B cells in MALT lymphoma, where it has been suggested to have a role in mediating B cell survival (Barone et al. 2008). The gene profiling analysis performed in this group has revealed that both BL cells and macrophages infiltrating BL tumours express CXCR4 – one of the receptors for CXCL12 (Sofia Petrova – unpublished results).

The migration of macrophages to BL is mediated by the cell death-associated forms of FKN (60 and 55 kDa). However, it is tempting to speculate that other forms of FKN that are present in BL could have additional, direct impacts on the tumour. FKN has been shown in other environments to carry out functions that could be potentially relevant also to BL. Boehme et al. have demonstrated that FKN acts as a strong survival factor for cultured microglia and is capable of even overcoming Fas-mediated death signalling (Boehme et al. 2000).

FKN has also been shown to provide survival signals for monocytes in culture (Landsman et al. 2009). Furthermore, both FKN- and CX3CR1-deficient mice have been shown to lose a Gr1^{low} CX3CR1^{high} subset of monocytes as a consequence of the increased rate of their apoptosis (Landsman et al. 2009). In sum, these observations suggest that FKN could also constitute an anti-apoptotic signal for macrophages that infiltrate BL tumours.

Moreover, apart from affecting viability of macrophages, FKN seems to be capable of moderating the type of immunological reaction that they mediate. It has been reported that FKN inhibited the release of TNF α , IL-6 and NO from LPS- and IFN- γ -treated microglia (Zujovic et al. 2000; Mizuno et al. 2003). This has been further confirmed by the observation that CX3CR1-deficient mice suffered from excessive neurotoxicity after LPS injection (Cardona et al. 2006). Following on from these observations it can be proposed that FKN might also suppress pro-inflammatory factors released from macrophages in BL and thus, would take an active part in establishing the immunosuppressive environment of the tumour.

In this respect, it is also possible that FKN can indirectly contribute to the inhibition of immune response by stimulating MFG-E8 production and inducing apoptotic cell clearance by macrophages (Miksa et al. 2007).

Finally, FKN is also likely to contribute to neoangiogenesis in BL tumours since its involvement in mediating endothelial cell proliferation and new vessels formation has been reported (Lee et al. 2006). It has also been shown to serve both as an anti-apoptotic and a mitogenic signal for vascular smooth muscle cells (White et al. 2010).

Notable differences were observed in the way that FKN was cleaved in the different BL cell lines used in this study, suggesting the existence of similar discrepancy between the individual cases of lymphoma. Rather strikingly, the division between the cells that were prone to produce (after staurosporine treatment) 60 kDa FKN (i.e. BL2 and L3055) and those that were able to produce 55 kDa FKN (also expressing calpain on their surface) (Mutu I and SAV), overlapped with the division for those that were EBV-negative (BL2 and L3005) and EBV-positive (Mutu I and SAV) (Gregory et al. 1990; Milner et al. 1993; Gordon et al. 2000). This suggested that EBV infection might affect the mechanism of FKN processing. EBV-positive type I latency BL lines are known to express only three of the virus components: EBV-determined nuclear antigen 1 (EBNA-1), EBV-encoded small RNAs (EBERs) and BamHI A rightward transcripts (BARTs) (Rowe et al. 1987; Rowe et al. 2009). While EBNA-1 has initially been associated with subsistence and replication of the EBV genome in cells, more recent reports provide evidence for its involvement in the regulation of various genes of the host cell (Wood et al. 2007; Flavell et al. 2008; O'Neil et al. 2008; Tsimbouri et al. 2008; Canaan et al. 2009). Notably, EBNA-1 has also been found to protect BL cells from apoptosis (Kennedy et al. 2003). Another study, however, suggests that it is not able to fulfil its role alone and it has to work in conjunction with EBERs in order to exert the anti-apoptotic effect (Komano et al. 1999). Furthermore, anti-apoptotic properties of both EBERs and BARTs acting independently have been reported (Nanbo et al. 2002; Choy et al. 2008). The observed interference of the virus components with the cell death signalling pathways indicates that they might have an effect on the way the cell reacts to a death stimulus, including activation of death-related proteases and in consequence, possibly also on the pattern of FKN cleavage.

Appendix A

Introducing point mutation into the FKN gene sequence

In order to confirm caspase involvement in the FKN processing during apoptosis and to identify a potential site of cleavage it was decided to introduce point mutation in the Asp residues of the FKN sequence (see sections 2.13.3 and 4.5.3.4 for details). The position of targeted residues in the FKN mucin stalk sequence and the outcome of the site-directed mutagenesis are presented here.

10	20	30	40	50	60	70
MAPISLSWLL	RLATFCHLTV	LLAGQHHGVT	KCNITCSKMT	SKIPVALLIH	YQQNQASCGK	RAIILETRQH
80	90	100	110	120	130	140
RLFCADPKEQ	WVKDAMQHLD	RQAAALTRNG	GTFEKQIGEV	KPRITPAAGG	MD ^D ESVVLEPE	ATGESSSLEP
150	160	170	180	190	200	210
TPSSQEAQRA	LGTSPPELPTG	VTGSSGTRLP	PTPKAQ ^D GGP	VGTELFVRVPP	VSTAATWQSS	APHQPGPSLW
220	230	240	250	260	270	280
AEAKTSEAPS	TQ ^D DPSTQAST	ASSPAPEENA	PSEGQRVWQ	GQSPRPENSL	EREEMGPVPA	HT ^D AFQ ^D WGP
290	300	310	320	330	340	350
GSMAHVSVVP	VSSEGTPSRE	PVASGSWTPK	AEEPIHATM ^D	PQRLGVLITP	VP ^D QAATR	QAVGLLAFLG
360	370	380	390			
LLFCLGVAMF	TYQSLQGCPR	KMAGEMA EGL	RYIPRSCGSN	SYVLVPV		

SIGNAL
 CHEMOKINE DOMAIN
 MUCIN STALK
 TRANSMEMBRANE
 CYTOPLASMIC

Figure A.1. The positions of Asp (orange) residues in the human FKN mucin stalk amino acid sequence.

1 ATGGCTCCGATATCTCTGTCGTGGCTGCTCCGCTTGGCCACCTTCTGCCATCTGACTGTCCTGCTGGCTG
71 GACAGCACCACGGTGTGACGAAATGCAACATCACGTGCAGCAAGATGACATCAAAGATACCTGTAGCTTT
141 GCTCATCCACTATCAACAGAACCAGGCATCATGCGGCAAACGCGCAATCATCTTGGAGACGAGACAGCAC
211 AGGCTGTTCTGTGCCGACCCGAAGGAGCAATGGGTCAAGGACGCGATGCAGCATCTGGACCGCCAGGCTG
281 CTGCCCTAACTCGAAATGGCGGCACCTTCGAGAAGCAGATCGGCGAGGTGAAGCCCAGGACCACCCCTGC
651 CGCCGGGGGAAT**GAC**GAGTCTGTGGTCCTGGAGCCCGAAGCCACAGGCGAAAGCAGTAGCCTGGAGCCG
421 ACTCCTTCTTCCCAGGAAGCACAGAGGGCCCTGGGGACCTCCCCAGAGCTGCCGACGGGCGTACTGGTT
491 CCTCAGGGACCAGGCTCCCCCGACGCCAAAGGCTCAG**GAT**GGAGGGCCTGTGGGCACGGAGCTTTTCCG
561 AGTGCCTCCCGTCTCCACTGCCGCCACGTGGCAGAGTTCTGCTCCCCACCAACCTGGGCCCAGCCTCTGG
631 GCTGAGGCAAAGACCTCTGAGGCCCCCGTCCACCCAG**GAC**CCCTCCACCCAGGCTCCACTGCGTCCTCCC
701 CAGCCCCAGAGGAGAATGCTCCGTCTGAAGGCCAGCGTGTGTGGGGTTCAGGGACAGAGCCCCAGGCCAGA
771 GAACTCTCTGGAGCGGGAGGAGATGGGTCCCGTGCCAGCGCACAC**GAT**GCCTTCCAG**GAC**TGGGGGCCT
841 GGCAGCATGGCCACGTCTCTGTGGTCCCTGTCTCCTCAGAAGGGACCCCCAGCAGGGAGCCAGTGGCTT
911 CAGGCAGCTGGACCCCTAAGGCTGAGGAACCCATCCATGCCACCAT**GAC**CCCCAGAGGCTGGGCGTCCT
981 TATCACTCCTGTCCCT**GAC**GCCCAGGCTGCCACCCGGAGGCAGGCGGTGGGGCTGCTGGCCTTCTTGGC
1051 CTCCTCTTCTGCCTGGGGGTGGCCATGTTACCTACCAGAGCCTCCAGGGCTGCCCTCGAAAGATGGCAG
1121 GAGAGATGGCGGAGGGCCTTCGCTACATCCCCCGGAGCTGTGGTAGTAATTCATATGTCCTGGTGCCCGT
1191 G

Figure A.2. The location of aspartic acid codons (bold) and nucleotides targeted for mutation (red) in the human FKN nucleotide sequence.

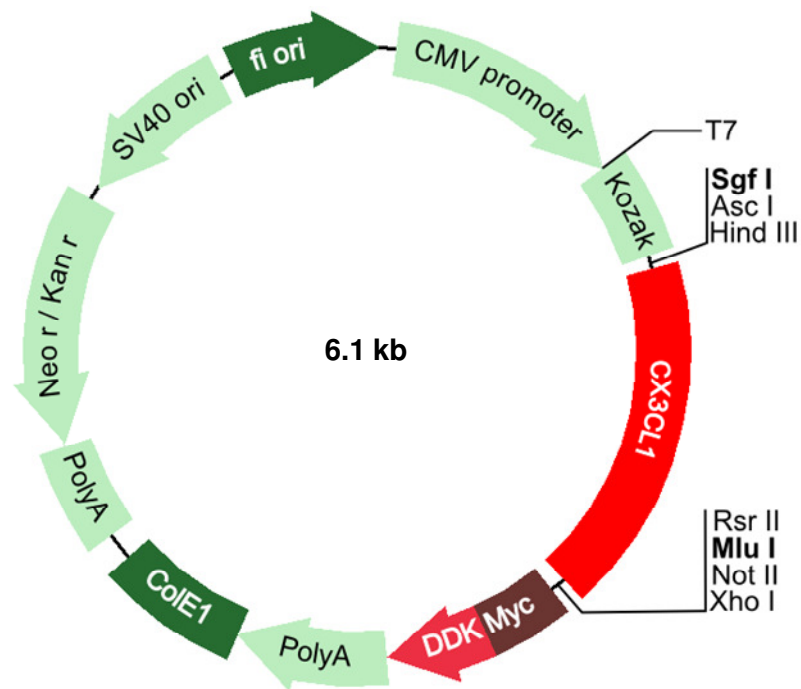


Figure A.3. Schematic picture of TrueORF pCMV6-Entry vector.

TrueORF pCMV6-Entry carrying FKN gene (C-terminal Myc and DDK Tagged) was used for site-directed mutagenesis. Supplied by Origene. Picture adapted from the supplier's product information (http://www.origene.com/cdna/trueorf/vector_detail.aspx?sku=PS100001).

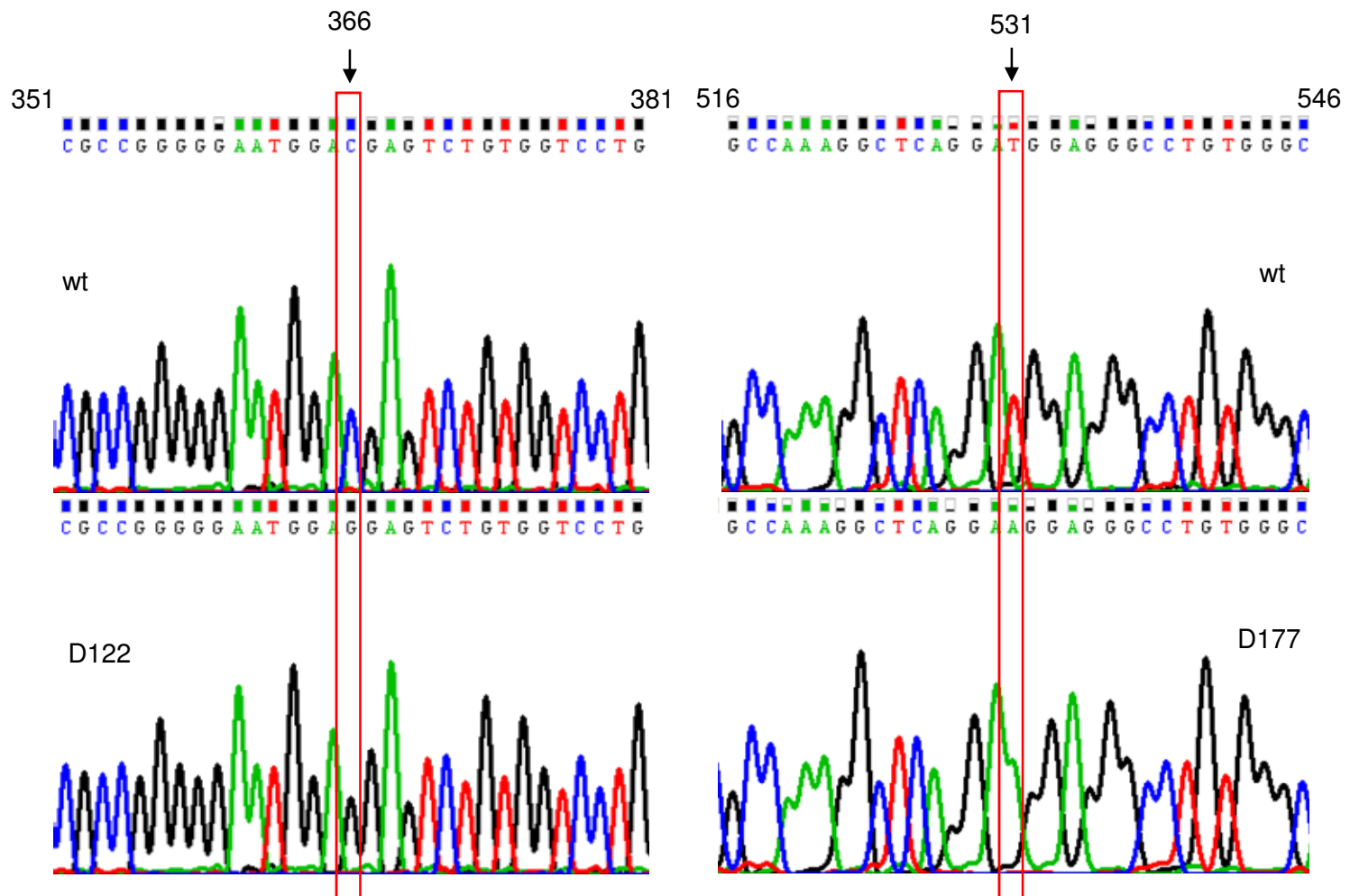


Figure A.4. Introduction of the point mutations in the FKX sequence.

Control sequences were placed on top of the mutated sequences. The mutated nucleotides were marked out. Asp: GAT, GAC; Glu: GAA, GAG

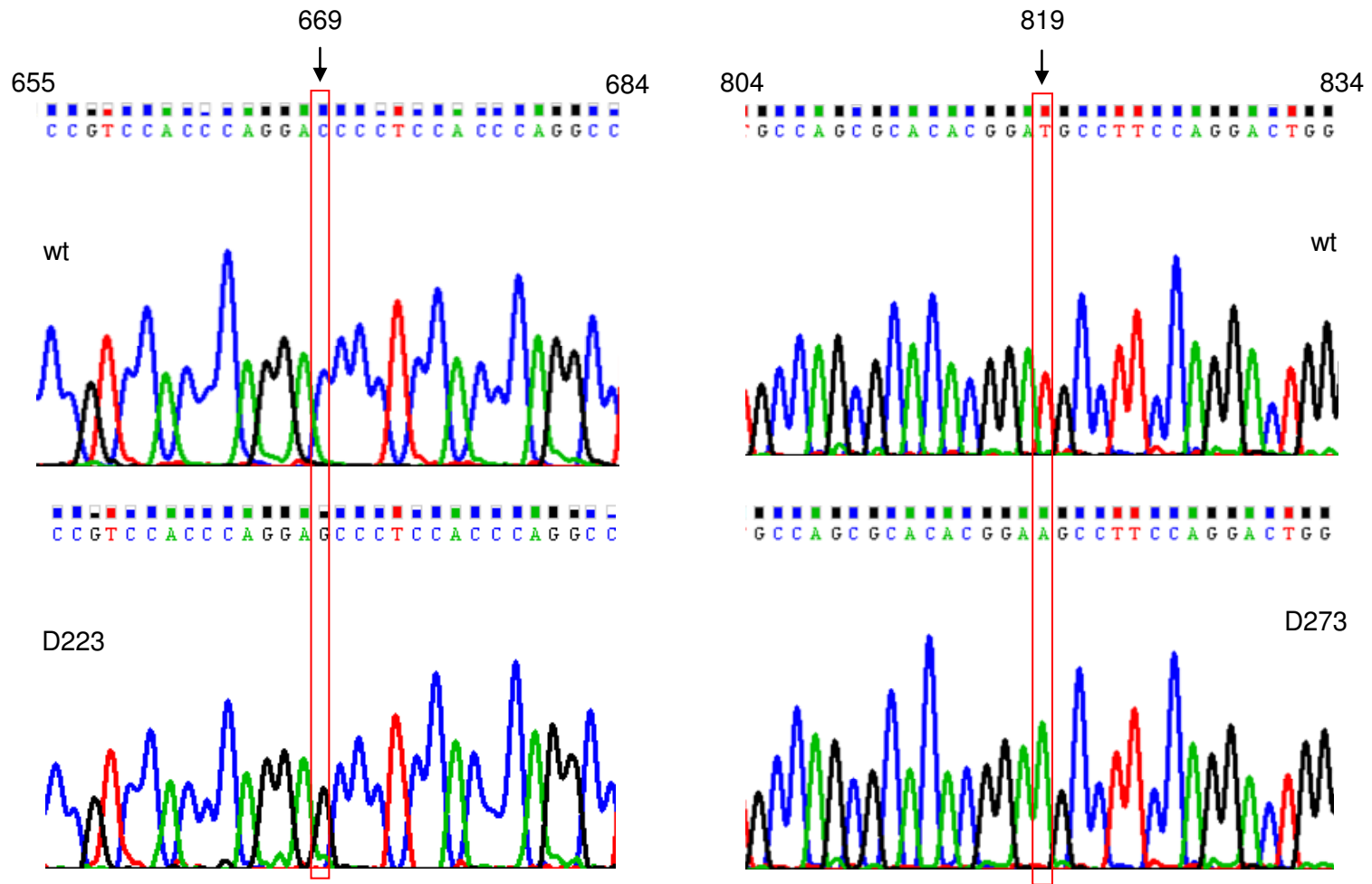


Figure A.4. continued

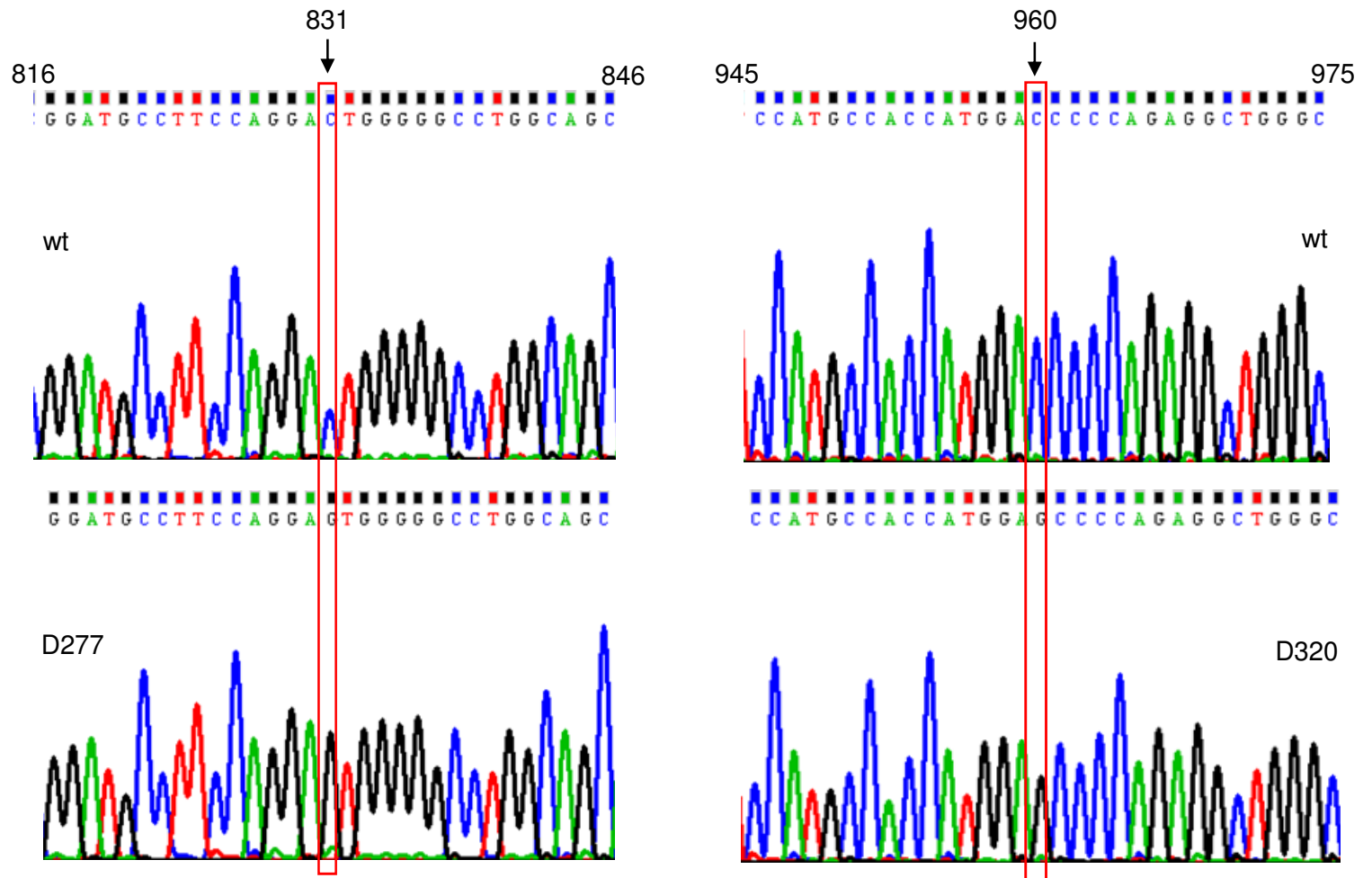


Figure A.4. continued

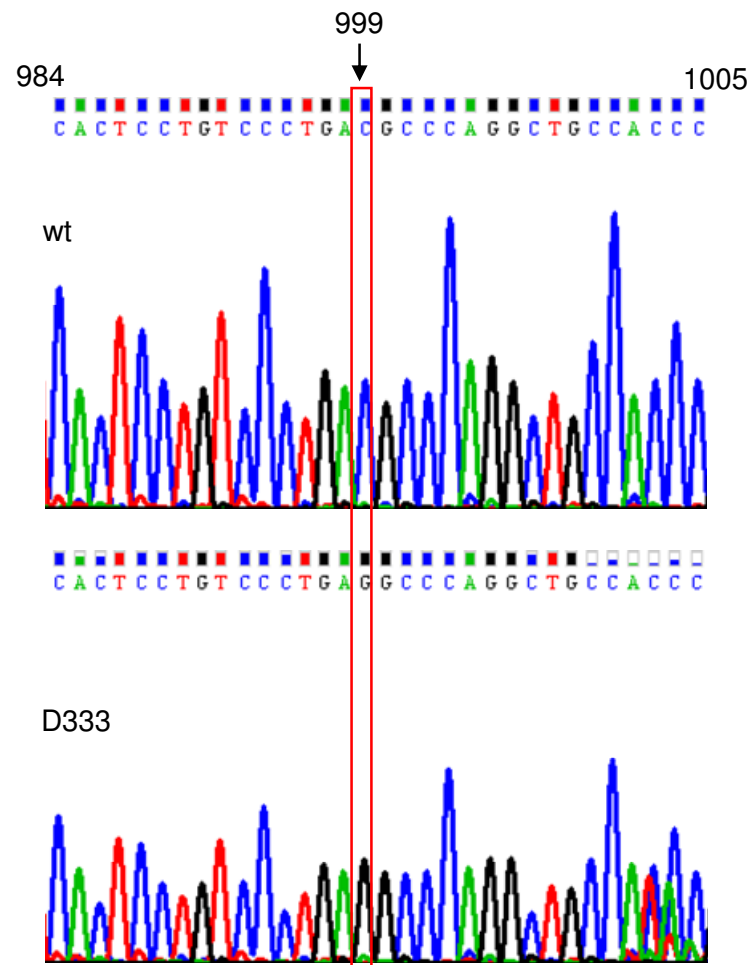


Figure A.4. continued

Appendix B

Time lapse imaging of MP release from apoptotic BL cells

The formation and release of MPs from staurosporine-induced BL2 cells were recorded using phase contrast widefield microscopy and a time lapse imaging system. BL2 cells were placed in POC mini chamber on a heated microscope stage (37°C) of an inverted microscope. A low-magnification image (10x objective) of the cells was collected every 15 sec, for 150 min, starting 30 min after the cells were exposed to staurosporine. The full image sequence can be found on the CD attached to the inside back cover.

Appendix C

Publication arising

CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis

Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, Melville L, Melrose LA, Ogden CA, Nibbs R, Graham G, Combadiere C, Gregory CD.

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Abstract

Cells undergoing apoptosis are efficiently located and engulfed by phagocytes. The mechanisms by which macrophages, the professional scavenging phagocytes of apoptotic cells, are attracted to sites of apoptosis are poorly defined. Here we show that CX3CL1/fractalkine, a chemokine and intercellular adhesion molecule, is released rapidly from apoptotic lymphocytes, via caspase- and Bcl-2-regulated mechanisms, to attract macrophages. Effective chemotaxis of macrophages to apoptotic lymphocytes is dependent on macrophage fractalkine receptor, CX3CR1. CX3CR1 deficiency caused diminished recruitment of macrophages to germinal centres of lymphoid follicles, sites of high-rate B-cell apoptosis. These results provide the first demonstration of chemokine/chemokine-receptor activity in the navigation of macrophages toward apoptotic cells and identify a mechanism by which macrophage infiltration of tissues containing apoptotic lymphocytes is achieved.

A copy of this paper is attached at the end of this thesis

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