

# Degree Course Life Technologies

## Option Biotechnology

# Diploma 2011

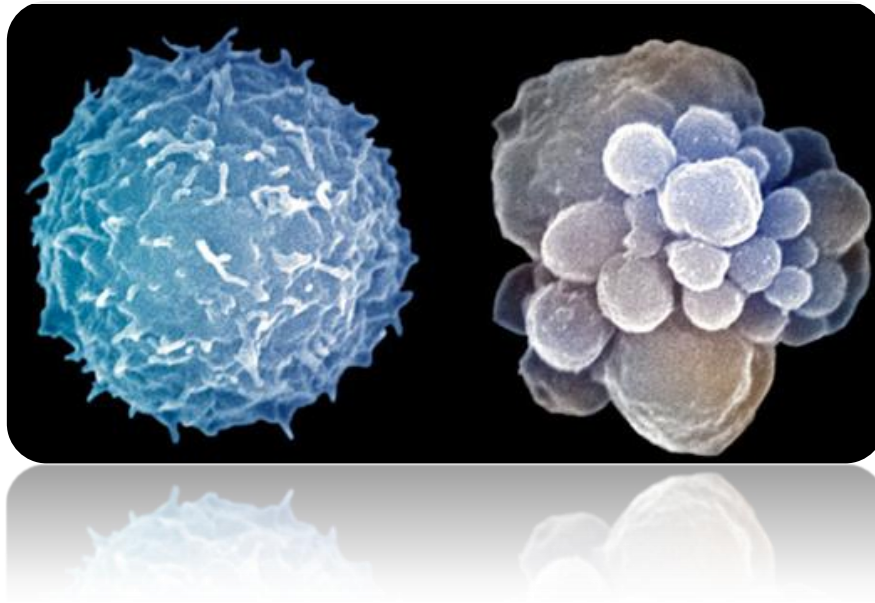
*Laetitia Roh*

*Role of the Bcl-2 family member Bok  
in proliferation  
or cell cycle control*

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# BCL-2 FAMILY



## ROLE OF BOK IN PROLIFERATION OR CELL CYCLE CONTROL

**Bachelor Thesis of Laetitia Roh  
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2. Mai - 19 August 2011**

HES-SO Valais

**Données du travail de diplôme**  
**Daten der Diplomarbeit**

FO 1.2.02.07.AB  
pof/31/01/2009

SI	TV
X	X

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Titre / Titel

**Role of the Bcl-2 family member Bok in proliferation or cell cycle control**

Description et Objectifs / Beschreibung und Ziele

The family of B-cell leukemia (Bcl-2) proteins are major regulators of apoptosis, the main (patho)physiological form of programmed cell death. Aberrant apoptosis is a cause or consequence of many diseases, most importantly of cancer and autoimmune disorders. In order to further study and analyse the cell cycle regulation of lymphocytic immune cells the following experimental approaches were designed.

- Primary cells and cell lines will be cultured and analysed in presence and absence of the *Bok* gene (gene ko mouse cells)
- Cell size increases of these cells during B and T cell blast formation (as a measure of activation status) are to be determined by FACS [mean forward scatter (FSC)] analysis
- Cells will be exposed to various doses of DNA-damaging treatments (etoposide, UV) or replicative stress-inducing agents (Hydroxyurea)
- Cell cycle arrest is then to be analysed by FACS and apoptosis by Western Blot analysis
- Cell colony formation assays, indicating long-term protective effects, are to be performed

Délais / Termine

Attribution du thème / Ausgabe des Auftrags:

02.05.2011

Exposition publique / Ausstellung Diplomarbeiten:

02.09.2011

Fin des travaux de diplôme dans les laboratoires de l'école /

Ende der Diplomarbeiten in den Labors der Schule:

15.07.2011

Défense orale / Mündliche Verteidigung:

dès la semaine 35 / ab Woche 35

Remise du rapport / Abgabe des Schlussberichts:

19.08.2011 | 12h00

Signature ou visa / Unterschrift oder Visum

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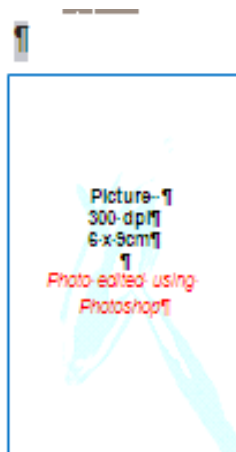
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pof/31/01/2009

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Mandant / Auftraggeber <input checked="" type="checkbox"/> HES—SO Valais <input type="checkbox"/> Industrie <input type="checkbox"/> Ecole hôte	Etudiant / Student <b>Laetitia Roh</b>	Lieu d'exécution / Ausführungsort <input type="checkbox"/> HES—SO Valais <input type="checkbox"/> Industrie <input checked="" type="checkbox"/> Ecole hôte
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# ABSTRACT



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[-2011-]

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Role of the Bcl-2 family member Bok in proliferation or cell cycle control

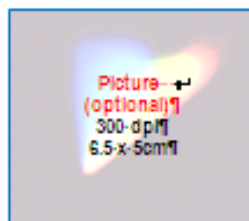
Graduate → Bok-Deletion

## Objectives

Bok, a protein of the Bcl-2 family, belongs to the pro-apoptotic group. Its exact role is unknown but may be open new ways for cancer therapy. The aim of this study is to define the function of Bok in proliferation or cell cycle control.

## Methods-Experiences-Results

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## ABREVIATIONS

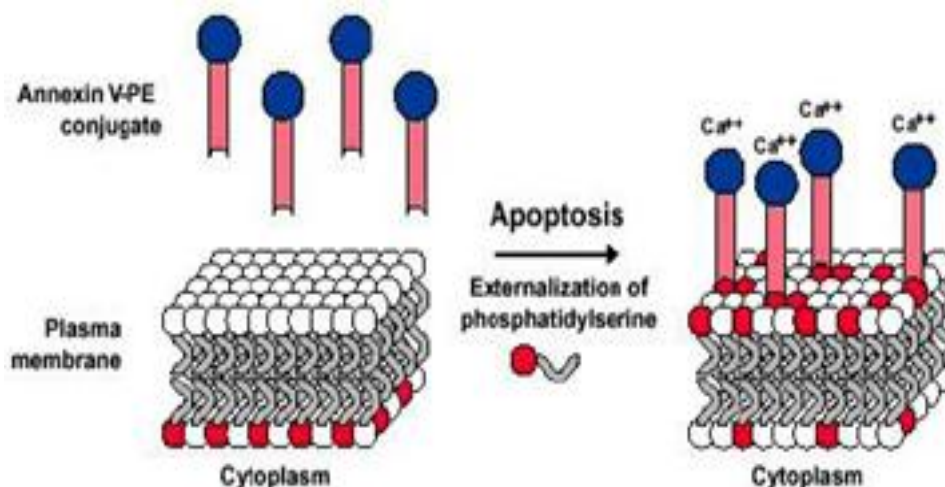
Ab	Antibody
APS	Ammonium persulfate
Bcl-2	B cell lymphoma 2
BCA	Bicinchoninic acid
BCR	B cell receptor
bp	basepair
BSA	Bovin serum albumin
CD	Cluster of differentiation
ConA	Concanavalin A
ER	Endoplasmatic reticulum
FACS	Fluorescence activated cell sorting
FCS	Fœtal calf serum
FITC	Fluoresceine isothiocyanate
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
kDa	Kilo Dalton
kHz	Kilo Herz
L-Asn	L-Asparagin
LEAF	low endotoxin, azide free
LPS	Lipopolysaccharide
MEF	Mouse embryo fibroblast
MW	Molecular weight
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
P/S	Penicillin & Streptomycin
PS	Phosphatidylserine
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
WT	Wildtype
2ME	2-Mercaptoethanol

# 1 INTRODUCTION

## 1.1 Apoptosis in general

The term apoptosis comes from the Greek and means "falling leaves of a tree". This natural and physiological phenomenon is essential for the proper functioning of a living organism. It is found both during embryogenesis and in the regression of the tail of the young frog. The mechanism of apoptosis also governs the cell death in order to shape the fingers and feet. It is also responsible of the elimination of nearly 95% of immature immune cells during T cell development in the thymus. [1] In other cases, aberrant regulation of apoptosis can result in a pathological event. When the balance between cell proliferation and apoptosis is broken, the disruption of the cell can lead to either an overactive apoptosis (autoimmune diseases, e.g. type I diabetes, neurodegenerative diseases like Alzheimer, Huntington's, Parkinson's) or conversely to an inhibited apoptotic activity (cancers, autoimmune diseases...).

The mechanism of apoptosis, also called programmed cell death, results in a cascade of enzymatic reactions that leads to physiological changes in cells such as decrease and condensation of the nucleus and cytoplasm, fragmentation of DNA. The cytoplasm is filled with vesicles and surface deformation is observed. [2] Another feature is observed on a microscopic level on the surface of the membrane, as phosphatidylserine (PS), a lipid initially present on the inner side of cell membrane, is expressed on the cell surface (flip-flop phenomenon). This gives possibility to differentiate apoptotic cells and healthy cells by labelling with Annexin V (Figure 1), a protein binding to PS. [1] At this stage, *in vivo*, the dead cell is phagocytosed by macrophages. Moreover, unlike necrosis, the cell membranes of apoptotic cells do not get ruptured, causing no damage to surrounding healthy cells and no inflammation.



**Figure 1 : During apoptosis, phosphatidylserine (PS) externalization is observed (represented here with red head) Annexin V which has affinity for PS can bind to them. By microscopy or flow cytometry, apoptotic cells can thus be easily differentiated from viable cells [3]**

Currently, two distinct pathways (Figure 2) are known to drive a cell to caspase activation, which are the key effector proteases of apoptosis. Importantly, both pathways culminate in the activation of the same set of effector caspases:

- ✱ The intrinsic pathway, also known as mitochondrial or stress-induced pathway. It is governed primarily by the proteins of the Bcl-2 family which regulates the permeability of the outer membrane of mitochondria. It is triggered by many cytotoxic factors such as viral infections, DNA damage and growth-factor deprivation.[4]
- ✱ The second possibility is the extrinsic pathway or death receptor pathway. In this case, a ligand binds so called death receptors on the cell surface, which then triggers the apoptotic cascade.

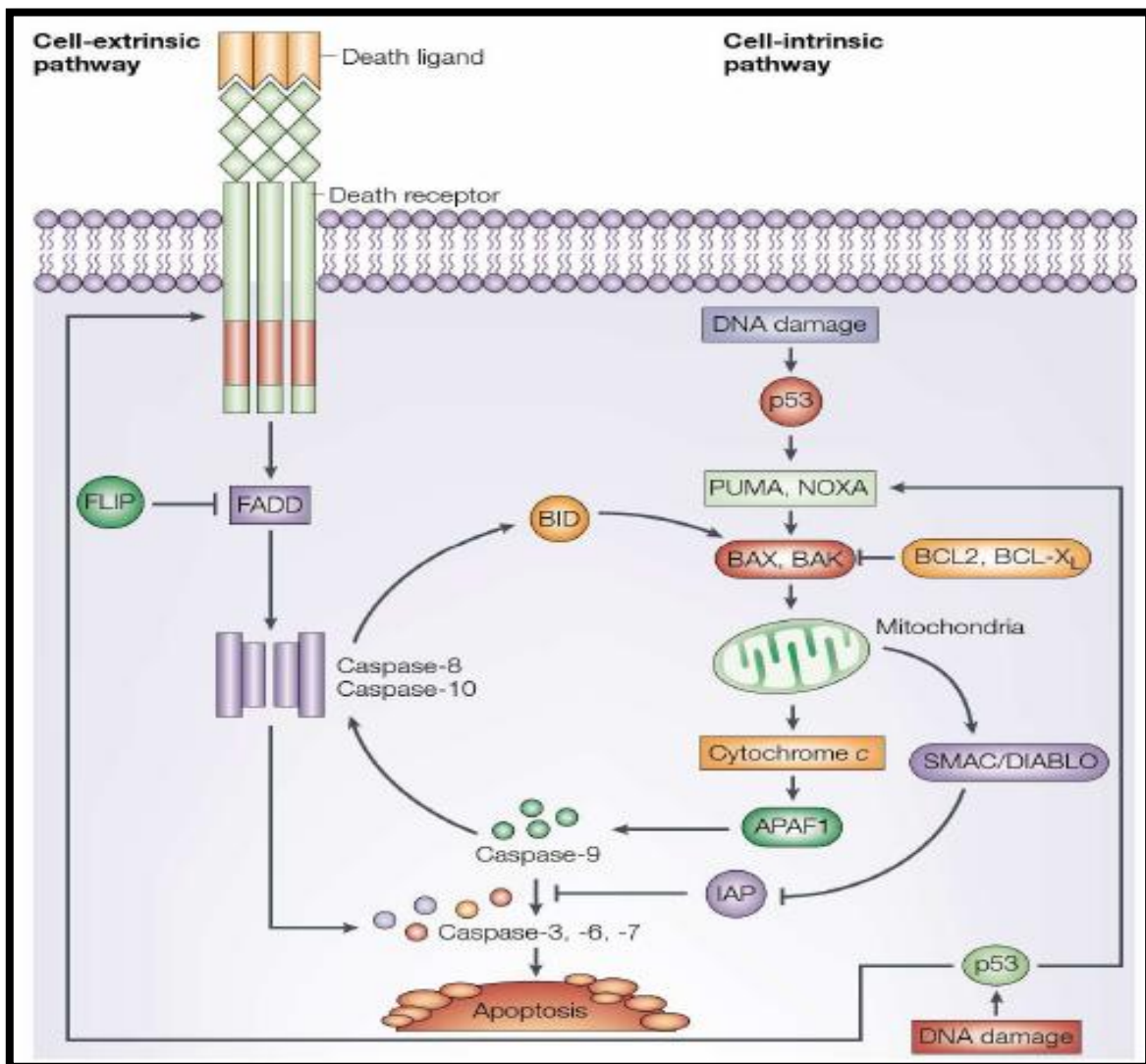


Figure 2 : Schema of different possible pathways in apoptosis (intrinsic and extrinsic pathways) [5]

## 1.2 The Bcl-2 family

The proto-oncogene Bcl-2 was identified for the first time in a follicular lymphoma type B with a translocation t(14;18). This protein family consists of at least 20 members. It is characterized by structures called Bcl-2 homology domain (BH) and each of them has at least one. In addition, most have a transmembrane domain to span the outer membrane of mitochondria (OMM), and thus regulate cell death. Bcl-2 proteins can also be found on the membrane of endoplasmatic reticulum (ER) and even on the external nuclear membrane.

A classification of the proteins was carried out according to their role in apoptosis. Activation of the pro-apoptotic proteins (e.g. Bax, Bak, Bok, Bad, Bid and Bim) induces the permeabilization of the OMM, drop in mitochondrial membrane potential and releases of the cytochrome c into the cytosol. Cyt. c is needed to trigger the apoptotic cascade. The anti-apoptotic (e.g. Bcl-2, Bcl-xL, Mcl-1, A1 and Bcl-2) are antagonists which directly neutralize the action of pro-apoptotic Bcl-2 family members and strongly inhibit apoptosis when over expressed. This is the reason why many chemotherapy resistant cancer cells heavily over express anti-apoptotic Bcl-2 proteins. [6][7][8]

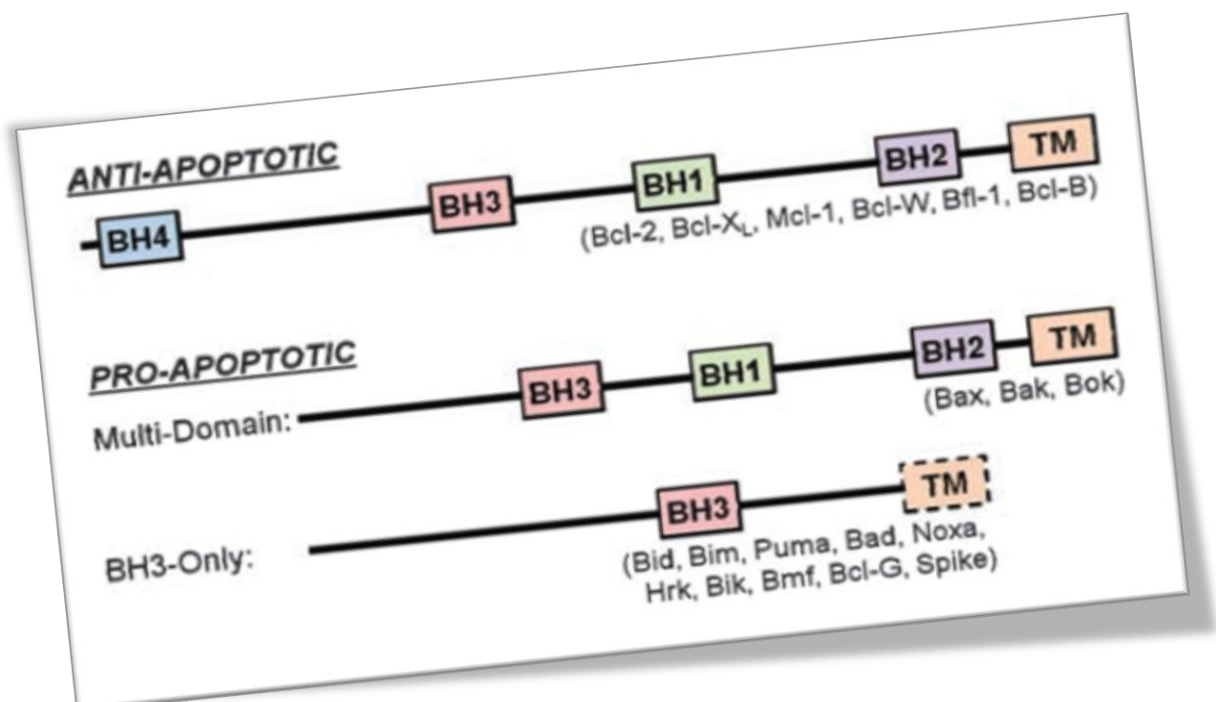
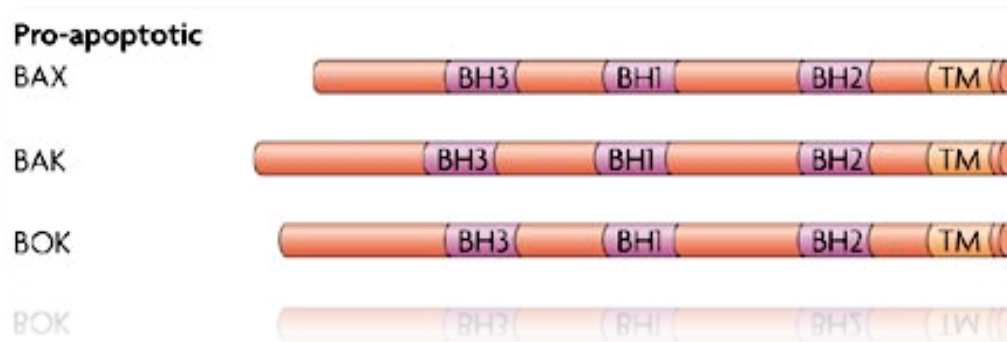


Figure 3 : Structure of the two different groups (anti-apoptotic and pro-apoptotic proteins) of Bcl-2 family [9]

### 1.3 Bok

*Bok* belongs to the family of proteins Bcl-2 and especially of the pro-apoptotic group. Its structure contains three Bcl-2 homology domains (BH1-3) necessary for the dimerization of the protein and a C-terminal transmembrane region which allows anchorage to the ER and mitochondrial membranes. [10]



**Figure 4 : Structure of pro-apoptotic proteins of the Bcl-2 family [11]**

Thus Bok looks very similar to Bax and Bak, two other members of this subgroup (Figure 4). Originally reported to be expressed mainly in the reproductive tissues (ovary, testis and uterus) [10], it is now clear that Bok is expressed in most tissues and cell type. However, its exact role is unknown. Indeed, recently discovered, much research is currently underway to determine precisely its function in the cell. This analysis is now facilitated by a recently developed *bok*-deficient mouse model (Kaufmann & Strasser, submitted).

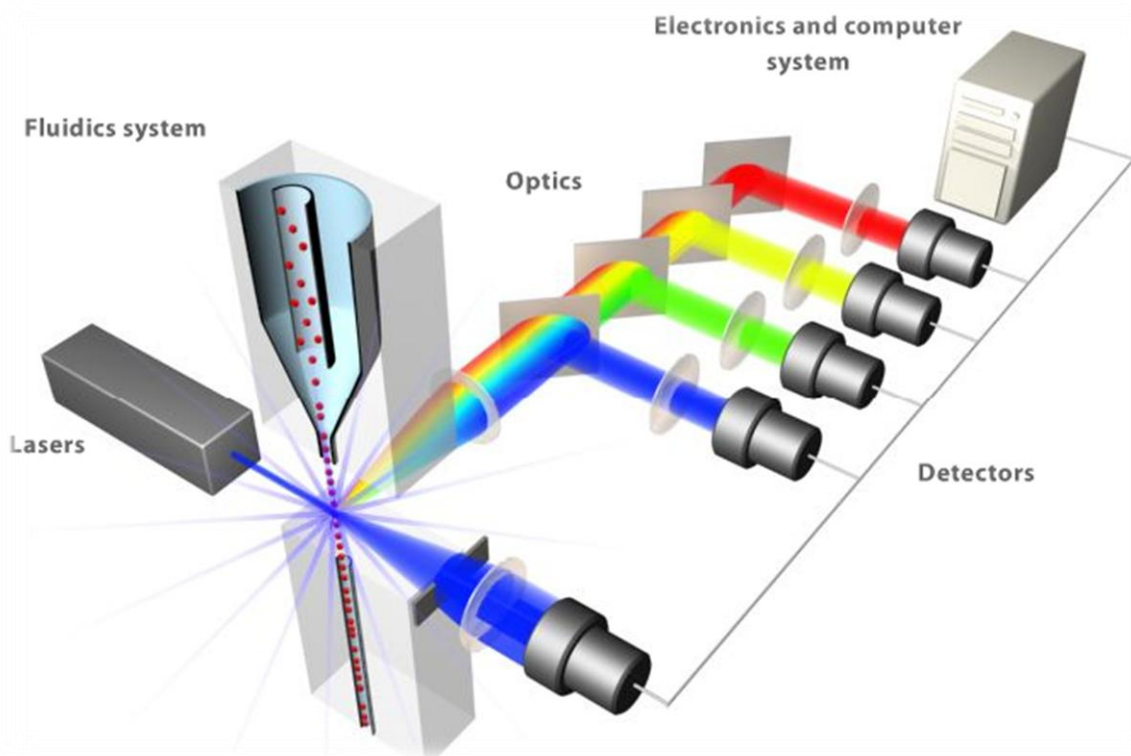
## 1.4 Flow cytometry (FACS)



**Figure 5 : Picture of the flow cytometry system (FacsCalibur, BD Biosciences)**

Flow cytometry (Figure 5) is an analytical method widely used in biomedical research. At the forefront of technology, it allows the detection of cells with monoclonal antibodies coupled to fluorochromes (Appendix 7). The antibody bound to the antigen of the cell of interest will issue a subsequent fluorescence to a specific wavelength in response to an excitation laser. For optimum performance, this unit was designed in 3 main parts. (Figure 6)

A fluid system transports the suspension of labeled cells in a liquid stream prior to the light source. The cells are then excited by the laser (optical part). Therefore, the cells emit fluorescence in turn corresponds to the wavelength of the fluorochromes attached to the surface. Converted into electronic signals and then digitized by the electronic system, they can be analyzed graphically by the operator [12].



**Figure 6 : Schema of the three main parts constituting flow cytometer: fluidic, optic and electronic system [13]**

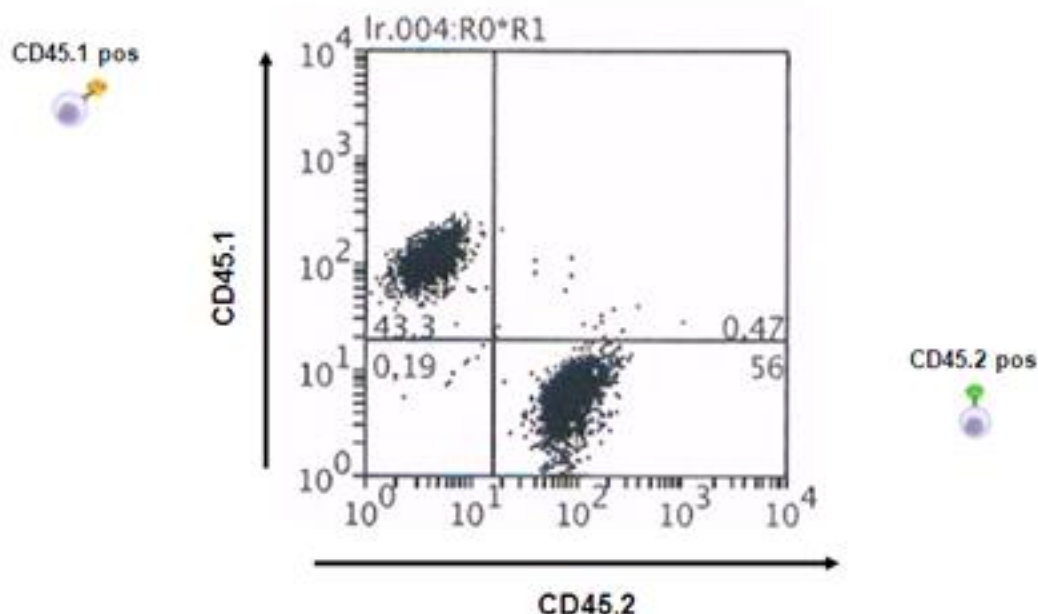
This instrument has a wide field of action. It confers many significant advantages such as the quantification of DNA content and used primarily in cancer's cytogenetic [14]. It is particularly characterized by its scanning speed average of 1000 cells per second depending on the model and its versatility allows you to work simultaneously on several parameters of a population while analyzing the cells individually. Moreover, its detection sensitivity and statistical significance of counting made him a major advantage over conventional optical microscopy.

## APPLICATION

Many applications of flow cytometry are now used in biomedical research, such as characterization of cellular subsets by cells surface staining, DNA count to determine the distribution of cell cycle phases, determination of cell viability with Annexin-V/PI or determining total cell numbers by the use of FACS reference beads. Each of the methods mentioned allows a different analysis of the cells on the same instrument.

### Surface staining

The surface staining allows the identification and distinction of cell types among the most used in the biomedical field. As each cell has its own set of surface antigens staining with specific monoclonal antibodies can identify those by FACS analysis. For example, monitoring of the blood of HIV patient, where a drop in T helper cells ( $CD4^+$  T cells) is observed, is one of the applications. In this practical work, this application will be particularly used to differentiate the CD45.1 and CD45.2 loci in the experiment of mast cells competition. (Figure 7)



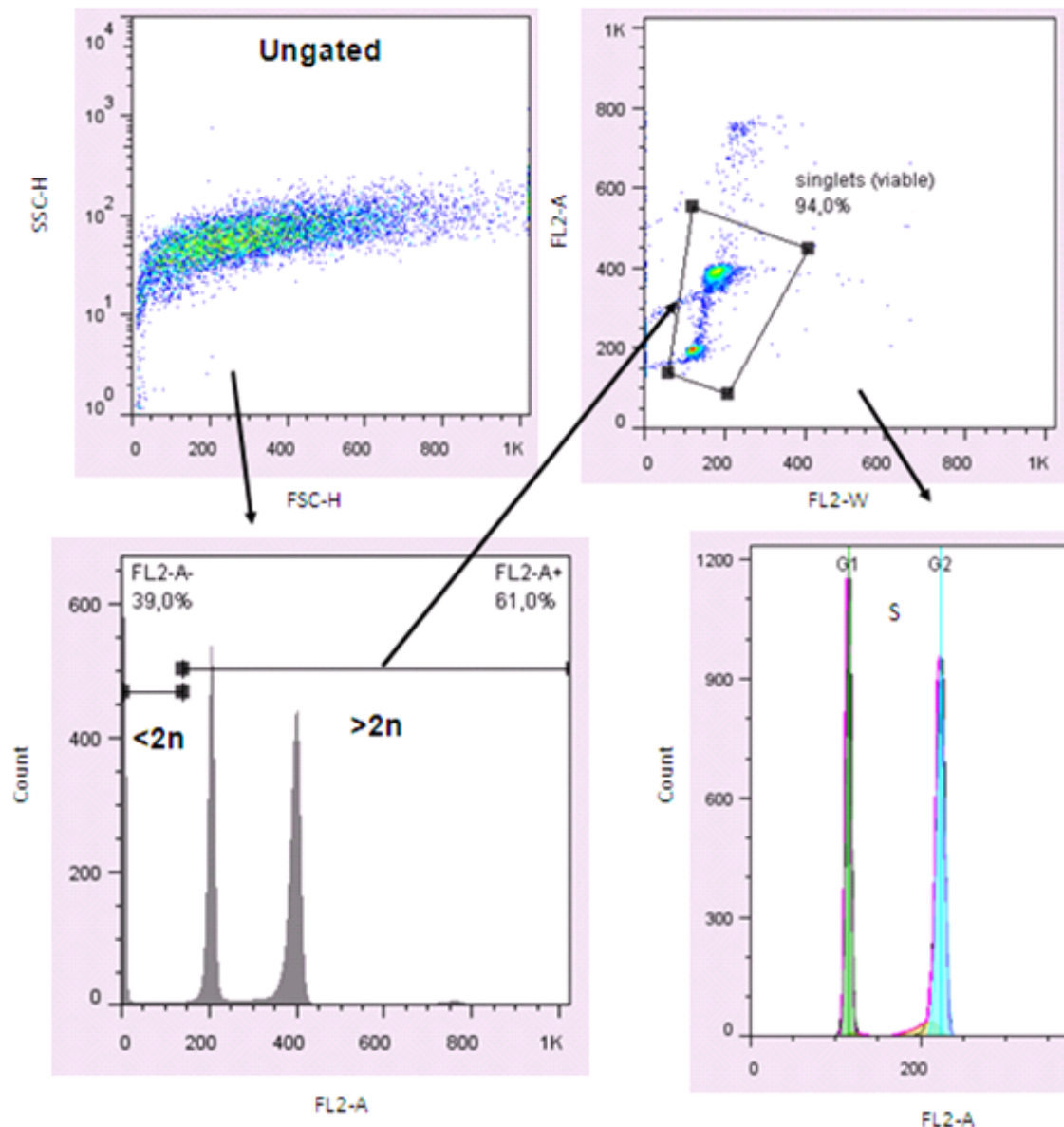
**Figure 7 : Scattergram (also called dot plot) representing mast cells competition results. At T0, 56% of viable cells are CD45.2 positive (using FITC-labeled anti-CD45.2), 43.3% are CD45.1 positive (using PE-labeled anti-CD45.1).**



### ***Cell cycle and DNA damage***

During cell renewal or development of a living organism, a whole series of steps must be respected to allow optimal cell division. The cell cycle also called generation time is the time taken to proceed from one cell division to the next [15]. The eukaryotic cell cycle can be divided in two main parts: Interphase and mitosis (M phase). Interphase is composed of three sub phases: G1, S and G2 whereas in the mitosis phase, duplicated chromosomes are segregated to give rise to two daughter cells. During the G1 phase, cells prepare intracellular components for the replication of the genome that will take place during the S phase. The same happens in the G2 phase to allow the cell to enter the M phase.

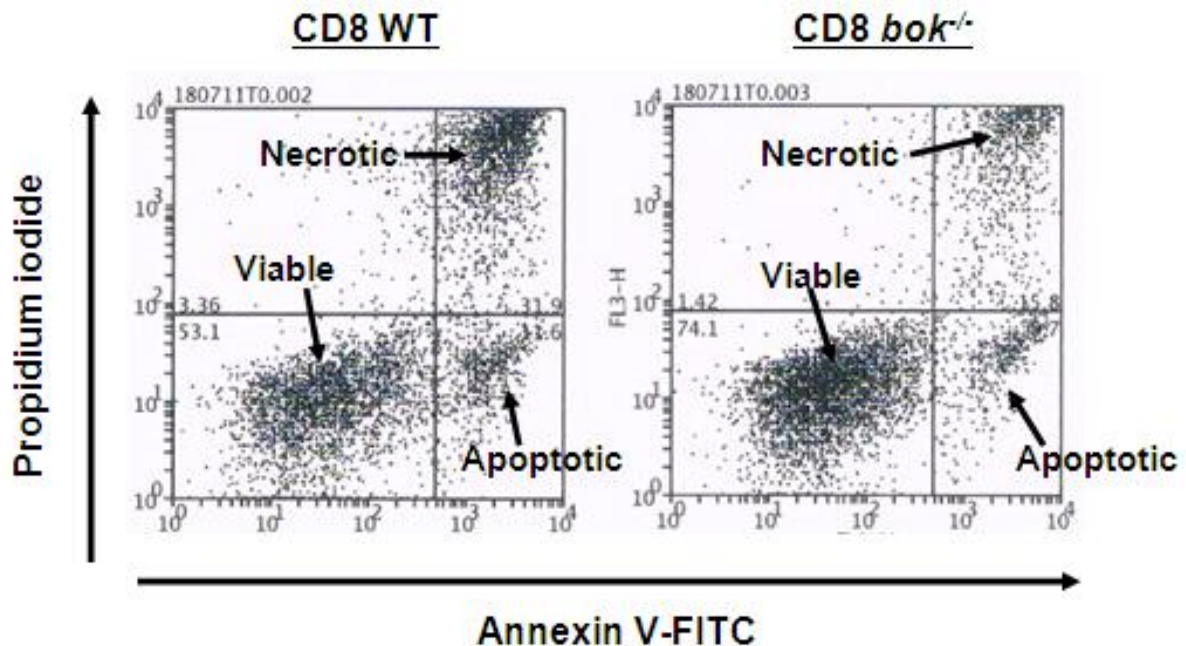
The amount of intracellular DNA can be used to accurately quantify the proportion of cells in each stage of the cell cycle. After the rupture of the outer cell membrane by the addition of a hypotonic buffer (as well as digestion of all RNA entities with RNase A, thereby reducing background noise), the detection of DNA is possible with propidium iodide, an intercalating agent that has particular affinity for DNA. Flow cytometry supports this kind of analysis and fluorescence is directly proportional to the amount of genomic DNA. Results can be plotted as a histogram (Figure 8). Furthermore, this method can also be used to quantify the percentage of dead cells, as their fragmented genomic DNA will be found in the subG1 peak ( $< 2N$ ) in a histogram.



**Figure 8 :** Example of histogram depicting phases of the cell cycle. This graph is realized with the FlowJo software. Mast cells were incubated during 72h in the presence of 1 $\mu$ g/mL etoposide. The first graph represents ungated cells before separating cells in two groups with less than 2n or more or equal to 2n chromosome content. Viable cells are then gated and finally histogram with 3 phases (G1, S and G2/M) is shown on histogram.

### Cells survival

In addition, the work of basic research is much facilitated by the differentiation between viable, early apoptotic and late apoptotic (secondary necrotic) cells. Cells are stained with Annexin V and propidium iodide, allowing the identification of the state of life or death of the cell. The result after flow cytometry is expressed in form of a scattergram (also called dot plot), as shown in Figure 9.



**Figure 9 : AnnexinV/PI scattergram of CD8 lymphocytes after cultivation in the absence of cytokines for 24 hours**

A recent study has shown, however, the limitations related to the Annexin V / PI double staining. Indeed, an overestimation of the number of apoptotic cells is possible if the binding of fluorochrome on double-stranded RNA rather than DNA fragmentation. To avoid this disagreement, pretreatment with RNase is highly recommended. [16]

### **Counting cells with reference beads**

This method determines directly the absolute count of any leucocytes population with low variability. A defined number of polystyrene FITC-labeled FACS reference beads is added to the sample containing cells of interest. Besides FITC-labeling the beads are very small and have a high side scatter profile, making it easy to distinguish them from cells. After analysis by flow cytometry, a ratio between the number of viable cells counted (PI exclusion) and the number of beads added will be calculated. In this example (Figure 10), the total number of cells in sample is calculated like this:

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Number of beads per well	:	8'000
Number of counted cells	:	4082
Number of counted beads	:	560

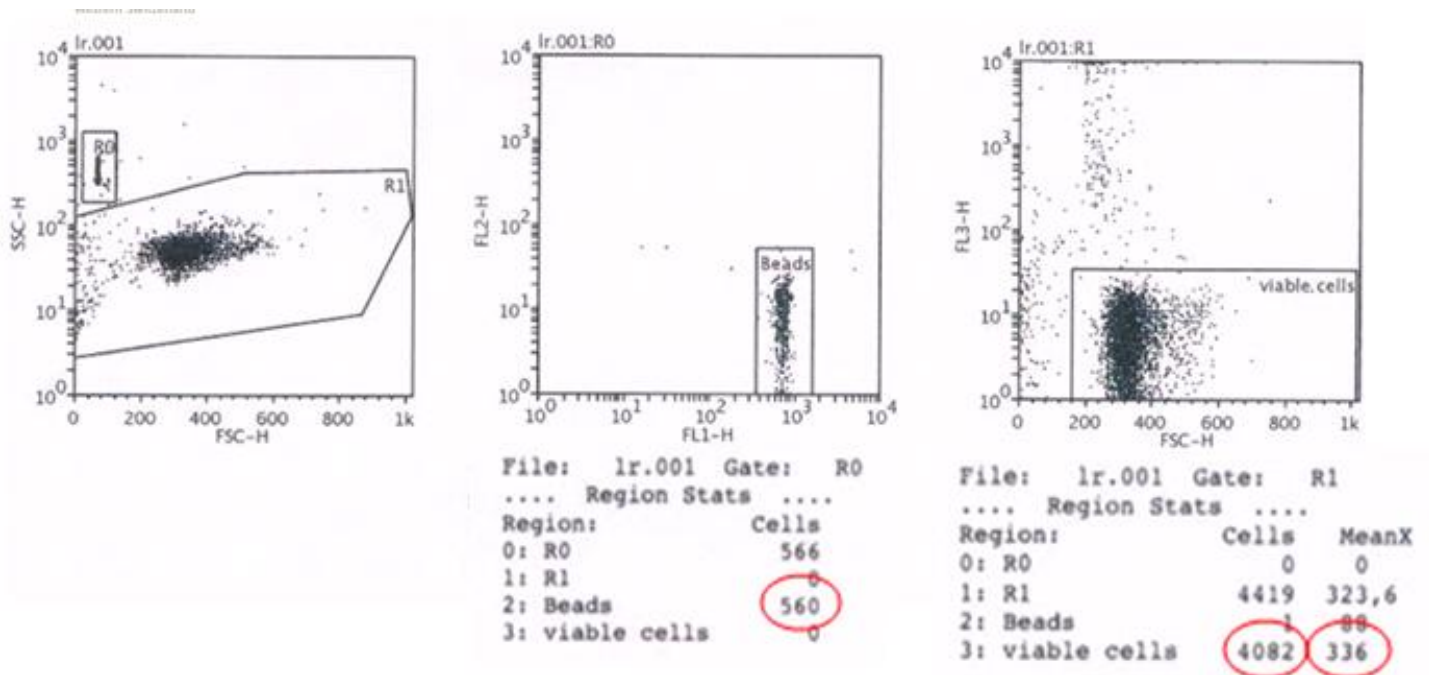
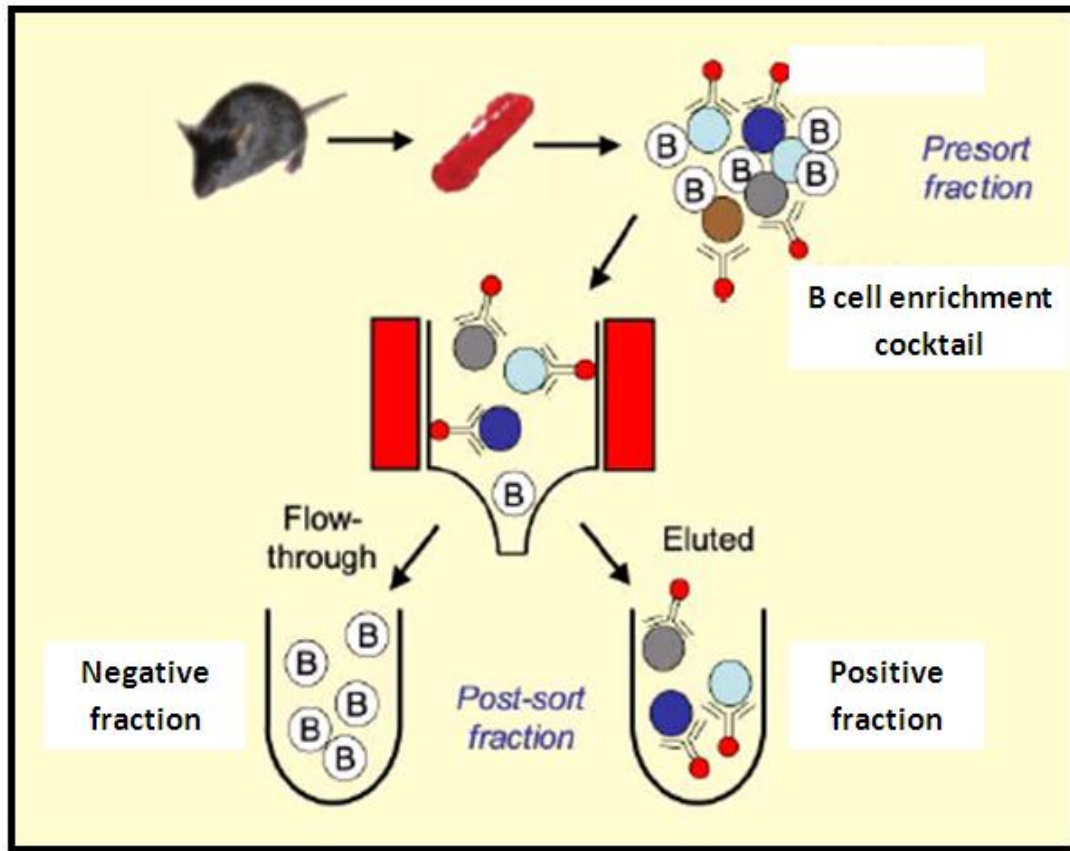


Figure 10 : Example of FACS analysis with reference beads for WT CD4 cells. Data were analyzed by Weasel 1.2.2 software. R0 gating represents reference beads and R1 viable cells.

### Cells isolation

Cell separation can be performed by FACS using a FACS sorter. Cell separation at the output of the controller is done with purity levels exceeding 99% but this application is slow (one hour to sort 10 million cells) and requires some experience. In this work, separations with magnetic beads are used. This is a newer and cheaper technique based on the conjugation of the ligand (e.g. antibody) to magnetic particles. [17] An antibody attached to the surface of magnetic beads allows labeling and magnetic separation of wanted (Figure 11). Thanks to the magnetism principle, the beads coupled to the cells (positive fraction) will be retained on the tube wall and thus recovered after removing the remaining solution containing the cells constituting the so-called negative fraction.



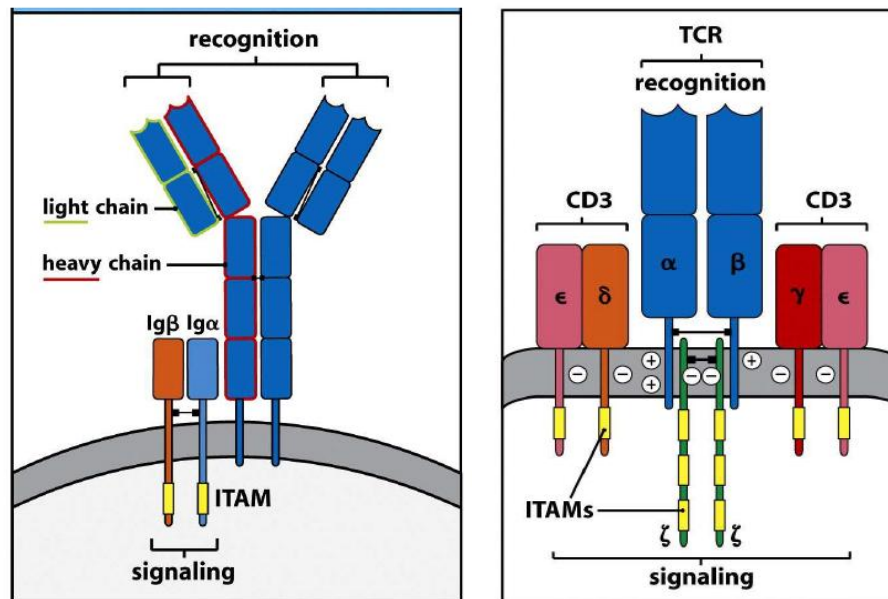
**Figure 11 : Schematic representation of the immunomagnetic purification of B by negative selection. After splenectomy, cells were washed and incubated with a cocktail of antibodies labeling all splenocytes except B cells. Since the B cells do not bind any of the antibodies present in the cocktail, they will not be retained on the magnetic wall and will be present in the negative fraction highly enriched for B cells. [18]**

This application can for example be used for allo- and autografts when it comes to selecting hematopoietic progenitors expressing the CD34 surface molecule which is the marker of stem cells [19]. In this work, it allows to obtain pure CD4<sup>+</sup> or CD8<sup>+</sup> T cell fractions (positive sort using an anti-CD4 or anti-CD8 antibody, respectively) and to obtain purified B cells by negative selection, all directly from the mouse spleen. According to the manufacturer (BD Biosciences), a purity of about 98% is obtained for the positive fraction and > 90% for the negative fraction.

This system is inexpensive and relatively simple. It still requires a long process of purification of the initial mixture. To cite just one step, the lysis of red blood cells is one of the most critical steps for the proper functioning of the assay. In addition and if possible, negative sorts (labeling and eliminating all the unwanted cells) is preferable to positive sorts, where the wanted cells are more manipulated by labeling with antibodies, which may trigger activation of cells or increased cell death.

## 1.5 Activation of T and B cells

T and B lymphocytes are part of the leukocytes, immune cells present in all mammals. They play a role in host defense. Membrane structure and particularly their receptor called T cell receptor (TCR) for T cells and B Cell Receptor (BCR) in B cells can recognize antigens. This characteristic allows the activation of these cells *in vivo* and *in vitro* with antigens or other activating factors.



**Figure 12 : B cell receptor structure with two co-receptor Igα and Igβ (left picture) and T cell Receptor structure with signaling complexe (CD3, dimere Zeta). ITAM: Immunoreceptor tyrosine-based Activation Motif [20]**

Activation of resting B cells and T cells leads to highly stimulated and proliferating cells, called B or T lymphoblasts. A net increase in their cytoplasmic contents as well as in overall cellular size can be observed. To activate T cells, concanavalin A (Con A) extracted from Jack Beans can be used and shows spectacular results for CD4<sup>+</sup> and CD8<sup>+</sup> T cells after 72 hours already. Stimulation with precoated anti-CD3 and a mix of anti-CD3/CD28 antibodies also give great results but is much more expensive than ConA. T cell blasts heavily depend on the cytokine Interleukin 2 (IL-2) for growth. Activated B cells need IL-2, IL-4 and IL-5 and are generally more difficult to cultivate *in vitro* than T cells. We can activate B cells using bacterial lipopolysaccharide (LPS) which is also necessary for growth and proliferation of these cells, probably due to its non-specific mitogenic power that drive them to the cells division rather than cells differentiation. [21]



## 2 AIM OF THE BACHELOR THESIS

Bok is a member of the Bcl-2 family and therein is part of the pro-apoptotic subgroup. Newly discovered, its exact role and function in apoptosis or other biological processes remain largely unknown. Besides its clear roles in regulating cell death, Bok has also been proposed to regulate cell cycle arrest and proliferation; but those experiments were only carried out in human cancer cell lines. Using *bok*-deficient cells, i.e. cells derived from a genetically manipulated mouse lacking the *bok* gene, it will help to understanding the mechanisms of Bok function. The following experiments, carried out during 16 weeks of this Bachelor thesis, are structured around the following main points

### ***Colony forming assay***

The aim of this experiment is to look for long-term protective effects in *bok*<sup>-/-</sup> MEFs compared to wildtype controls upon addition of apoptotic stress stimuli (DNA damage or replicative stress).

### ***Direct competition growth assay in bone marrow derived mast cells***

According to previous data in the Kaufmann lab *bok*<sup>-/-</sup> mast cells grow slower than WT cells when cultured *in vitro* (in separate wells). Now, experiments will be performed with respect to cell growth and cell survival if WT and *bok*<sup>-/-</sup> are mixed together in the same wells.

### ***T and B cell work***

The differences in activation *in vitro*, as measured by increase in cell size modification and fold increase in total cell number, will be compared between *bok*-deficient and WT CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and B cells.. Furthermore, differences in cell cycle arrest upon DNA damage will be evaluated by FACS analysis, (Hypo PI profiling) and anti-Bok Western blotting of activated T and B cells performed.



### 3 MATERIALS AND METHODS

Methods used for all these experiments are described in Appendix 1. During this project, cells were isolated from organs dissected from 8 to 14 weeks old male or female C57BL/6 WT and *bok*-deficient (*bok*<sup>-/-</sup>) mice. They were housed in individually ventilated cages (IVC) at the 'Zentrale Tierställe' of the Inselspital (Bern, CH). Regarding statistical analysis, the results are represented as mean +/- standard deviation (SD). Values from different experiments were compared using the Student's t test. Significance was defined for p values <0.05.

#### 3.1 Media

##### **Complete DMEM Medium**

DMEM/Glutamax 4.5 g/L D-glucose with Pyruvate (Invitrogen), supplemented with 5% fetal calf serum (FCS, PAA Clone) and 1% Penicillin/Streptomycin (100x solution, Invitrogen)

##### **FMA Medium**

RPMI 1640 + glutaMAX<sup>TM</sup>-1 (Invitrogen) supplemented with 10% fetal calf serum (FCS) (PAA Clone), 1% Penicillin/Streptomycin (P/S) (100x solution, Invitrogen), 50µM 2-Mercaptoethanol (5000x stock (250mM), Sigma) and 250µM L-Asparagin (100x stock (25mM), Sigma)

##### **Freezing Medium**

90% fetal calf serum (FCS, PAA Clone) + 10% DMSO (Sigma)

##### **RPMI 1640 Medium**

RPMI 1640 + glutaMAX<sup>TM</sup> Medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, PAA Clone), 1% Penicillin/Streptomycin (P/S) (100x solution, Invitrogen), 50µM 2-Mercaptoethanol (Sigma) and 10% of IL-3 conditioned WEHI-3B medium as a source of murine IL3.

## 3.2 PCR

### Cells

Tail biopsies from two C57BL/6 and one *bok*<sup>-/-</sup> mouse were collected from sacrificed animals.

### Genomic DNA extraction

Genomic DNA was purified from mouse tail biopsies using 500 µl of tail lyses buffer and 25 µl of Proteinase K (20 mg/ml; Roche) to digest them overnight at 56°C in an Eppendorf thermomixer at 650rpm. The digest was centrifuged at 13000rpm during 5 minutes and 450 µl of the cleared supernatant transferred to a new Eppendorf tube. Genomic DNA was precipitated by addition of 500µl isopropanol (Sigma) and subsequently centrifuged at 13000rpm for 15 minutes. The DNA pellet was washed once with 500µl of 70% ethanol (Merck/Grogg) and let to dry upside down. The dry DNA pellets were resuspended in 150µl of TE Buffer (pH 8.0) by incubated at 37°C during 30 minutes or overnight at room temperature. Before using samples for PCR reaction, genomic DNA was well vortexed.

### *Bok*<sup>-/-</sup> genotyping using a 3-primer PCR based reaction

1 µL of purified genomic DNA template was used per reaction. Note that the 'Green Mix' is ready to use and contains everything required for PCR apart from the DNA template and the primers (i.e buffer, dNTP mix, Taq polymerase), and further already includes the loading buffer/dye for subsequent agarose gel electrophoresis.

### Bok (del535) Genotyping Primers

- ***bok* 17 (*bok*5's)**

5' cgggtttgaatggaagggtc      20mer, %GC:55%, T<sub>m</sub>=54°C

- ***bok* 18 (*bok*5'as)**

5' tgttcccatgggtgctacatcc      21mer, %GC:52.4%, T<sub>m</sub>=54°C

- ***bok* 20 (*bok*3'as)**

5' gagctagctagctatgtgtg      20mer, %GC: 50%, T<sub>m</sub>=52°C

*Figure 13 : Structure of the primers used for genotyping*

The master mix was prepared as follows:

**Table 1 : Mastermix for 7 PCR reactions for a final volume of 25 $\mu$ l**

<b>Reagents</b>	<b>Reagents volume for 1 reaction [<math>\mu</math>l]</b>	<b>Reagents volume for 7 reaction [<math>\mu</math>l]</b>
<b>Green Mix</b>	20	140
<b>Primer bok 17 [10<math>\mu</math>M]</b>	2	14
<b>Primer bok 18 [10<math>\mu</math>M]</b>	1	7
<b>Primer bok 20 [10<math>\mu</math>M]</b>	1	7

All amplification reactions were carried out in a total volume of 25  $\mu$ L. The cycling parameters for PCR were as follows: a preincubation step at 94°C for 2 minutes then 35 cycles were performed as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds. PCR products were stored at 4°C until analysis. A negative sample called negative control was included by replacing the DNA template by water. Three positive controls were added to the samples to confirm the location of bands on the gel. The size of the WT allele is 150 bp and the size of the mutated (deleted) *bok* allele of 383 bp.

### ***Analysis of the amplification product***

The DNA fragments obtained following PCR were analyzed by agarose gel electrophoresis. For direct analysis of the fragments, the amplified PCR products were loaded on a 2 % agarose gels. Electrophoresis was performed in TAE buffer for about 2 hours at 80V, and the DNA was visualized on an ultraviolet transilluminator following RedSafe<sup>TM</sup> (IntRON biotechnology) staining. The size of the amplified fragments was estimated by comparison to a 100 bp ladder marker set (Fermentas).

### ***Reagents***

- |   |                      |
|---|----------------------|
| • Agarose                                     | Sigma Aldrich        |
| • Ethanol 100%                                | Sigma Aldrich        |
| • Isopropanol 100%                            | Sigma Aldrich        |
| • GeneRuler 100pb DNA Ladder PLUS, SM0321/2/3 | Fermentas            |
| • Primers TK bok 17                           | Sigma Aldrich        |
| • Primers TK bok 18                           | Sigma Aldrich        |
| • Primers TK bok 20                           | Sigma Aldrich        |
| • Proteinase K , stock 10mg/mL                | Roche                |
| • RedSafe <sup>TM</sup>                       | IntRON biotechnology |

### 3.3 Colony forming assay in SV40 Large T antigen (LT) immortalized mouse embryo fibroblasts (MEF)

#### Cells

Multiple independent SV40 LT Ag immortalized MEF lines; each derived from a different embryo: 4 for WT (E4, E5, E6 and CB) and 3 for *bok*<sup>-/-</sup> (E2, E5 and E9).

#### Experiment

1x10<sup>3</sup> mouse embryo fibroblasts were seeded per well in 6-well culture plate (CellStar®, Greiner Bio-one) in a volume of 2mL complete DMEM Medium. After a minimum of 2 hours to let the cells attach to the plates, apoptotic stress stimuli were added, including endoplasmatic reticulum-stress inducers Tunicamycin (Enzo LifeSciences), Thapsigargin (Enzo LifeSciences) and Brefeldin A (Sigma Aldrich), as well as the DNA damage inducing agents Etoposide (Ebewe, Pharmacy Inselspital), hydroxyurea (Sigma Aldrich) and irradiation with UVB (UVB Stratalinker 1800, Stratagene). After 7 to 8 days of incubation at 37°C for primary MEF and 6 to 7 days for SV40 MEF, cells were washed with PBS (pH 7.4), fixed with 1mL cold ethanol 70% (Sigma Aldrich) for 5 minutes on ice and then stained with 2mL of a 0.1% Crystal Violet solution (Sigma Aldrich) under slow agitation. Finally cells were washed with 1mL ddH<sub>2</sub>O and air-dried. Counting of cells was realized manually upon a white light table and the following formula was used to calculate percentage of cells:

---

*Equation 1*

#### Reagents

- |                       |                             |
|-----------------------|-----------------------------|
| • Brefeldin A 5mg/mL  | Sigma                       |
| • Crystal violet      | Sigma Aldrich               |
| • Gentamicin          | Sigma                       |
| • Ethanol 100%:       | Sigma Aldrich               |
| • Etoposide 20mg/mL   | Ebewe, Pharmacy Inselspital |
| • Hydroxyurea 500mM   | Sigma Aldrich               |
| • Dulbecco's PBS 1x   | PAA Laboratories GmbH       |
| • Thapsigargin 1mM    | Enzo LifeSciences           |
| • Trypsin-EDTA 1x     | Invitrogen                  |
| • Tunicamycin 10mg/mL | Enzo LifeSciences           |

### 3.4 Direct competition growth of bone marrow derived mast cells

#### Cells

Three mast cells lines were differentiated *ex vivo* from total bone marrow isolated from the femur of C57BL/6 WT (CD45.1), WT (CD45.2) and *bok*<sup>-/-</sup> (CD45.2) mice. Aliquots of primary bone marrow were frozen in liquid nitrogen at -196°C in 1mL of Freezing Media (90% FCS + 10% DMSO) for future use.

#### Cultivation of mast cells

Bone Marrow cells were quickly thawed, washed once in prewarmed PBS and plated at a density of  $0.5-1 \times 10^6$  cells/ml in RPMI1640 Medium (Invitrogen). Fresh medium was added weekly for 3 weeks and suspension cells placed in new wells in order to eliminate unwanted adherent cells. After 4 weeks of culture, 10% of stem cell factor (SCF) conditioned medium (derived from CHO/muSCF cell line) was added to the culture to increase proliferation of mast cells. Cells were subsequently maintained in complete RPMI1640 medium plus 10% of IL-3 and 10% SCF. Established mast cell lines could be used for experiments approx 4 weeks before they stopped to grow.

#### Experiment

All results are derived from cells that had been in culture for less than 4 weeks after full maturation of mast cells. 1:1 Mixes between  $2.5 \times 10^4$  WT(CD45.1) and  $2.5 \times 10^4$  WT(CD45.2) mast cells as well as  $2.5 \times 10^4$  WT(CD45.1) and  $2.5 \times 10^4$  *bok*<sup>-/-</sup> (CD45.2) mast cells were prepared in 48-well plates (CellStar®, Greiner Bio-one), in a total volume of 1 mL Limiting concentrations of cytokines, i.e. 10%, 1% and 0.1% of each, IL3 and SCF were tested. The percentages of CD45.1- and CD45.2-positive cells were quantified over time by flow cytometry (FACS).

#### Flow cytometric analysis

At each time point (including t=0 h), the cells were transferred to a 5mL FACS tube, topped with FACS Buffer (buffered saline solution + 2% FCS + 10mM NaN<sub>3</sub>), spun at 1500 rpm for 5 min at 4°C and decanted. The cells were then stained in a total volume of 100 µl (20 min on ice, in the dark) with an anti-CD45.1-PE (1/500) and an anti-CD45.2-FITC (1/500) antibody, diluted in FACS Buffer + 1% normal rat serum (NRE) + 10% hybridoma supernatant of FcγRII/III blocking antibody (antiCD16/CD32, clone 2.4G2)). Cells were washed with FACS buffer and resuspended in 200µl of FACS buffer plus 2 µg/mL propidium iodide (PI, Sigma Aldrich) and analyzed on a flow cytometer (BD FACS Calibur™). Data were analyzed by Weasel 1.2.2 software.

#### Reagents

- |  |  |
|--|--|
| • Ab mouse anti-CD45.1-PE, Clone A20, 0.20 mg/mL   | Biologend  |
| • Ab mouse anti-CD45.2-FITC, Clone 104, 0.50 mg/mL | Biologend  |
| • Normal Rat serum (NRS)                           | Promocell  |
| • Propidium iodide (PI), stock 500µg/mL            | Sigma Aldrich                                    |
| • Blocking antibody Anti-FcγRII/III                | HB-197 hybridoma cell supernatant<br>Clone 2.4G2 |
| • SCF  | CHO/muSCF conditioned supernatant                |



### 3.5 Mast cells HYPO PI cell cycle analysis

#### **Cells**

Mast cells were differentiated *ex vivo* from murine bone marrow as described earlier. Three mast cell lines were used for this experiment: WT CD45.1, WT CD45.2 and *bok*<sup>-/-</sup> CD45.2; all of which are on a C57BL/6 genetic background

#### **Experiment**

1x10<sup>5</sup> Mast cells were cultured in the presence or absence of apoptotic stress (etoposide, 1, 0.5 and 0.1 µg/mL) in 48-wells culture plates in a final volume of 1mL of complete RPMI 1640 Medium. . After 48 or 72 h cells were harvested and analyzed for cell cycle distribution as described below.

#### **Cell cycle analysis by flow cytometry**

The percentage of Mast cells in G0/G1, S and G2/M phase of the cell cycle after treatment with different concentrations of etoposide (Ebewe, Pharmacy Inselspital) was determined by “hypotonic PI Buffer” staining, as described above for T and B cells

#### **Reagents**

- |                              |   |
|------------------------------|---|
| • Etoposide "Ebewe"          | Inselpital Pharmacy                     |
| • Tween-20                   | Sigma Aldrich                           |
| • RNase A, DNase inactivated | AppliChem                               |
| • PI                         | Sigma Aldrich                           |
| • IL3                        | WEHI-3B conditioned supernatant         |
| • Dulbecco's PBS 1x          | PAA Laboratories GmbH                   |
| • SCF                        | conditioned medium from CHO/muSCF cells |

### 3.6 T and B cell activation

#### **Cells**

T and B lymphocytes were purified from the spleen of C57BL/6 WT and *bok*<sup>-/-</sup> mice.

#### ***B cell-enrichment from Murine Spleen by Magnetic Bead Sorting***

Splenic B cells were purified using magnetic bead based cell separation technology (BD IMag<sup>TM</sup>, BD BioSciences). The spleen was surgically excised from sacrificed mice and a single cell suspension was prepared using a 70 µl cell strainer (BD Biosciences). After lysing of red blood cells with 1x red cell removal buffer (150mM NH<sub>4</sub>Cl, 12mM NaHCO<sub>3</sub>, 0.1mM EDTA), mouse B lymphocyte enrichment antibody cocktail (BD BioSciences) were added to cells and negative magnetic sort performed following the manufacturer's protocol (BD Biosciences). The resulting negative fraction, consisting of a highly purified resting B cell population, was used for subsequent experiments. Viable cells were resuspended in FMA Medium (Invitrogen).

#### ***Magnetic Labeling and Separation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from Murine Spleen***

A splenic single cell suspension was prepared as described above. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were isolated by positive sorting following the manufacturer's instructions (BD Biosciences). Purified T cells were resuspended in FMA Medium and cells were counted using a Neubauer chamber diluting (10-20\*10<sup>6</sup> cells/mL) and Trypan blue to exclude dead cells.

#### ***B Cell Activation***

B cells (2.5x10<sup>4</sup>) were cultured in a total of 200µL per well (96 well plate) in FMA Medium and activation followed up to 6 days. B cells were activated with either 20µg/mL lipopolysaccharides (Sigma Aldrich) or with 20µg/mL monoclonal anti-mouse anti-IgM (Jackson ImmunoResearch), all in the presence of 10ng/mL IL-2, 50ng/mL IL-4 and 50ng/mL IL-5 (all recombinant cytokines, Peprotech).

#### ***T Cell Activation***

T cells (2.5x10<sup>4</sup>) were incubated in a total of 200µL in FMA Medium in 96-well culture plates (CellStar<sup>®</sup>, Greiner Bio-one) and activation followed up to 7 days. T cells were activated with either 2 µg/mL Concanavalin A (ConA) (Sigma), with 10 µg/mL pre-coated anti-CD3 (Clone 145-2C11, Stock 1mg/ mL, low endotoxin azide free, LEAF) or 10 µg/mL pre-coated anti-CD3 plus 10 µg/mL pre-coated anti-CD28 (Clone 37.S1, Stock 1mg/ mL, LEAF), in the presence of 10ng/mL of IL-2 (Peprotech)

#### ***Large Scale T Cell Activation***

Purified CD4<sup>+</sup> or CD8<sup>+</sup> T cells were seeded in a total volume of 5 mL in 25 cm<sup>2</sup> flasks at a density of 2\*10<sup>6</sup> cells/mL in FMA Medium in the presence of 2µg/mL Concanavalin A (Sigma Aldrich) and 10 ng/mL IL2 (Peprotech). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 3 days. Concanavalin A was then removed by at least 3 wash steps with PBS. Activated T



cells were seeded at  $5\text{-}10 \times 10^4$  cells/ml and expanded for 2-3 more days in the presence of 10 ng/mL IL2

### ***Determination of viable cell number using FACS reference beads***

Cells were harvested into FACS tubes and 100 $\mu$ l of FACS buffer containing 8000 FITC-labeled FACS reference beads (Invitrogen) added (1xKDS.BSS + 2% FCS + 10mM  $\text{NaN}_3$  +  $8 \times 10^4$  FACS reference beads/mL + 4 $\mu$ g/mL PI). Cells were then analyzed by flow cytometry on a FACS instrument (FACSCalibur, BD BioSciences). To obtain the total number of cells in sample, the following formula was used (Equation 2). Data were analyzed by Weasel 1.2.2 software.

---

***Equation 2***

### ***Cell cycle analysis***

The percentages of B and T cells in G0/G1, S and G2/M phase of the cell cycle after treatment with different concentrations of etoposide (0.5  $\mu$ g/mL, 1.0  $\mu$ g/mL, 2.5  $\mu$ g/mL, 5  $\mu$ g/mL, 10  $\mu$ g/mL) was determined by hypotonic propidium iodide (PI) staining of genomic DNA content. Cells were lysed in 200 $\mu$ l of hypotonic PI Buffer (0.1% sodium citrate, 0.03% Tween-20, 25  $\mu$ g/ml RNase A and 50  $\mu$ g/mL PI) for at least 30 minutes on ice in the dark. Cells were analyzed in a FACS at a flow rate of <200 events/second and PI staining intensity was plotted on a linear scale (FL2A). Cell-cycle profiles were analyzed using the Weasel 1.2.2 software and FlowJo software (Dean Jett Fox model) after exclusion of cellular doublets and dead cells (subG1).

### ***Quantification of Cell Viability***

Viability of B cells was assessed by FACS by exclusion of propidium iodide (PI) (Sigma) and absence of cell-surface staining with EGFP-labeled AnnexinV (self-made). At least  $5 \times 10^4$  cells were incubated in  $\text{Ca}^{2+}$  containing FACS buffer with GFP-AnnexinV (ca 0-5  $\mu$ g/mL) for 20 min on ice in the dark. Cells were washed 1x and then resuspended in 200  $\mu$ L FACS buffer containing 2  $\mu$ g/ml PI.

### ***Freezing of cells***

$1\text{-}5 \times 10^6$  B cells were centrifuged at 1500 rpm during 5 minutes at 4°C, washed and resuspended in 1mL of freezing medium and transferred to cryovials. Before cells were stocked in liquid nitrogen at -196°C, tubes were put at -80°C in a freezing box containing isopropanol (Sigma) to make sure cells were cooled down at a constant optimal rate of 1 grade Kelvin per hours.

### ***Reagents***

- |   |   |
|---|---|
| <ul style="list-style-type: none"> <li>• Monoclonal anti-mouse CD3</li> </ul>   | Stock 1mg/mL<br>Clone 145-2C11<br>BioLegend |
| <ul style="list-style-type: none"> <li>• Monoclonal anti- mouse CD28</li> </ul> | Stock 1mg/mL<br>Clone 37.S1<br>BioLegend    |

- |  |   |
|--|---|
| • Rat biotinylated anti-mouse CD4 and CD8    | Stock 0.5mg/mL<br>Clones GK1.5 + 53-6.7,<br>BioLegend |
| • Blocking Anti-FcγRII/III (= antiCD16/CD32) | HB-197 hybridoma cell<br>supernatant<br>Clone 2.4G2   |
| • SCF  | conditioned medium from<br>CHO/muSCF cells            |
| • Gentamicin                                 | Sigma Aldrich   |
| • Anti-IgM                                   | Jackson ImmunoResearch                                |
| • B cell enrichment cocktail                 | BDBiosciences   |
| • Concanavalin A                             | Sigma Aldrich   |
| • Lipopolysaccharides, E.coli O55:B5         | Sigma Aldrich   |
| • Dulbecco's PBS 1x                          | PAA Laboratories GmbH                                 |
| • Normal Rat serum (NRS)                     | Bioconcept/Promocell                                  |
| • Streptavidin particles Plus-DM             | BDBioSciences   |
| • MT.PBS plus trypan blue                    | Carl Roth   |
| • NH <sub>4</sub> Cl: Ammonium chloride      | Sigma Aldrich   |
| • NaHCO <sub>3</sub> : Sodium bicarbonate    | Sigma Aldrich   |
| • BSA: 7.5% Stock                            | Invitrogen  |
| • NaN <sub>3</sub> : 1M Stock in PBS         | Sigma Aldrich   |
| • EDTA: 0.5 M pH8.0                          | Sigma Aldrich   |
| • Propidium iodide (PI), stock 500µg/mL      | Sigma Aldrich   |

### 3.7 Protein extraction

#### Cells

Cells used for extraction were WT and *bok*<sup>-/-</sup> mast cells and activated T and B cells (see above)

#### Protein extraction

Total protein extracts were obtained by lysing cells in 50-100 µl (depending on the size of the cellular pellet) ONYX Buffer (20 mM Tris/HCl pH7.4, 135 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1% Triton-X100, 10% glycerol plus protease inhibitor cocktail (Roche)). Cell pellets were well resuspended by pipetting and vortexing before tubes were placed for 15 minutes on ice. The lysate was then cleared by centrifugation for 10 min at 14000rpm, 4°C and the supernatant were transferred into a new Eppendorf tube.

#### Protein quantitation with bicinchoninic acid (BCA)

The concentration of total protein was determined by using the BCA kit following the manufacturer's protocol (Pierce Chemical Co., Rockford, IL). Protein samples were diluted 1:10 like the protocol recommended for microplate procedure. Absorbance was read at 562 nm in a multiplate spectrophotometer (SpectraMax M2, Bucher biotech)

#### SDS-PAGE Gel Electrophoresis + Immunoblotting

4x Lämmli buffer (0.25M Tris/HCl pH 6.8, 40% glycerol, 8% SDS, 0.016% Bromophenol Blue in <sub>dd</sub>H<sub>2</sub>O) plus DTT to a final concentration of 100 mM was added to the protein samples before denaturation at 95°C for 5 minutes. Samples were loaded onto to a 12.5% SDS-PAGE and electrophoresis performed at 100 V for approx 2 h (until front reached the bottom of the gel). The proteins were transferred to a methanol activated PVDF membrane (Immobilon-P, 0.45µm, Millipore) at 4°C, 50V during 1 hour.

For immunoprobng, the membranes were first blocked for at least 1 h in Blotto solution (5% skin milk powder in PBS + 0.1% Tween-20(PBS-T) and 10mM NaN<sub>3</sub>). After 3 wash steps in PBS-T, the membranes were probed with appropriate dilutions of primary antibody for 2h at room temperature. Membranes were washed 3x and then incubated with appropriate horse-radish peroxidase (HRPO) coupled secondary antibodies (diluted 1:20'000 in PBS-T + 5% milk). Membranes were very well washed (at least 3x 10 min) and the signals were detected by chemiluminescence (ECL kit, Pierce, ECL Luminata Forte, Millipore) and exposure to photosensitive films (ECL hyperfilm, GE Healthcare).

#### Reagents

• Protease Inhibitor cocktail 50x	Roche
• Pepstatin 500x: Aspartate protease inhibitor	Sigma Aldrich
• Dulbecco's PBS 1x	PAA Laboratories GmbH
• Glycerol	Sigma Aldrich
• SDS	Sigma Aldrich
• Bromophenol Blue	Carl Roth

- Milk powder fat free
- Tris (= Trizma)
- Glycine
- Tween-20
- Ammonium Persulfate for Electrophoresis
- Tetramethylethylenediamine (TEMED)
- PageRuler™ PLUS, prestained protein

Migros  
Sigma Aldrich  
Sigma Aldrich  
Sigma Aldrich  
Sigma Aldrich  
Sigma Aldrich  
Fermentas

- SDS PAGE Running buffer 10x

1L  
0.25M Tris  
1.92M glycine  
1% SDS

- SDS PAGE Transfer buffer 10x

1L  
250mM Tris  
1.92M glycine

## 4 INSTRUMENTS AND KITS

The specific instruments and kit used in this project are listed in alphabetic order:

- BD IMag<sup>TM</sup>, Cell Separation Magnet, 552311, Lot 04214, BD Biosciences
- Cytospin3, Shandon, Dako Diagnostics AG
- Developer machine for X-ray films, Kodak
- FacsCalibur, BD Biosciences
- Microcentrifugeuse Sorvall, Legend Micro 17, Thermo Scientific
- Microscope Zeiss Axioplan, Carl Zeiss
- Pierce<sup>®</sup> BCA<sup>TM</sup> Protein Assay Kit, 23227, Lot JC121778, Thermo Scientific
- Power pack, Consort
- SDS-PAGE and Western blot equipment, Hoefer Inc
- SpectraMax M2, Bucher Biotech, Basel
- Thermocycler TECHNE, Genius
- Thermomixer compact, Eppendorf
- UVB Stratalinker 1800, Stratagene

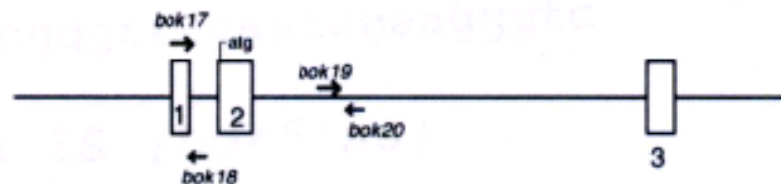
## 5 RESULTS

The main objective of this project was to highlight the role of Bok in cell proliferation and regulating cell cycle arrest. This approach was carried out on different types of primary or cultivated murine cells and several methods of analysis were performed. In addition, to obtain a statistical analysis, experiments were performed if possible at least in triplicates.

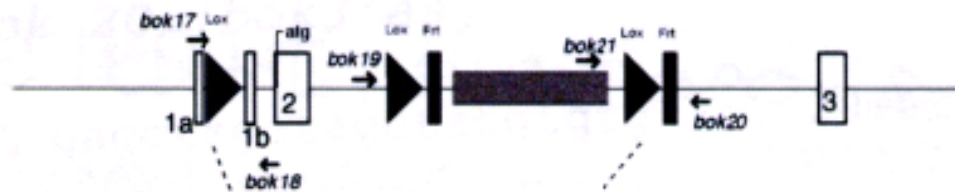
### 5.1 Genotyping by polymerization chain reaction (PCR)

The aim of this manipulation is to amplify a specific WT and mutant allele of *bok* to confirm the mice's genotypes. To ensure the correct genotype of the mouse which was used for the experiments, tail-biopsies were collected *post mortem* and tested by PCR with 3 different primers: *bok* 17, *bok* 18 and *bok* 20. (Figure 14)

*bok* wildtype allele



recombined allele: *bok<sup>fl</sup>*



*bok<sup>fl</sup> x Cre => bok<sup>-</sup>*

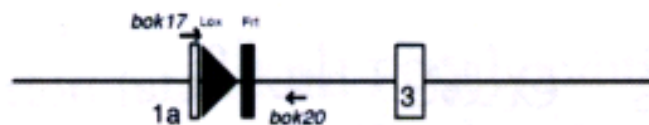
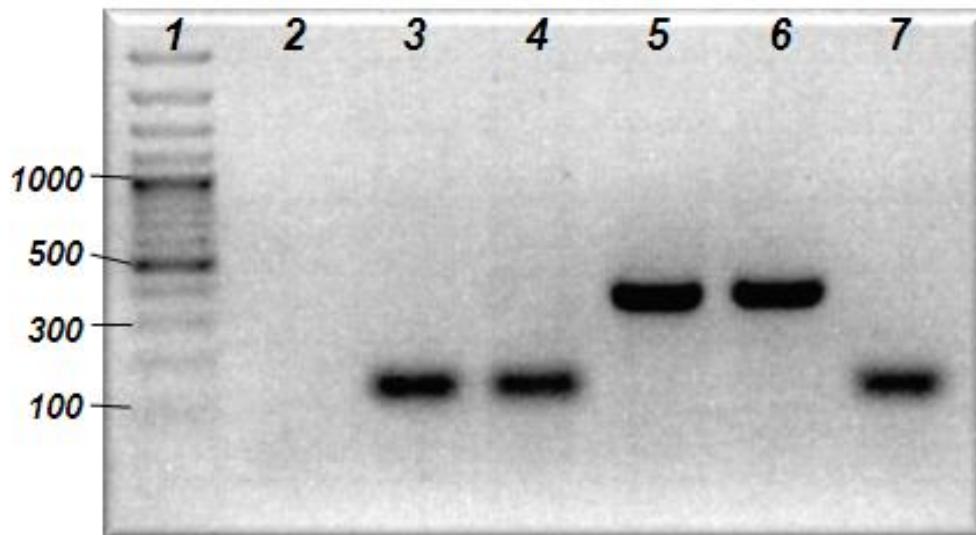


Figure 14 : Schematic representation of the mouse *bok* locus of WT and *bok*-deficient mice with the position and orientation of the primers used for genotyping.

Because of mice's DNA, the usual rules of sterility are not as strict as for human DNA (hood, adequate local). In addition, as the genotyping is only qualitative and not quantitative, the DNA quantification by spectrophotometer has not been performed.



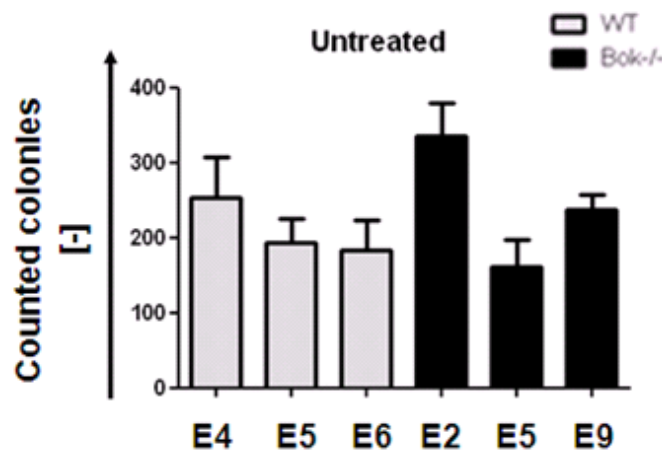
**Figure 15 : Bok PCR genotyping results from 1.5% agarose gel electrophoresis.** 1. GeneRuler™ 100pb Plus DNA Ladder (Appendix 2) – 2. Negative control – 3. Wild Type Tail sample of 25.05.11 – 4. CS7/BL6 (positive control for WT) – 5. Bok<sup>-/-</sup> Tail sample of 31.05.11 – 6. Bok#2 (positive control for bok<sup>-/-</sup>) – 7. Wild Type Tail sample of 31.05.11

PCR amplification of genomic DNA extracted from Wild-Type and *bok*<sup>-/-</sup> animals confirmed the correct genotypes. On the gel (Figure 15), a clear-cut band was observed in 150pb for the Wild-Type allele and a band of 383 bp for the deleted allele of the *bok*<sup>-/-</sup> mice. In addition, these bands are perfectly similar to those of positive controls.

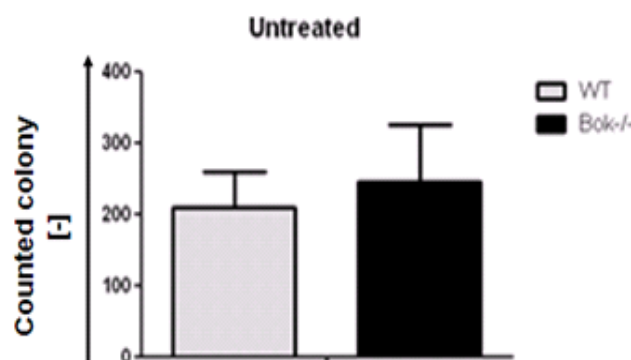
## 5.2 Colony forming assay in mouse embryo fibroblasts (MEF)

In these experiments, the identification of colonies formed after 5 days was performed on 6-well plates. Three fibroblast lines, each derived from different embryos, were used for each genotype. These are all immortalized with an oncoprotein, SV40 large T antigen and have the characteristic to adhere to the plate after 2 hours already. Wildtype MEF represented by three separate embryos (E4, E5 and E6) served as control. The *bok*<sup>-/-</sup> MEF lines were derived from embryos E2, E5 and E9. The following experiments were realized four times for each concentration of drugs (etoposide, hydroxyurea) or treatment (UV irradiation).

According to Figure 16, a significant variation between independent lines of the same genotype is already visible before treatment. To obtain the survival colonies number, a relationship between treated and untreated cells is performed for each drug / irradiation. Therefore, this variation inter-embryo and inter-genotype (Figure 17) plays a significant role to interpret results.

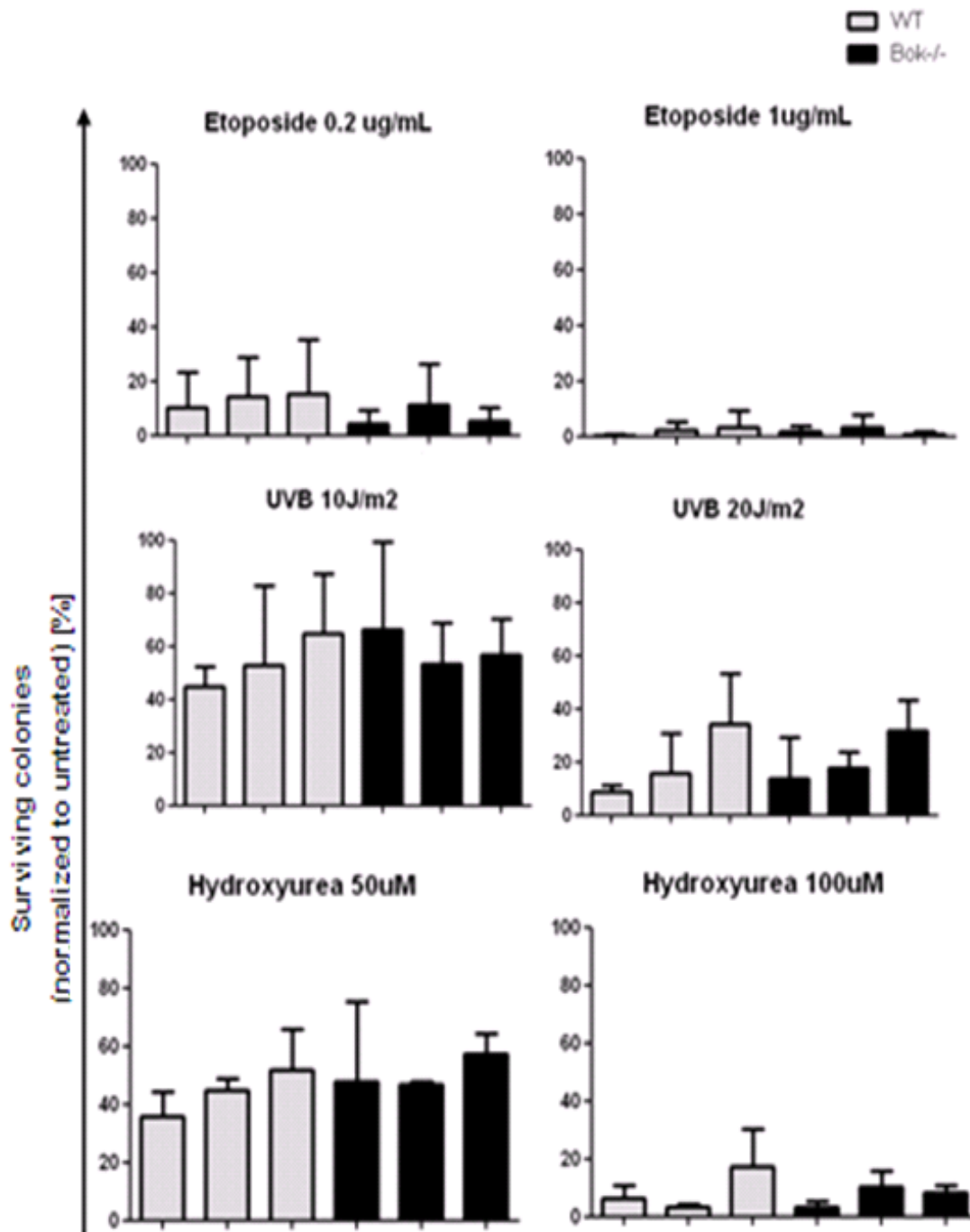


**Figure 16 :** *bok*<sup>-/-</sup> or WT SV40 LT immortalized MEF were seeded at  $1 \times 10^3$  cells per well (6-well plate). After 5 days, cells were fixed, stained with crystal violet and counted manually. Data are presented by means  $\pm$  SD of four experiments with three independent embryos of each genotype.



**Figure 17 :** Comparison between untreated cells coming from WT and *bok* embryos. Data are represented by means  $\pm$  SD of four experiments with three independent embryos of each genotype.

Regarding the results obtained after normalization of the number of colony treated in function of the number of untreated colonies, the results of these four experiments with three independent embryos are represented graphically (Figure 18).

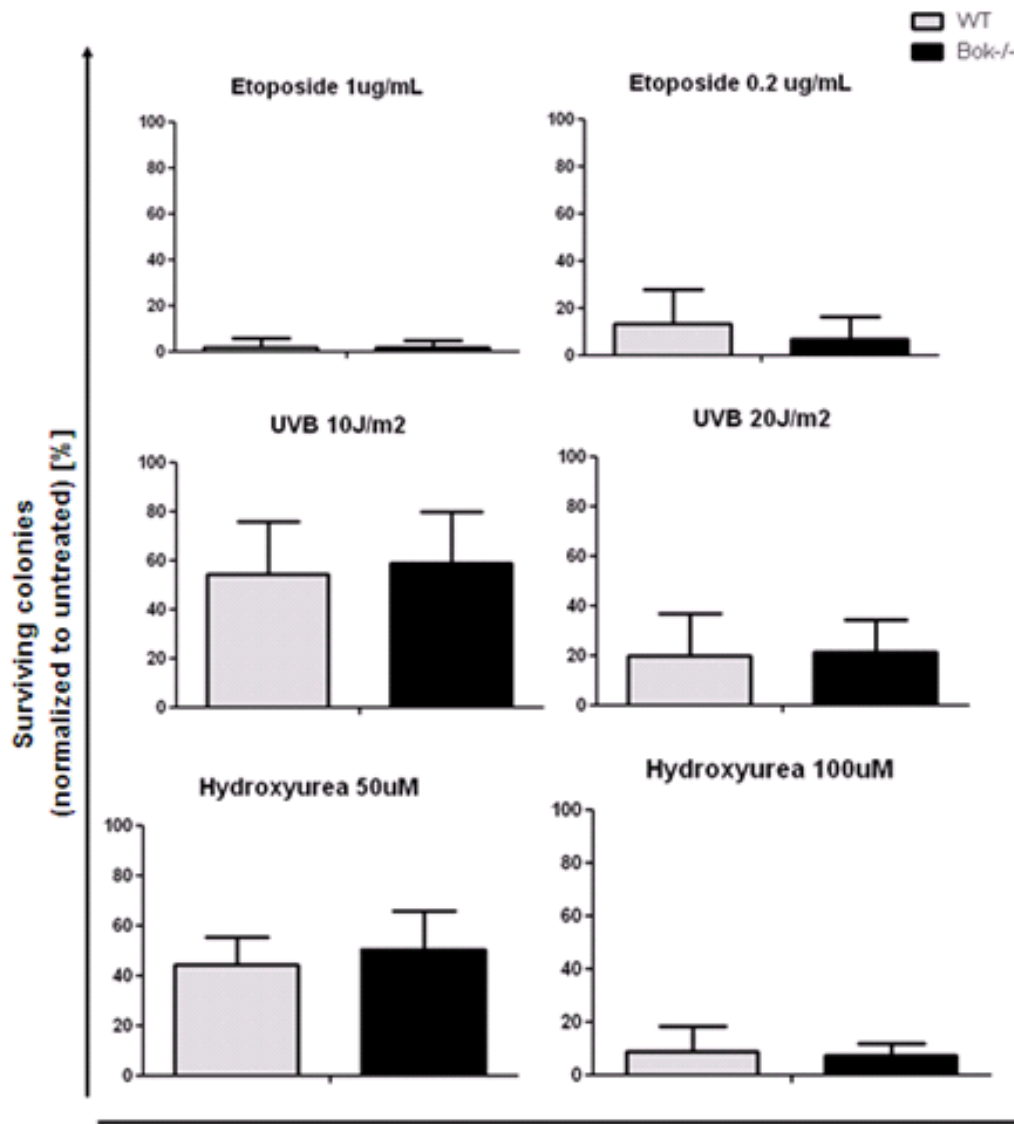


**Figure 18 :** *bok<sup>-/-</sup>* or WT SV40 immortalized MEF were seeded at  $1 \times 10^3$  cells per well (6-well plate) the day prior to UVB-irradiation with two different doses. The other wells were treated with two different doses per drug, including the DNA-damaging drug etoposide and the replicative stress inducing drug hydroxyurea..

After 5 days, cells were fixed, stained with crystal violet and counted manually. Data are presented as percentage of surviving colonies compared to the untreated controls; data are represented by means  $\pm$  SD of four experiments with three independent embryos of each genotype.

In Figure 18, two replicative stress-inducing drugs were tested on MEF cells. These substances are etoposide with a concentration of 0.2 and 1  $\mu\text{g/mL}$  and hydroxyurea with a concentration of 50 and 100  $\mu\text{M}$ . In view of the obtained results, there are more survival cells for WT cells than for *bok*<sup>-/-</sup>, except for the low concentration of hydroxyurea which is confirmed by Figure 19. In other words the loss of Bok does not increase survival after replicative stress-inducing drugs.

DNA damage on MEF cells was also evaluated after UVB irradiation of 10 and 20  $\text{J/m}^2$ . Both intensity of irradiation show no difference between the two genotypes although three embryos of the same genotype showed a wide variation between them. The same observation as replicative stress inducing drugs applies equally to this analysis that bok does not increase survival after DNA damage.



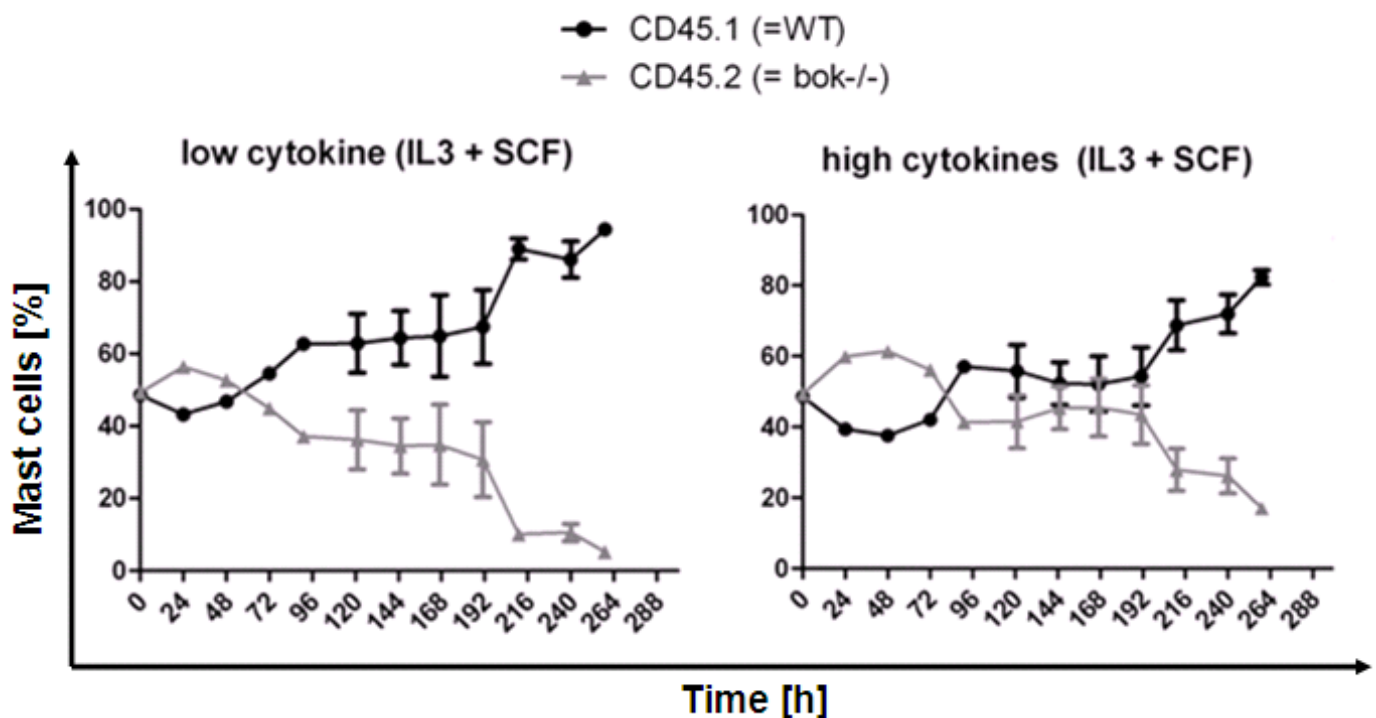
**Figure 19 : Comparison between cells coming from WT and bok embryos after different treatment: UVB irradiation, etoposide and hydroxyurea. Data are presented as percentage of surviving colonies compared to the untreated controls; data are represented by means  $\pm$  SD of four experiments with three independent embryos of each genotype.**

In general, derived cells derived from embryos *bok<sup>-/-</sup>* survive less well than WT cells to radiation and drugs. Regarding the results obtained after UVB irradiation, the loss of Bok does not increase cell survival following DNA damage. The same observation applies equally to replicative stress-inducing drugs like etoposide and hydroxyurea. However, these observations are subjective, because of the large variation between embryos.

### 5.3 Direct competition growth in bone marrow derived Mast cells

Mast cells are commonly involved in adaptive immune responses, especially during allergic and inflammatory reactions. *In vivo*, while they are still immature, they leave the bone marrow prior to localize in tissues where, under the influence of stem cell factor (SCF), they will differentiate and become mature. [22] In this experiment, these conditions were reproduced *in vitro*. With growth factors IL-3 and SCF, bone marrow derived progenitor cells were differentiated into mast cells *in vitro* for 4 weeks before using them for experiments.

Previous data from Kaufmann's lab indicated that *bok*<sup>-/-</sup> mast cells grow slower than WT cells when cultured in separate wells. In this experiment, this observation was also verified by mixing mast cells of two different genotypes in the same well. Cells can be distinguished from each other by their CD45 surface locus using FACS, as mast cells were derived from either CD45.1 or CD45.2 mice. The sum of CD45.1 and CD45.2 cells in a well always constitutes a total of 100%.



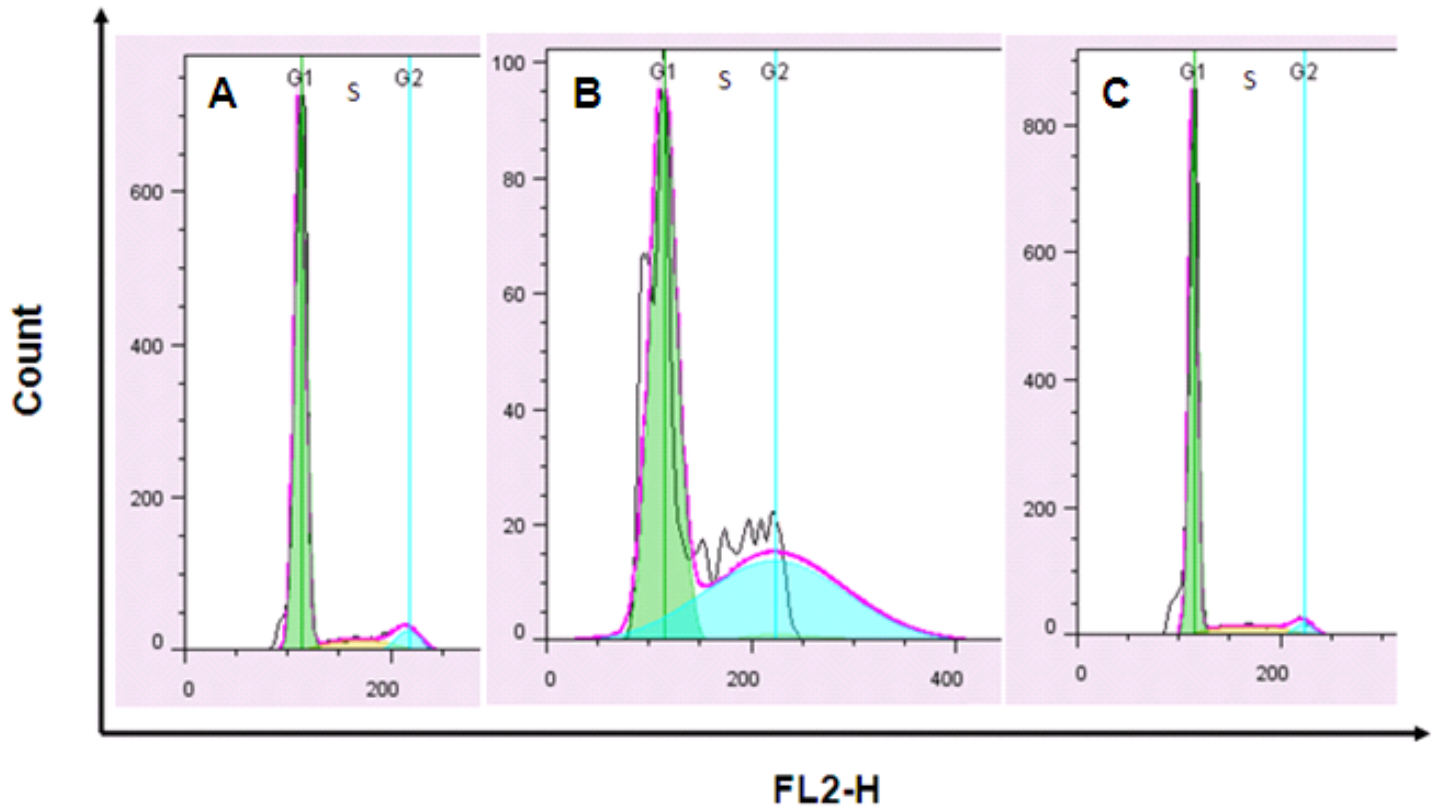
**Figure 20 :** Mast cells competition result with 1% IL3 plus 1% SCF (left graph) and with 10% IL3 plus 10% SCF (right graph). Each mix contained 50% mast cells of each genotype at time 0. After various times of culturing, cells were washed and labelled by PE-labelled anti-CD45.1 and FITC-labelled anti-CD45.2 antibodies and analyzed by FACS. Dead cells were excluded by PI staining.

These experiments aimed at understanding the relationship between competition and cell growth. Figure 20 shows the evolution of the growth of mast cells over time with a cytokine concentration of 1% and 10%. A 50:50 starting mixture reveals first a preferential growth for CD45.2 phenotypes (*bok*<sup>-/-</sup>) during the first 48 hours. Indeed, the graph shows a faster growth for mast cells *bok*<sup>-/-</sup> than for WT during this period.



After one day, a difference of nearly 15% is observed between *bok*<sup>-/-</sup> and WT mast cells with the low concentration of cytokine. This difference is more pronounced with the high cytokines concentration (about 20%). For both cytokine concentrations, this trend is reversed after about 60 hours for low cytokine concentration and after more than 3 days for the 10% cytokine concentration. In this situation, WT grow faster than *bok*<sup>-/-</sup>. When the concentration of cytokines becomes limiting, *bok*<sup>-/-</sup> cells grow more slowly or are more sensitive to death induced by cytokine removal. This is supported by the observation that the decrease in growth for *bok*<sup>-/-</sup> at low cytokine concentrations (1%) is much more pronounced than at high cytokine concentrations (10%).

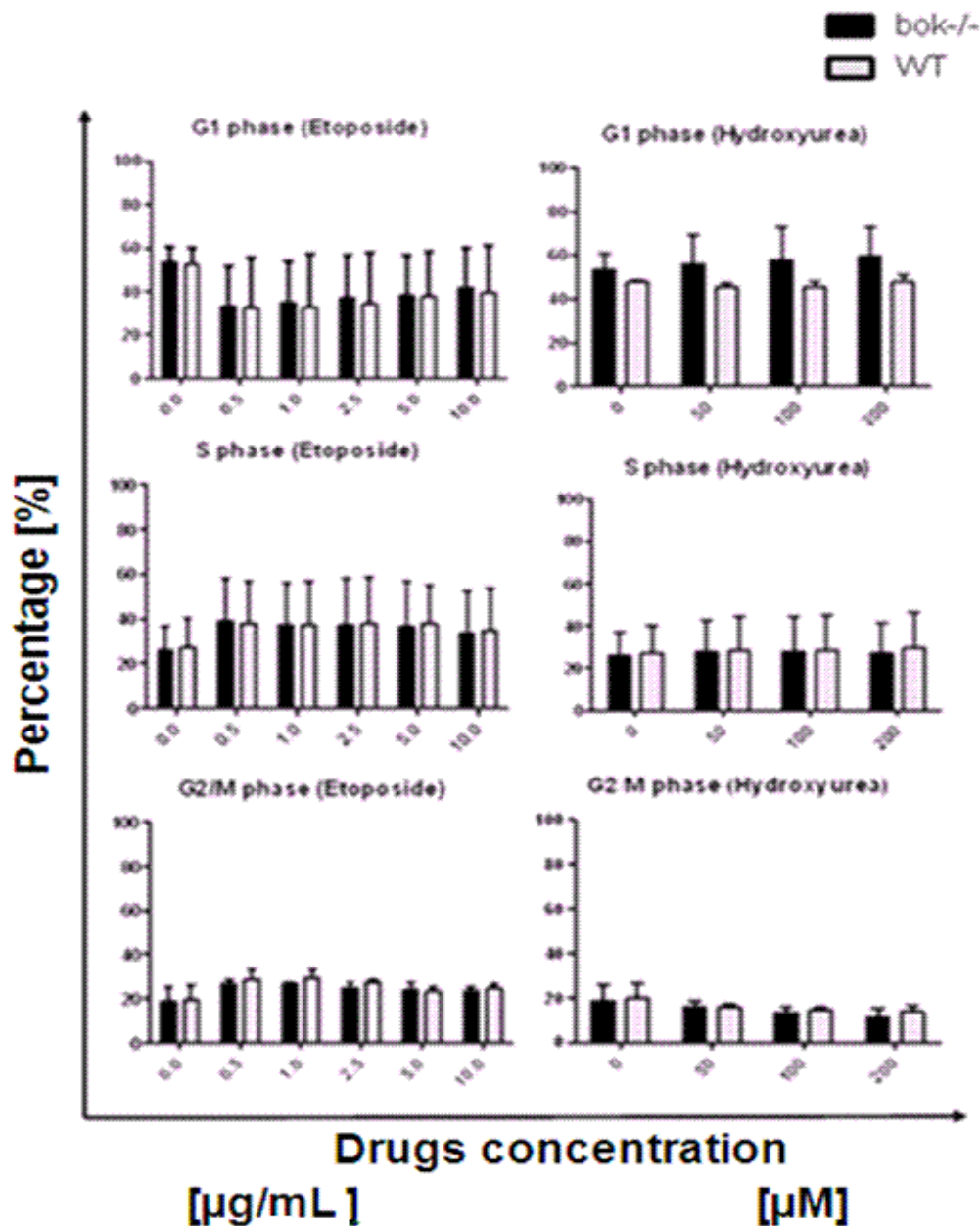
## 5.4 Cell cycle analysis



**Figure 21 : Results obtained for T cells hypo PI cell cycle analysis after 5 hours. (A) Untreated – (B) 10 µg/mL etoposide – (C) 100 µM hydroxyurea**

The addition of drugs such as hydroxyurea plays a role in DNA synthesis and thus blocks the cell in S phase of the cell cycle. This substance is also used in the medical community to treat certain cancers or sickle cell anemia. [23] An accumulation of cells in G1 phase is then observed. The other substance tested here is etoposide, which affects topoisomerase II. This drug prevents the relinking step, initially performed by this enzyme and causes a definitive break through DNA strands. The cell cycle will be inhibited at the premitotic phase (late S and G2). [24]

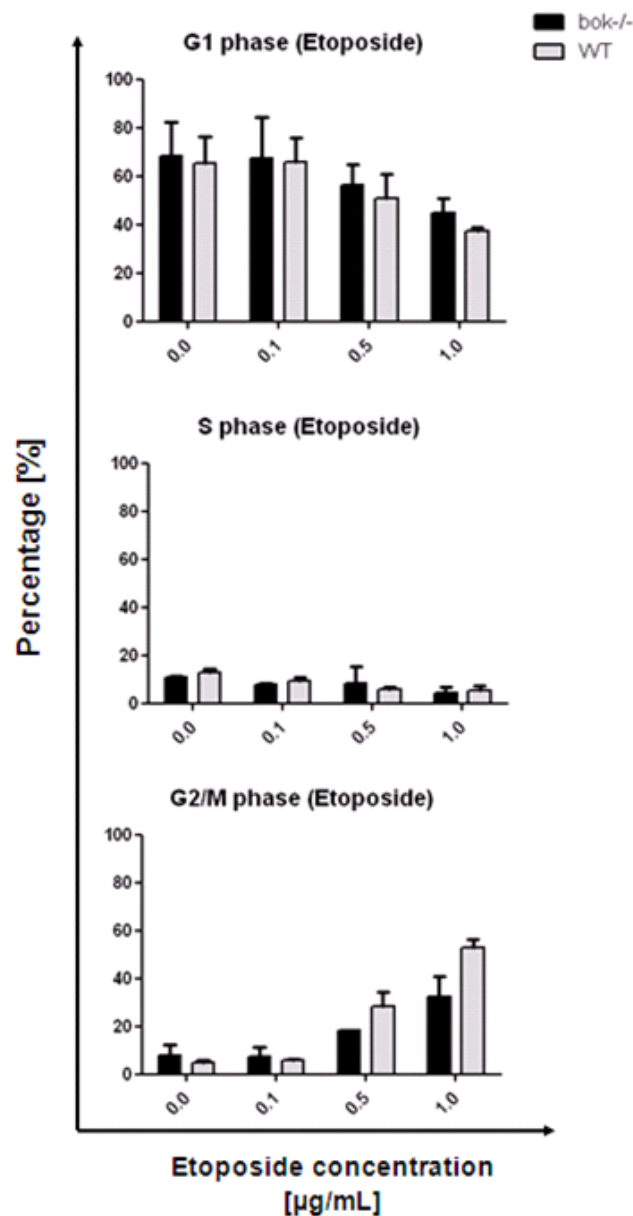
In this experiment, ConA activated T cells were tested with five different concentrations of etoposide (0.5, 1, 2.5, 5 and 10 µg/mL) and three hydroxyurea concentrations of 50, 100 and 200 µM. These tests were always performed in parallel to an untreated sample manipulated similarly to the others. Obtained results are shown in Figure 22.



**Figure 22 :** *bok*<sup>-/-</sup> and WT T cells, activated by ConA, were left untreated, treated with different concentration of etoposide and hydroxyurea during 5 hours. Nuclear DNA was then stained with PI (using hypotonic buffer), and cell cycle profiles were analyzed by flow cytometry. Data represent means  $\pm$  SD of two independent experiments for each genotype

To determine whether Bok is required for DNA damage-induced cell cycle arrest, analysis of T cells response with etoposide is realized. In Figure 22 cell arrest is slightly observed in the S or G2/M phase but no difference is observed between both genotypes. The cell cycle arrest was induced but it's not very flagrant. The concentration of drugs is probably too low to affect cells. This does not allow concluding categorically that Bok is not required for DNA damage-induced cell cycle arrest. For the treatment of cells with the replicative-stress hydroxyurea, no arrest is really observed in S phase. Higher dose of drug must be tested.

Like for the T cells cycle experiment, the same analyses were performed on mast cells using etoposide concentrations of 0.1, 0.5 and 1  $\mu\text{g/mL}$ . Like T cells, an untreated sample was made in parallel to other analysis to compare changes.

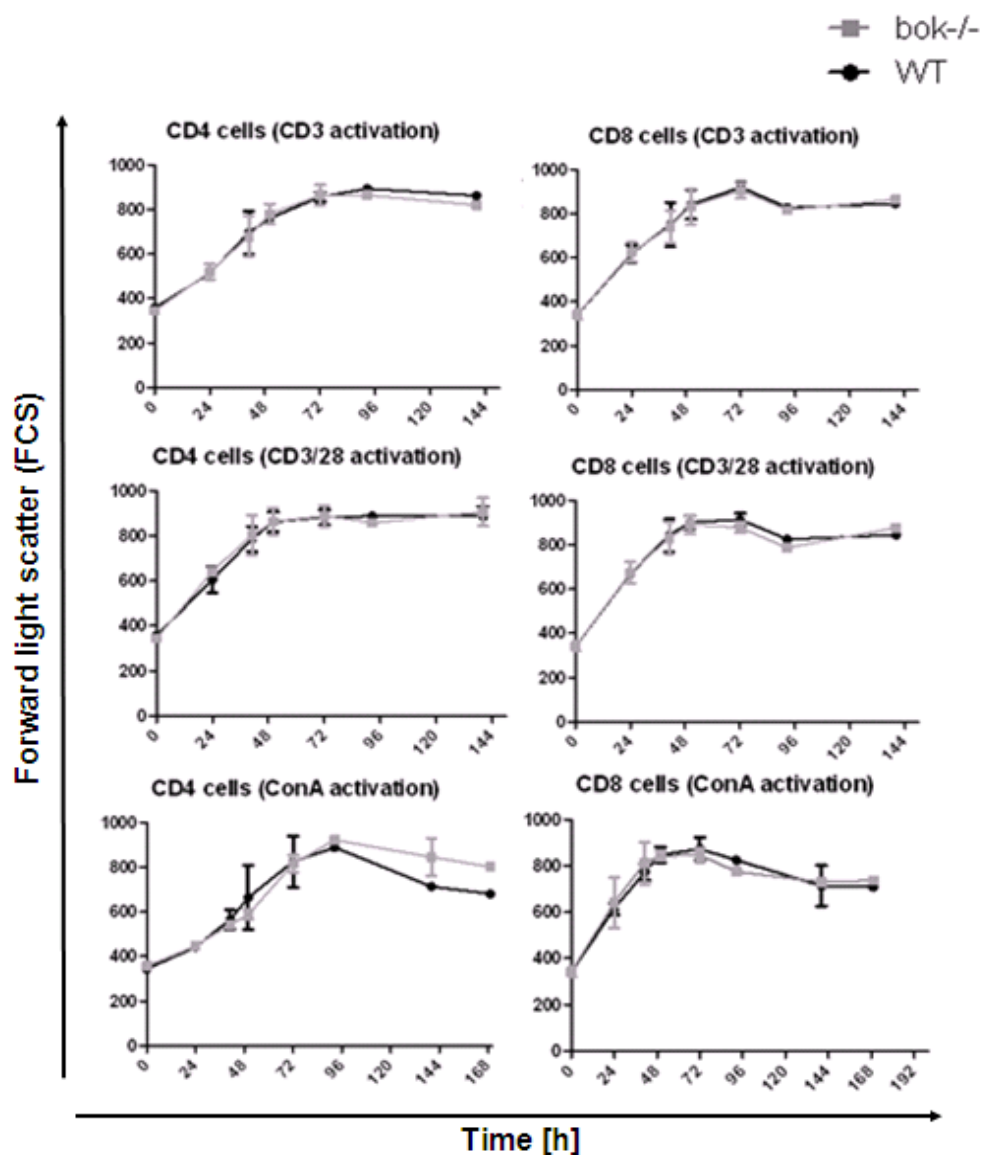


**Figure 23 :** *bok*<sup>-/-</sup> and WT mast cells were left untreated, treated with different concentration of etoposide during 72 hours. Nuclear DNA was then stained with PI (using hypotonic buffer), and cell cycle profiles were analyzed by flow cytometry. Data represent means  $\pm$  SD of three independent experiments for each genotype.

Figure 23 shows that mast cells arrested in the G2/M phase after DNA damage exposure with etoposide. In return, percentage of cells in the G1 phase decreases following expansion of G2/M phase. .

## 5.5 T cell activation

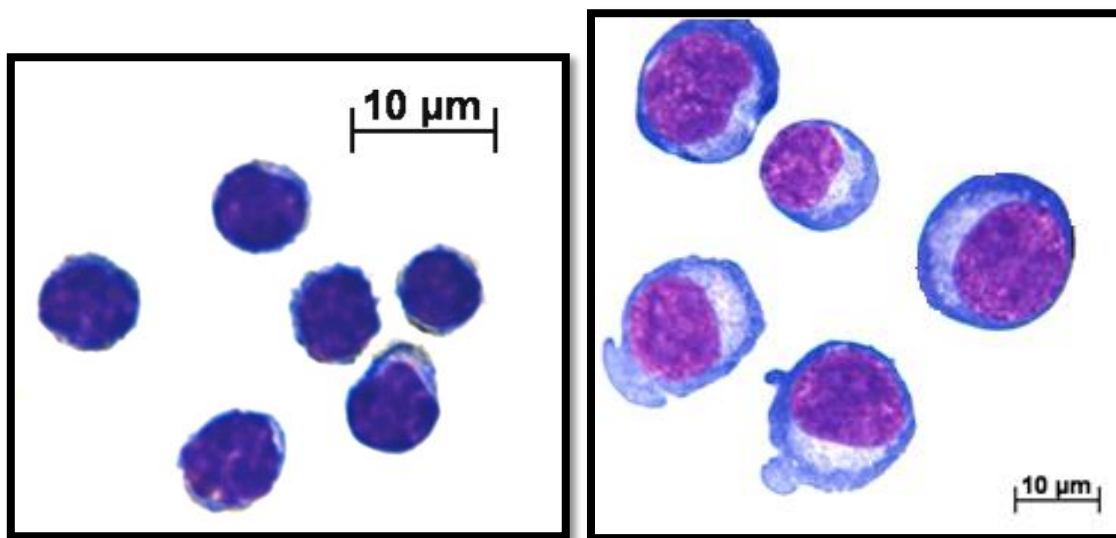
This experiment is realized with splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells stimulated *in vitro* by different methods: mitogenic anti-CD3 antibody, anti-CD3 plus anti-CD28 antibodies or concanavalin A (ConA). The concentration of all stimuli was 10µg/mL. After a few hours, cells increased and eventually doubled in size regardless of cells type CD4<sup>+</sup> or CD8<sup>+</sup> (Figure 24). However, after 4 days, the size of CD8<sup>+</sup> T cells decrease independently with the type of stimuli. A decrease in size is also observed with CD4<sup>+</sup> cells after activation with ConA after the same period of culture. This can be explained by the depletion of the stimulus in the medium. In addition, T cell activation seems to occur more rapidly in the CD8 T cells than in CD4 T cells.



**Figure 24 :** *bok*<sup>-/-</sup> and WT resting T cells were activated with different stimuli:  $\alpha$ -CD3 antibody,  $\alpha$ -CD3 plus  $\alpha$ -CD28 antibodies or ConA. The relative cell size (forward light scatter: FSC) of activated T cells was determined by flow cytometry and is represented as mean FSC. Data show means  $\pm$  SD of two independent mice of each genotype

Regarding the two phenotypes, no difference between the increase of mean size (mean forward scatter of viable cells) of cells is observed during the early stages of activation between *bok*<sup>-/-</sup> and WT T cells.

As mentioned above, following the activation, cells size can double or even triple. So, this phenomenon can be observed by light microscopy after performing cytopspin (Figure 25). Fixation of cells is made with paraformaldehyde. As for staining, it requires two solutions: haematoxylin and eosin. Eosin, an acidic dye, highlights the basic parts such as the cytoplasm and haematoxylin stains the acidic parts of the cell (eg the nucleus) because it is a basic dye.



**Figure 25 : Pictures of T cells taken by microscopy before (left side) and after activation (right side)**

This time, figure 26 shows the number of cells after activation by the same stimuli as before. An enhanced growth for *bok*<sup>-/-</sup> T cells than WT after anti-CD3 and anti-CD3/CD28 activation is observed. With concanavalin A, stimulation takes more time but the fold increase in cell number is higher. This results means that Bok can play an essential role in mitogen-induced cell proliferation but it is difficult to say precisely because the tests have been tried only 2 times. Triplicate experiments are necessary to obtain a reliable statistical study.

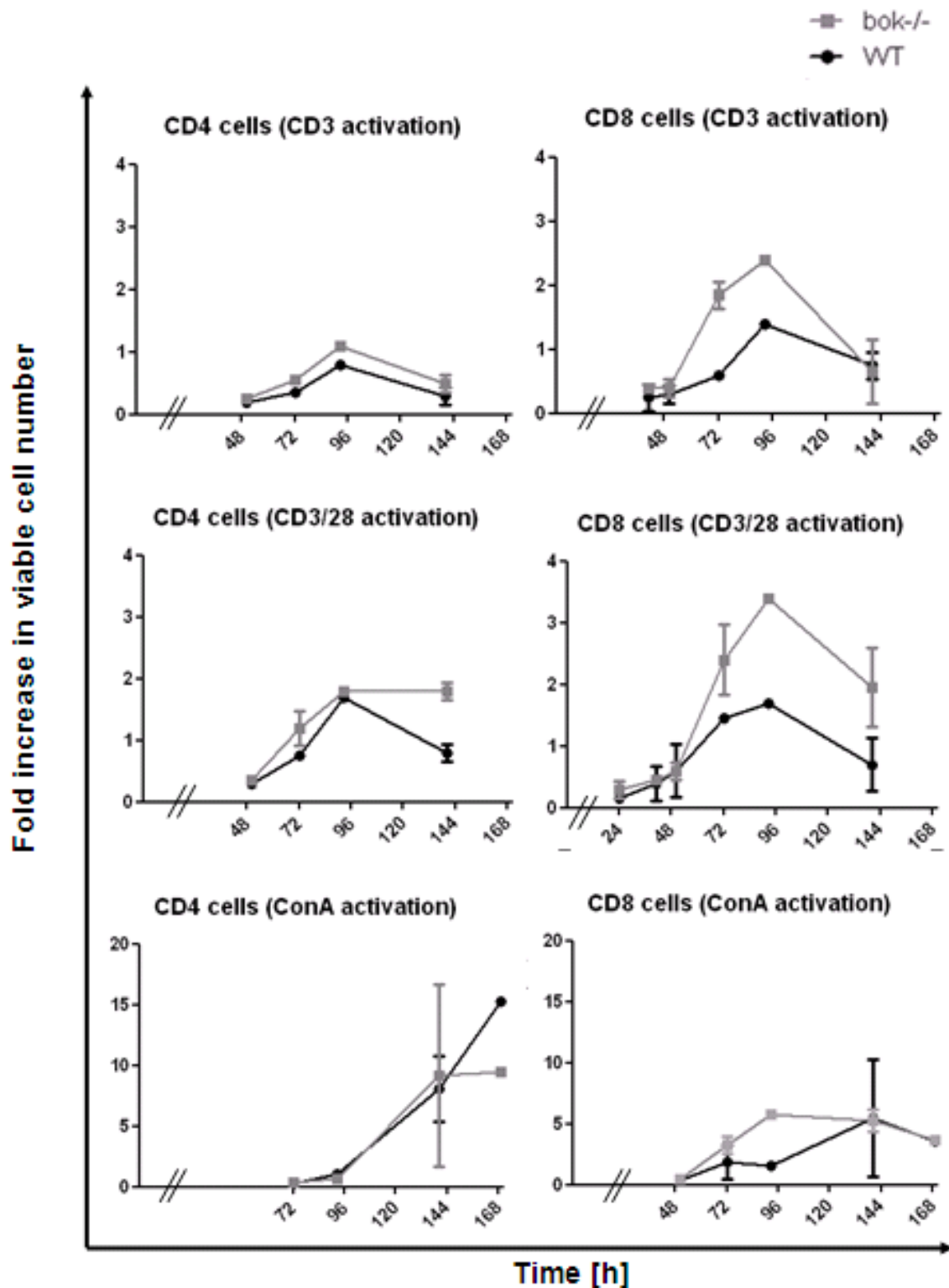


Figure 26 : bok<sup>-/-</sup> and WT activated T cells were activated with different stimuli:  $\alpha$ -CD3 antibody,  $\alpha$ -CD3/CD28 antibody or ConA. Percentages of cells were counted over time with reference beads by flow cytometry. Data represent means  $\pm$  SD of two independent experiments

## 5.6 B cell activation

For B cells, similar experiments were realized in parallel of the T cell activation. In this case, different stimuli are used like LPS and IgM with a concentration of 20 µg/mL. Results are shown in Figure 27.

Like T cells, B cells increased in size with a factor of about 2 already after 24 hours incubation. Forward light scatter watch a reduction of cell size after 96 hours in both cases, probably due to the exhaustion of the stimuli.

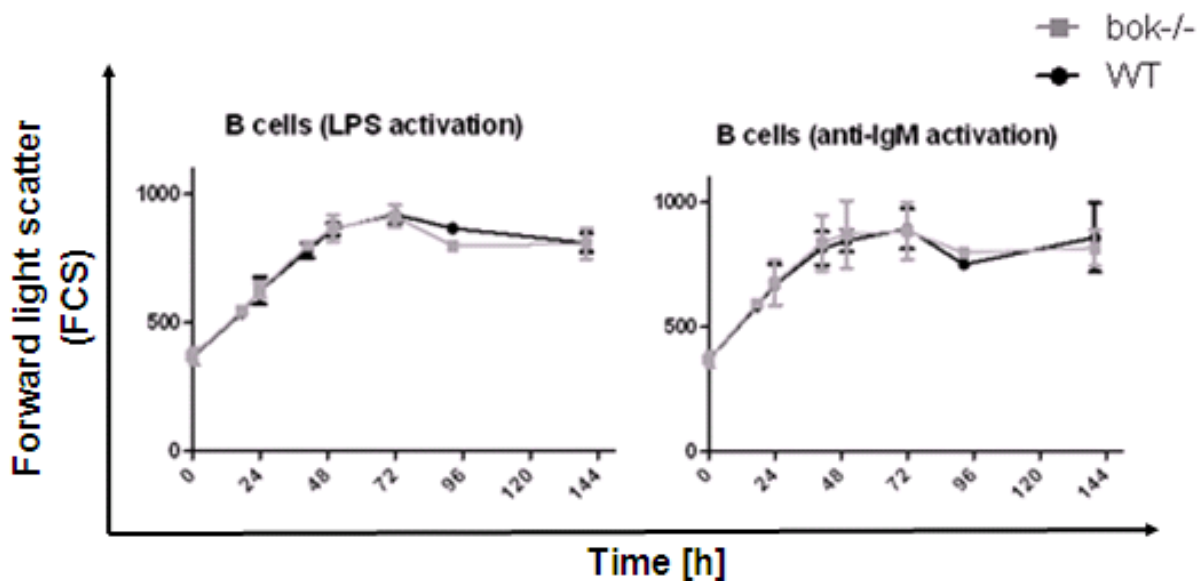


Figure 27 : *bok*<sup>-/-</sup> and WT activated B cells were activated with different stimuli: LPS or  $\alpha$ -IgM. The relative cell size (forward light scatter: FCS) of activated B cells was determined by flow cytometry and is represented as mean FCS. Data show means  $\pm$  SD of two independent mice of each genotype

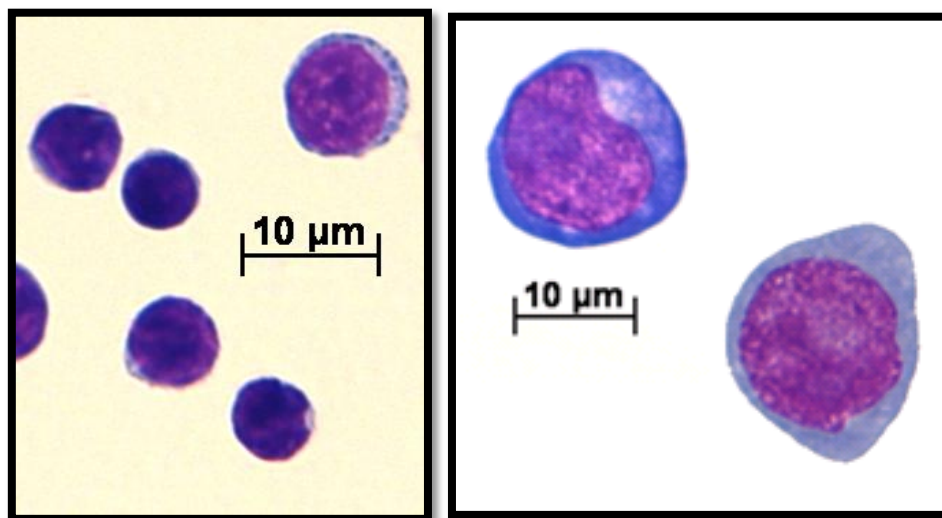
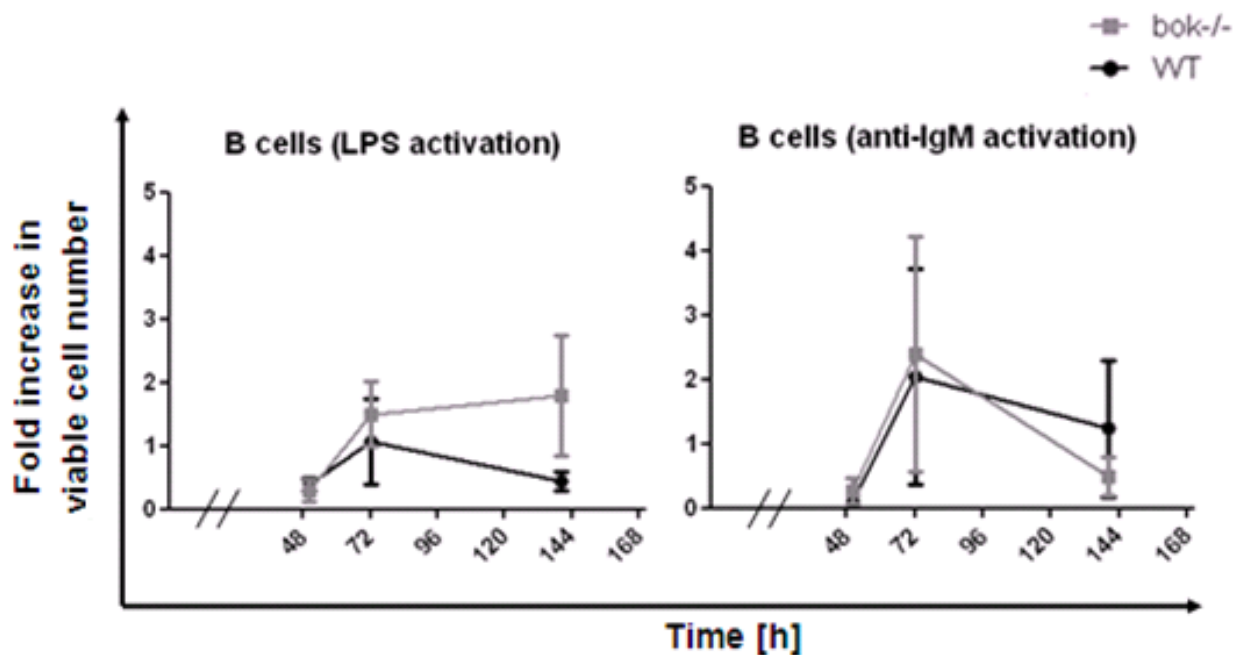


Figure 28 : Pictures of B cells taken by microscopy before (left) and after activation (right)

After *in vitro* stimulation, a dramatic expansion of cells occurs and modification of nucleus and cytoplasm can be observed after coloration of a cytospin. (Figure 28)  
The cell size can increase up to threefold.

Like for T cell activation, viable cell number appears to increase more in *bok*<sup>-/-</sup> cells than other genotype (Figure 29). But unlike the experience with the LPS activation, a net growth peak is visible after 72 hours during B cell activation by anti-IgM before declining slowly. However, the standard deviation is quite high. To confirm these statements, it would be preferable to repeat the analysis at least once more to obtain 3 independent readouts (n = 3).



**Figure 29 :** *bok*<sup>-/-</sup> and WT activated B cells were activated with different stimuli: LPS and  $\alpha$ -IgM. Percentages of cells were counted over time with reference beads by flow cytometry. Data represent means  $\pm$  SD of two independent experiments



## 5.7 Proteins extracts

In order to visualize the protein Bok, proteins from B lymphocytes and mast cells were extracted directly from cultured cells. The extraction was done for each cellular pellet in a volume of extraction solution between 50 and 75  $\mu$ L depending on the size of the pellet. In order to load the same amounts of samples on SDS-PAGE gels, protein concentrations of the extracts was quantified by a spectrophotometric method (BCA assay) at a dilution of 1/10 in duplicates. A calibration curve (standard curve using BSA) was also performed. The calculated calibration slope gave an equation of  $0.00119x - 0.0133$  with a correlation coefficient of 0.99. With this calibration curve (Appendix 3) the concentrations of various samples can be defined with their absorbance (Table 2).

**Table 2: Results of quantification of proteins samples by spectrophotometry using the BCA assay**

Date	Cell type	Genotype	Culture time [h]	Concentration [ $\mu$ g/mL]
28.06.11	B cell	WT	0	1413.17
28.06.11	B cell	<i>bok</i> <sup>-/-</sup>	0	1714.56
29.06.11	B cell	WT	24	1172.06
29.06.11	B cell	<i>bok</i> <sup>-/-</sup>	24	1409.80
30.06.11	B cell	WT	48	2005.84
30.06.11	B cell	<i>bok</i> <sup>-/-</sup>	48	2234.72
17.06.11	Mast cell	WT 5.1	0	2614.94
17.06.11	Mast cell	WT 5.2	0	5886.80
17.06.11	Mast cell	<i>bok</i> <sup>-/-</sup>	0	3596.24
27.06.11	Mast cell	<i>bok</i> <sup>-/-</sup>	0	9100.49
28.06.11	Mast cell	<i>bok</i> <sup>-/-</sup>	24	10435.88
29.06.11	Mast cell	<i>bok</i> <sup>-/-</sup>	48	9333.60
30.06.11	Mast cell	<i>bok</i> <sup>-/-</sup>	72	9234.54
27.06.11	Mast cell	WT	0	10041.70
28.06.11	Mast cell	WT	24	7806.84
29.06.11	Mast cell	WT	48	9160.35
30.06.11	Mast cell	WT	72	8218.25

In order to achieve homogeneity in the amount of loaded sample and therefore compare these bands, they were diluted to a concentration of 2 $\mu$ g/mL. 15  $\mu$ L of each samples, corresponding to 30  $\mu$ g per lane, were then subjected to electrophoretic separation on a 12.5% SDS-polyacrylamide gel. The different results obtained are illustrated in Appendix 4.

The first two gels were loaded with the extracts from B cells and mast cells. The primary anti-Bok antibody revealed many non-specific bands between 28 and 55 kDa with a sharp band around 28 kDa. The theoretical molecular weight of Bok protein is 22kDa. It is impossible to confirm its presence because no positive control is present but the nature of the sample used to look for another protein because it is as much as WT sample than *bok*<sup>-/-</sup> sample.

The anti-tubulin antibody allows verifying the similar protein loading. According to the comparable intensities of these bands, it can be concluded that similar amounts of protein extracts were loaded per lane, except for mast cells WT5.1 extract from 17.06.2011, mast cells *bok*<sup>-/-</sup> extract from 17.06.2011 and mast cells *bok*<sup>-/-</sup> extract T72h from 30.06.2011 (Figure 34 Appendix 6) where no band is detectable. The bands correspond to 50 kDa and confirm the presence of the protein tubulin. Earlier incubation on the same gel with an anti-Bcl-2 antibody recognizes however a protein band at 25kDa, corresponding to the molecular weight of the protein Bcl-2. It can be concluded that the missing tubulin bands were likely degraded by proteolysis, either during sample preparation or in dying cells. [25]

During the first test with the anti-Bok antibody, the results showed a band at approximately 28 kDa and no positive control could confirm the presence of Bok on the membrane. A second gel was run with extracts from B cells and a sample of hepatocytes has been added as a positive control. No sample gave a signal for Bok and the positive control only showed a low intensity band at around 22kDa, which corresponds to the size of Bok. Finally, the anti-Bim antibody was tested on the same membrane. The identification of a 25 kDa band confirms the presence of the protein Bim in the extracts [26], especially in mitogen-activated B cells. One residual gel could be stained and observed. Stained with Coomassie blue (Appendix 4), it shows again a homogeneity between the different extractions.

Finally, a lack of protein may explain the absence of observed signal. Indeed, it is necessary to load a high amount of protein extract to see a strong band for Bok. The reason is that the rabbit polyclonal Bok-antibody, which was developed in the Kaufmann lab, has a rather low binding affinity and quite a few cross-reactive bands. However, it is to our knowledge the only antibody available that reliably detects Bok protein.



## 6 CONCLUSION AND PREVIEW

At the end of these 16 weeks of experimental lab work, the requirements in the specifications have been performed successfully. This research is not yet complete but offers new ways for the role of Bok in the cell proliferation and cell cycle control based on obtained results. Observations of *bok*-deficient fibroblasts to DNA damage like UVB irradiation and other apoptotic stimuli (etoposide and hydroxyurea) show normal sensitivity. Although there is a significant variation between independent lines derived from different embryos of the same genotype, no difference appears between the averages of each genotype. It is likely that the observed variation between single lines of the same genotype is caused by the procedure of SV LT Ag transformation. This could be verified by performing similar experiments in primary MEFs (which can only be cultured in vitro for max 5 passages).

Regarding the mast cells competition assay, it seems that *bok*<sup>-/-</sup> cells grow faster than WT during the first 2 or 3 days but when cytokine concentration is limiting, *bok*<sup>-/-</sup> cells either grow slower than WT or become more sensitive to death than WT cells. This faster growth (at early stage) is confirmed in the T and B cell experiments, where the fold increase of *bok*<sup>-/-</sup> activated lymphocytes is significantly greater than in WT controls. Moreover, cell cycle arrest analyses show that Bok is not necessary for DNA Damage-induced cell-cycle arrest or replicative stress in the cell types analyzed. This could also be explained by low endogenous of Bok in the cell types analyzed. Western blot analysis from proteins extracts of mast cells or activated B cells showed no detectable signal for Bok, indicating that Bok levels are indeed very low in those cell types. Given very low endogenous Bok protein expression in the cells analyzed, it is thus surprising and very interesting that some clear differences (e.g. in cell proliferation) could be identified in this project. Bigger differences might be found when analyzing cells from tissue like uterus, ovaries, brain or gastrointestinal tract (e.g. stomach, intestines, liver and pancreas) because qPCR analysis performed in the Kaufmann lab revealed that major *bok* expression is found there.

Generally, it can be difficult to compare a knockout strain with a wild strain. Indeed, the total removal of a protein may result in a compensatory up- or downregulation of related (e.g. other Bcl-2 family members) or unrelated genes. The influence is difficult to quantify because the mechanism is unclear. That's why it would be beneficial to either re-introduce WT *bok* into *bok*-deficient cells (and use those as "WT controls") and/or to knockout *bok* conditionally in the adult mice. Alternatively, approaches with siRNA or shRNA, leading to a partial "knockdown" rather than complete "knockout" would be valuable tools to confirm the obtained findings.

Other prospects for analysis of cellular stress are possible in the same focus, such as glucose deprivation in MEF and ER-stress in general (MEF, activated T and B cells, mast cells), as Bok has recently been identified to localize mainly to the membranes of the ER and associated membranes of the secretory system (unpublished observations by Nohemy Echeverry, Kaufmann lab).

## 7 ACKNOWLEDGMENTS

I would like to sincerely thank the following people of the Institute of Pharmacology and HES-SO for giving me the opportunity to carry out my diploma work in Bern and for supporting me during this time. I'm sincerely grateful to Doctor Thomas Kaufmann for his availability and implications throughout this work, for encouraging me and for his corrections and to Professor Bruno Schnyder who was also always keen to answer to my questions. Last but not least, I would like to thank the staff of the Kaufmann lab for their kindness and their precious help during this work.

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## 9 APPENDIX

### Appendix 1: Protocols used during this project

TK, 10.05.11

#### Laetitia: T and B cell Activation Exp#1

**Aim:** compare increase in cell size and cell number over time after activation of splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cells of WT and *bok<sup>-/-</sup>* genetic background.

**Start Date:** 17.05.2011

**Mice:** 1x C57BL/6 WT, 1x *bok<sup>-/-</sup>*

#### Procedure:

**Day -1 (Mon):** pre-coat 96-well plates with anti-CD3 and anti-CD3/antiCD28 monoclonal antibodies (10 µg/ml, each, in PBS); leave at 4°C over night

**Day 0 (Tue):**

- Sacrifice mice, remove spleen and collect in 3ml of RPMI medium; Collect tail biopsies for genotyping
- Prepare single cell suspension using 70 µm cell strainers. Wash 1x, spin
- Perform red blood cell lysis: add 1ml of 1x red cell removal buffer (RCRB) to cell pellet, vortex and leave on ice for 5'; top with PBS, spin
- Resuspend pellet in 1 ml, determine cellularity (dilute 1:10 with Trypan blue to count)
- **perform MACS separation after separate protocols:**

Positive sort for CD4<sup>+</sup> T cells (starting number: ca 10-20x10<sup>6</sup> splenocytes)

Positive sort for CD8<sup>+</sup> T cells (starting number: ca 10-20x10<sup>6</sup> splenocytes)

Negative sort for B cells (starting number: ca 10-20x10<sup>6</sup> splenocytes)

#### Activation of lymphocytes:

-> Seed 2.5x10<sup>4</sup> cells, each, per well (96-well plates; 200 µl per well)

Stimulation: CD4 and CD8 cells:	i) anti-CD3	} All + IL-2
	ii) anti-CD3/CD28	
	iii) Concanavalin A (2 µg/ml)	
B cells:	i) LPS (20 µg/ml)	} All + IL-2, IL-4 and IL-5
	ii) anti-IgM (20 µg /ml)	

**FACS Measurement:**

Collect cells (200 µl) in FACS tubes, add 100 µl of FACS reference beads (in FACS buffer) containing PI

**Important:** prepare enough 'beads mix' for all timepoints of the experiment

-> Analysis: determine a) Mean FSC ( $\Leftrightarrow$  cellular size)

b) Viable (PI-negative) cell number

**Timepoints** (FACS time; collect cells 15-30 min before)

t0: Tues, 15:00

t24: Wed, 15:30

t41: Thu, 8:30

t50: Thu, 18:00

t72: Fri, 15:30

for later timepoints: maybe necessary to dilute cells -> transfer into 48 well, 0.5 ml; in presence of cytokines

t96: Sat (evtl Thomas)

t144: Mon

t168: Tue

## CD4<sup>+</sup> and CD8<sup>+</sup> Magnetic Labeling and Separation (MACS) from Murine Spleen

### Reagents & Materials:

- Staining Buffer = PBS + 3% FCS + 0.1% NaN<sub>3</sub>
- IMag wash buffer = PBS + 0.5% BSA (SIGMA 7.5% stock, endotoxin-free) + 2mM EDTA + 0.1% NaN<sub>3</sub>
- Rat biotinylated anti-mouse CD4 and CD8 antibodies, c=0.5 mg/ml (Biolegend, clones GK1.5 and 53-6.7, 4°C box 2C2 and 2C5)
- 2.4G2 anti-Fc $\gamma$ RII/III blocking antibody from HB-197 hybridoma cell supernatant (4°C - cold room)
- Streptavidin particles Plus -DM = Streptavidin magnetic beads (BD Biosciences #557812, 4°C Ab box 2)
- Normal rat serum (NRS) [Bioconcept/PromoCell], aliquots at -20°C
- IMag magnet
- Capped + sterile FACS tubes (5 ml)
- Plugged + sterile glass Pasteur pipettes
- Growth Medium: FMA medium (DMEM high glucose/ Glutamax + P/S + 50 $\mu$ M 2ME + L-Asn + 10% FCS)

### Protocol:

1. Collect spleen (determine weight!) in Staining Buffer; sieve through 70  $\mu$ m filter (BD)
2. Count cells (determine total cellularity) and resuspend in staining buffer at a concentration of  $2 \times 10^6$  cells/ml in 2.4G2 supernatant, containing 1% NRS.
3. Incubate 15 min on ice.
4. Split in 2 equal parts
5. Add biotin-CD4 and biotin-CD8 antibodies to one tube each, both at 0.0625  $\mu$ g per  $10^6$  cells (= 0.125  $\mu$ l/1x10<sup>6</sup> cells)
6. Incubate 15 min on ice.
7. Add 10x volume of IMag wash buffer.
8. Centrifuge and suck off all supernatant.
9. Add Streptavidin Magnetic beads (vortex well before use) at 2.5  $\mu$ l per 1x10<sup>6</sup> cells.
10. Mix well by pipetting) and incubate 30 min at 6-12°C (put in fridge).
11. Add 1ml of IMag wash buffer.
12. Transfer to sterile, capped FACS tube
13. Place the tube onto the BD IMagnet and incubate for 6-8min.
14. With the tube on the BD IMagnet, carefully aspirate the solution at the bottom of the tube (= negative fraction), using a plugged + sterile glass Pasteur pipette, and transfer to a new tube.
15. Remove the original tube from the BD IMagnet and add 1ml IMAG wash buffer. Gently resuspend the cells by pipetting up and down and return the tube to the BD IMagnet for another 2-4 minutes. Collect solution and combine with negative fraction
16. Repeat step 15.
17. Remove the tube from the BD IMagnet. Resuspend the CD4 or CD8 positive fraction in 1ml FMA medium.
18. Count the cells in the CD4 or CD8 positive fractions and use for experiments.

## Counting Cells by FACS with Reference Beads

**Beads:** Peak flow Flow Cytometry Reference Beads (FITC, 6µm) Molecular  
Probes # P-14828. Supplied @  $\sim 1.7 \times 10^7$  beads/ml; use @ 40  
beads/µl  
Have high SSC, low FSC and high FITC

**Make stock solution -> USE SAME SOLUTION FOR ALL TIMEPOINTS OF YOUR  
EXPERIMENT!**

e.g.

5 mL FACS buffer (= KDS.BSS + 2% FCS + 10mM  $\text{NaN}_3$ ; filtered)

$2 \times 10^5$  beads = 11.76 µL (vortex beads well before use)

4 µg/ml PI (add 40 µL of 500 µg/ml PI stock solution to 5 ml)

- Add 100 µL of this to 100 µL cells (96 well plate) - i.e. 4000 beads per well
- Remark: it is important to add beads first to wells and then mix and transfer  
cells/ beads mix into FACS tube

FACS- gate on live cells, count these. Then use the ratio of cells to beads as to  
calculate the number of cells in the sample.

Total no cells in sample =  $4000 \times (\text{cells counted}) / (\text{beads counted})$

## Protease & Phosphatase Inhibitors for Protein Extract Preparation

Add fresh to your lysis buffer (e.g. ONYX buffer or RIPA buffer); all inhibitors in the same box at -20°C/ Biochemistry drawer)

*Alternatively:* prepare lysis buffer with all inhibitors in advance and freeze down aliquots (only thaw once)

- **Roche Complete protease inhibitor cocktail (containing EDTA)**  
**50x STOCK** (in H<sub>2</sub>O), 20 µl aliquots -> 1 aliquot/ml lysis buffer
- **Pepstatin (Asp protease inhibitor -> mainly for Cathepsin D!)**  
 0.5 mg/ml = **500x STOCK** (in MeOH) -> add 2 µl/ml buffer
- **Optional: phosphatase inhibitors** (sodium fluoride + sodium vanadate)
  - **NaF:** 1M (in H<sub>2</sub>O) = **20x Stock**; use at 50 mM <=> 50 µl/ml
  - **Na<sub>3</sub>VO<sub>4</sub>:** 200 mM (in H<sub>2</sub>O) = **100x Stock** use at 2 mM <=> 10 µl/ml

Example: preparation of 5 ml 1x ONYX buffer

2x ONYX buffer:	2.5 ml
Protease Inhibitor 50x:	0.1 ml
Pepstatin A 500x:	0.01 ml
NaF 20x:	0.25 ml
Na <sub>3</sub> VO <sub>4</sub> 100x:	0.05 ml
ddH <sub>2</sub> O:	2.09 ml
-----	-----
Total:	5.00 ml

## Bok (del535) Genotyping Primers

- *bok 17 (bok5's)*

5' cggggtttgaatggaagggtc      20mer, %GC: 55%, T<sub>m</sub>=54°C

- *bok 18 (bok5'as)*

5' tgttcccatggtgctacatcc      21mer, %GC: 52.4%, T<sub>m</sub>=54°C

- *bok 20 (bok3'as)*

5' gagctagctagctatgtgtg      20mer, %GC: 50%, T<sub>m</sub>=52°C

## GENOTYPING PCR

3- Primer reaction (standard PCR conditions; V<sub>tot</sub> 20-50 µl). Use double amount of common primer (bok17). Final primer concentrations:

bok17:	50 nM
bok 18:	25 nM
bok 20:	25 nM

DNA template: 1 µl (genomic DNA from tail biopsies in ~ 200 µl TE buffer)

PCR program:

94°C / 2 min

94°C/ 30 sec

55°C/ 30 sec

72°C/ 45 sec


 35 cycles

72°C / 5 min

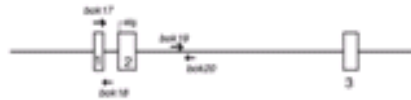
25°C / forever

Wt allele (+):      **150 bp** (bok17/bok18)

Deleted allele (bok):      **383 bp** (bok17/bok20)

## GENOTYPING PRIMERS

*bok* wildtype allele



recombined allele: *bok<sup>f</sup>*



*bok<sup>f</sup> x Cre => bok<sup>-</sup>*



## Genomic DNA from Mouse Tail Biopsies:

Tail lysis buffer:	50 mM Tris/HCl pH 8.0	-> 25 ml 1M Tris/HCl pH 8.0
	200 mM NaCl	-> 20 ml 5M NaCl
	0.5% SDS	-> 12.5 ml 20% SDS
	5 mM EDTA	-> 5 ml 0.5M EDTA
		-> 437.5 ml H <sub>2</sub> O to 500ml

- add 500 µl of tail lysis buffer to tail (in safe lock Eppli)
- add 25 µl of Proteinase K (20 mg/ml; Roche)
- digest o/n at 56°C in Eppendorf thermomixer (+ shaking)

Next day:

- spin 13'000 rpm/ 5 min
- transfer 450 µl into new Eppli (make sure to leave hair back)
- add 500 µl of iPrOH (isopropanol)
- mix until DNA precipitates
- spin 13'000 rpm/ 15 min -> discard SN
- wash with 500 µl 70% EtOH -> discard SN and tip tube on tissue
- let tubes dry "upside down"
- add 150 µl of TE buffer to dried DNA pellets
- let resuspend at 37°C/30 min or o/n at RT
- vortex DNA well prior to pipetting for PCR
- use 1 µl for genotyping PCR reaction

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## Mast Cell - CD45.1/CD45.2 Competition, Exp#1

Date: 03.05.2011 -

### A) Seeding of cells:

1. harvest cells in 15ml Falcon tube, centrifuge (1'500 rpm, 5 min), discard supernatant
2. resuspend pellet in 0.5 ml RPMI medium count cells: WT (CD45.1), WT (CD45.2) and bok-/- (CD45.2)
3. dilute to  $2.5 \times 10^6$ /ml in RPMI medium
4. Prepare following mixes: **a)** WT (5.1) + WT (5.2): 400 ul + 400 ul  
**b)** WT(5.1) + bok-/-: 400 ul + 400 ul
5. Add 20 ul of mix per well in 48-well plate. Seed 3 x 8 wells per mix. Keep remaining mix on ice for  $t_0$  measurement
6. Add 480 ul of
  - RPMI + 10% IL3/10% SCF: wells 1-8 (of each mix)
  - RPMI + 1% IL3/1% SCF: wells 9-16
  - RPMI + 0.1% IL3/0.1% SCF: wells 17-24
 (prepare 10 ml of each RPMI+cytokine mix)

### B) CD45.1/CD45.2 FACS Analysis:

Staining buffer: FACS buffer + 1% NRS + 10% 2.4G2

Antibodies (keep sterile): anti-CD45.1-PE: use 1/500  
 anti-CD45.2-FITC: use 1/500

**$t_0$  measurement:** - use one of the CD45.1/CD45.2 mixes for the following  
 control: C1) CD45.1-PE single stain  
 C2) CD45.2-FITC single stain  
**samples:** 1) WT + WT -> CD45.1-PE/CD45.2-FITC  
 2) WT + bok -> CD45.1-PE/CD45.2-FITC

**further timepoints:** every 24 h for up to 7 days(?)

Staining:

- harvest cells in FACS tube, top with FACS buffer, spin
- decant supernatant
- antibody staining in 100 ul, 20-30 min on ice (covered from light)
- wash 1x in FACS buffer
- decant + tipp off excess liquid in Kimwipe
- add 200 ul FACS buffer + 25 ul **PI** (25 ug/ml) -> ready to measure

## Gel recipes

### Laemmli gel (1mm)

4% Stacking Gel	1 Gel	2 Gel	3 Gel	4 Gel	4 gel caster
30 % Acrylamide stock	0.4 ml	0.8 ml	1.2 ml	1.6 ml	2 ml
0.5 M TrisCl pH = 6.8 (Stacking Gel)	0.75 ml	1.5 ml	2.25 ml	3 ml	3.75 ml
20% SDS	15 µl	30 µl	45 µl	60 µl	75 µl
Deionized H2O	1.805 ml	3.61 ml	5.415 ml	7.22 ml	9.025 ml
10% APS 2x	15 µl	30 µl	45 µl	60 µl	75 µl
TEMED (1:10) 2x	15 µl	30 µl	45 µl	60 µl	75 µl
Final Volume	3 ml	6 ml	9 ml	12 ml	15 ml

12.5% Separating Gel	1 Gel	2 Gel	3 Gel	4 Gel	4 gel caster
30 % Acrylamide stock 4°C	3.333 ml	6.667 ml	10 ml	13.333 ml	16.667 ml
1.5 M TrisCl, pH = 8.8 (Separating Gel)	2 ml	4 ml	6 ml	8 ml	10 ml
20% SDS	40 µl	80 µl	120 µl	160 µl	200 µl
Deionized H2O	2.5605 ml	5.121 ml	7.6815 ml	10.242 ml	12.8025 ml
10% APS	40 µl	80 µl	120 µl	160 µl	200 µl
TEMED (1:10) 4°C	26.5 µl	53 µl 30 µl 6x10µl	79.5 µl	106 µl	132.5 µl
Final Volume	8 ml	16 ml	24 ml	32 ml	40 ml

## Appendix 2: PCR marker

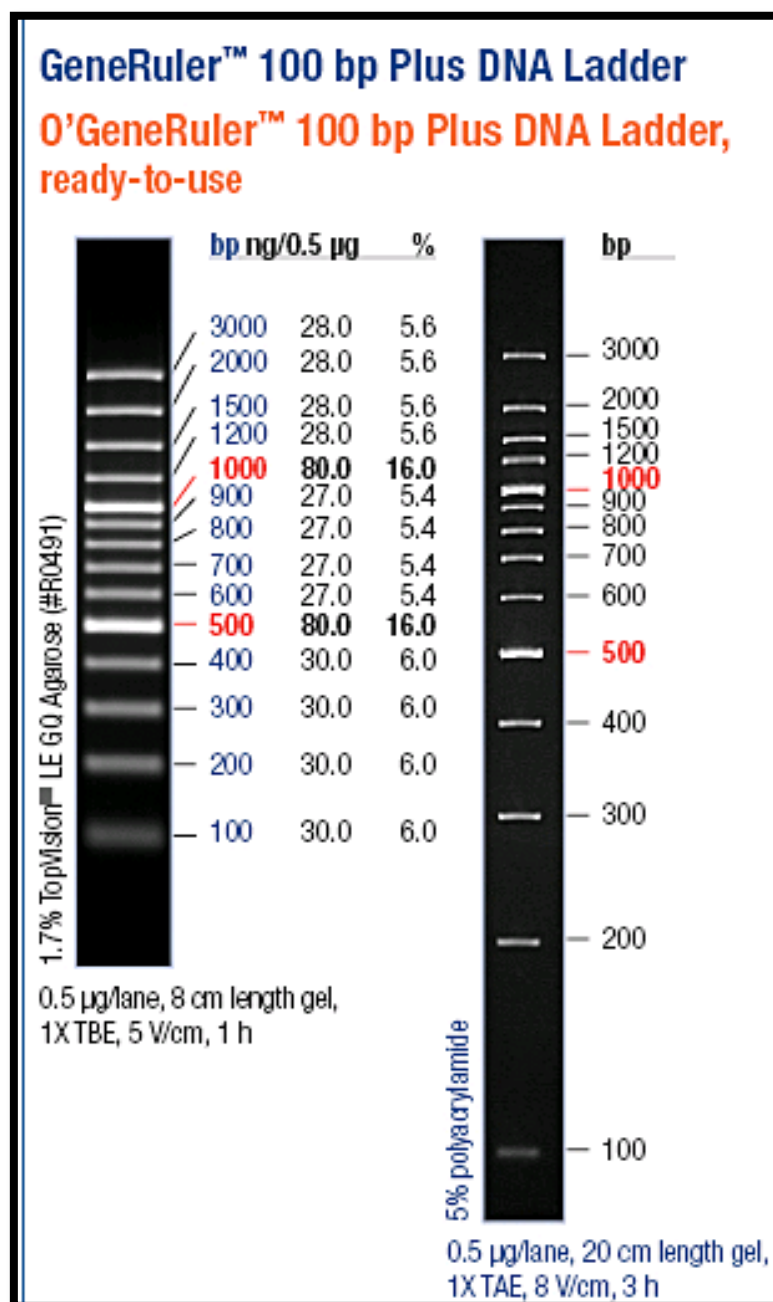
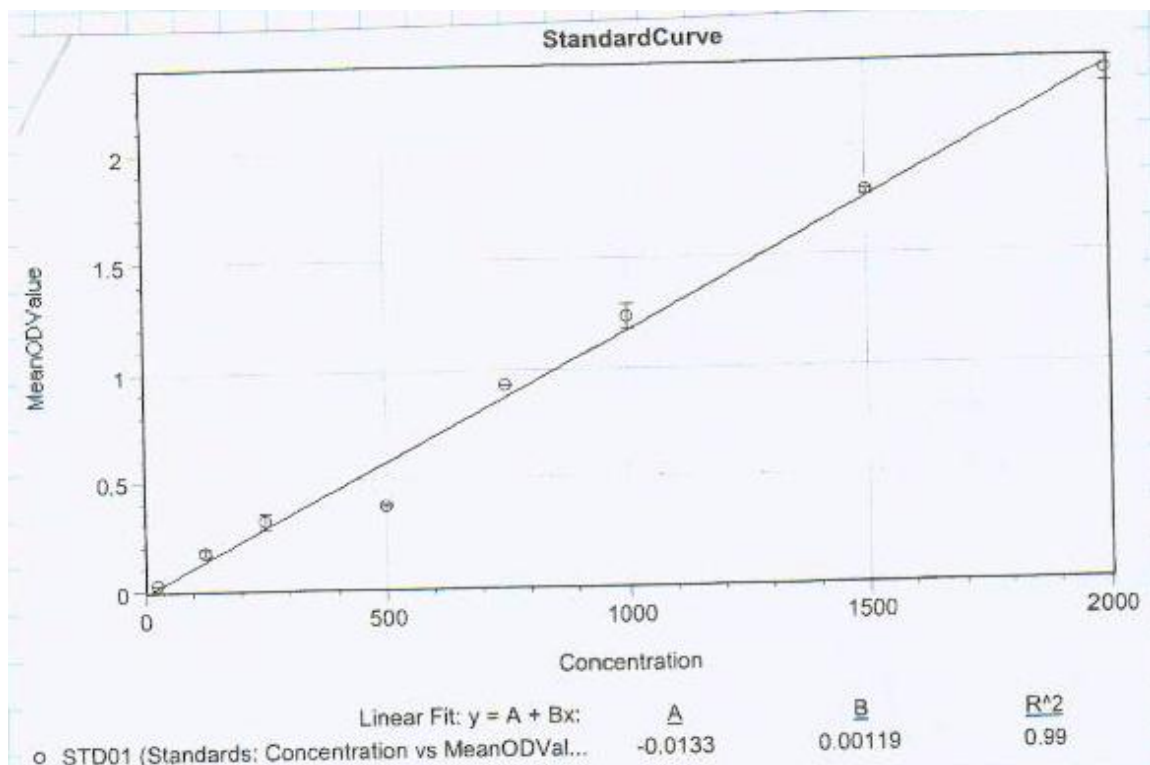


Figure 30 : GeneRuler™ 100pb Plus DNA Ladder used for genotyping [27]

## Appendix 3: Calibration curve for proteins quantification

**Table 3: Results of the calibration curve**

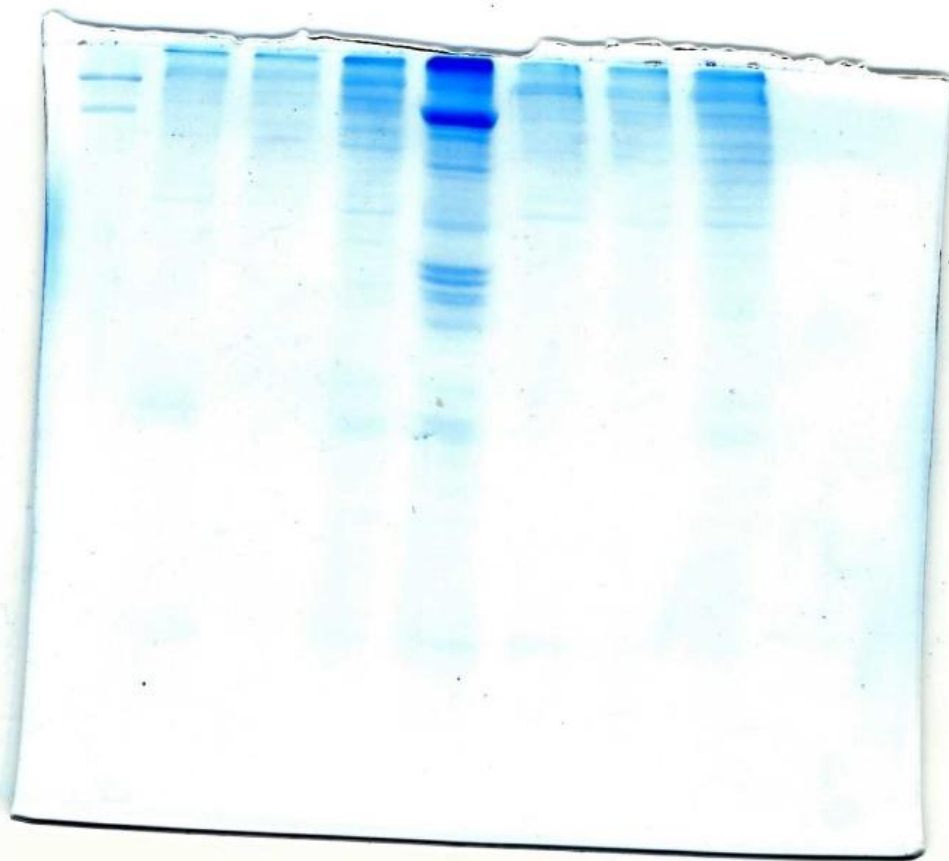
	Concentration [µg/mL]	Absorbance
Standard 1	2000	2.333
Standard 2	1500	1.786
Standard 3	1000	1.239
Standard 4	750	0.930
Standard 5	500	0.385
Standard 6	250	0.315
Standard 7	125	0.175
Standard 8	25	0.026



**Figure 31 : Standard curve of bovin serum albumin (BSA) used for proteins quantification by spectrophotometry**

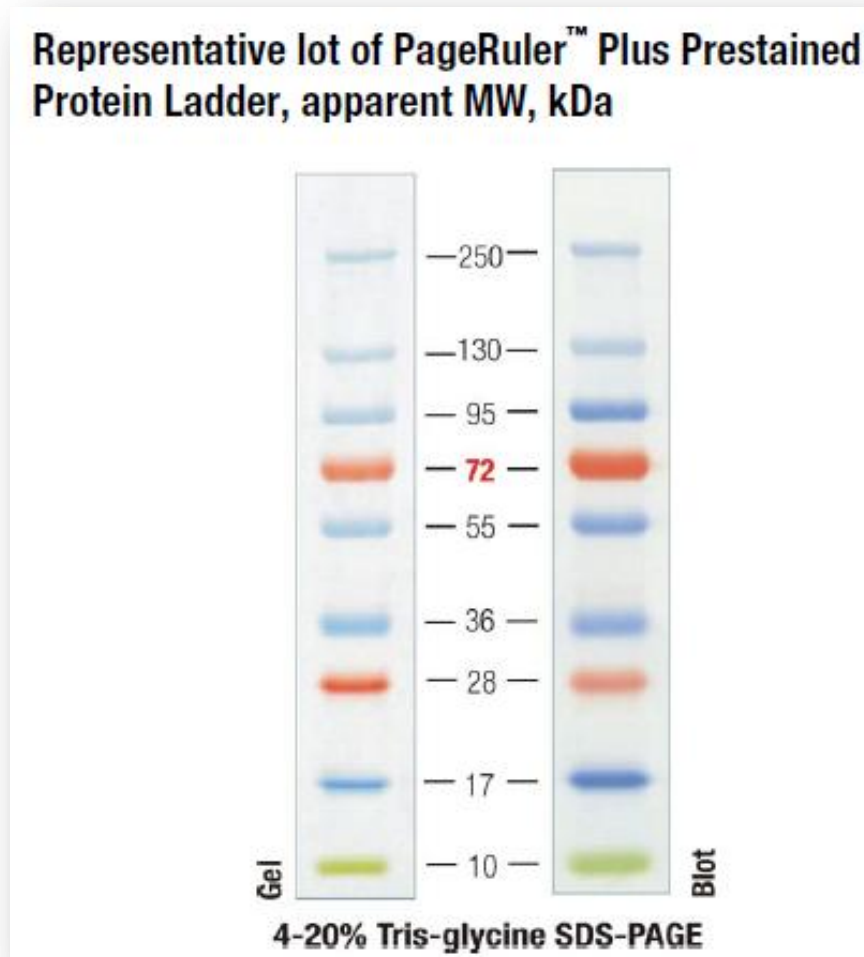


## Appendix 4: Coomassie gel from protein extracts



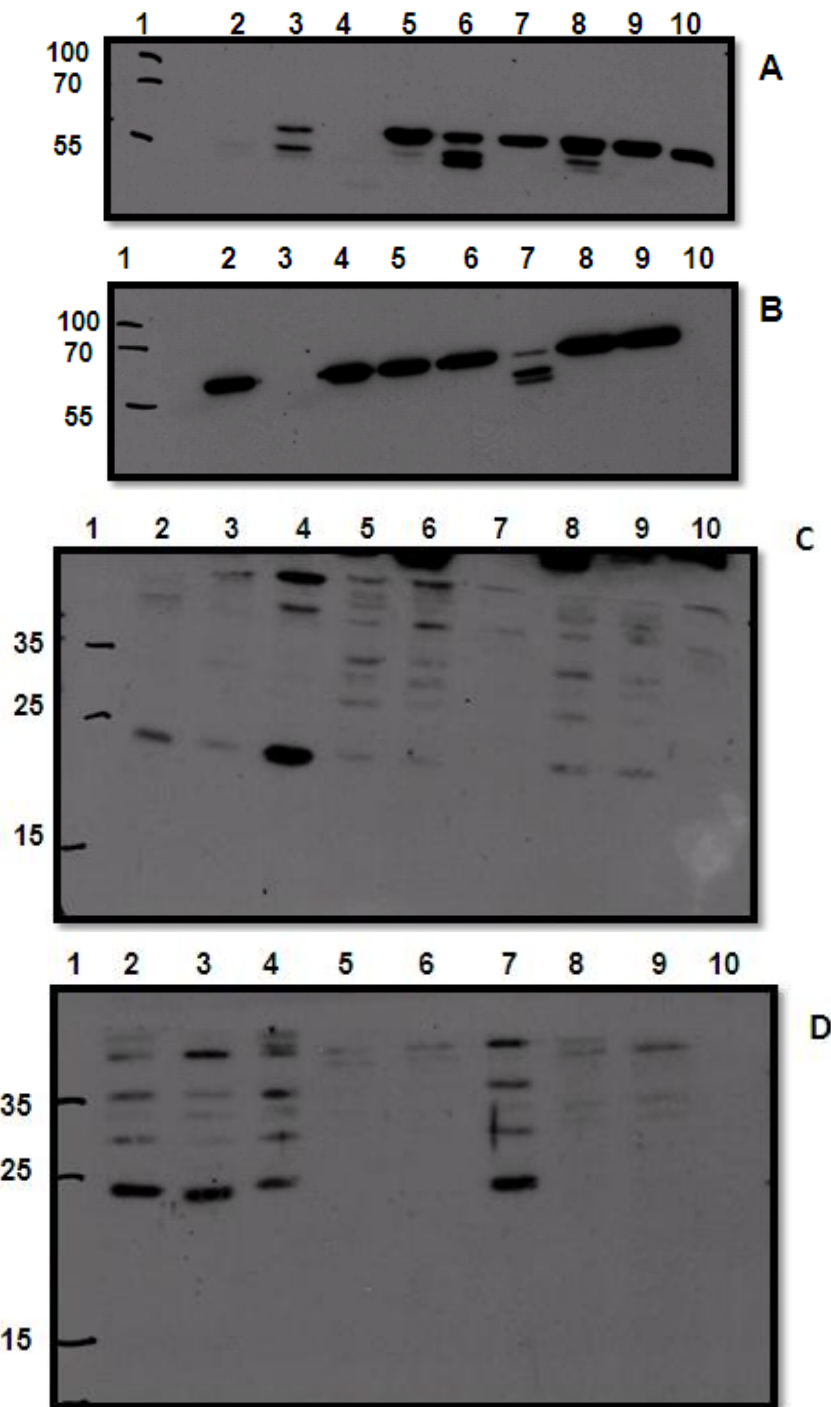
*Figure 32 : Gel control from B cells protein extract colored with Coomassie blue, 19.07.2011*

## Appendix 5: Western blot marker



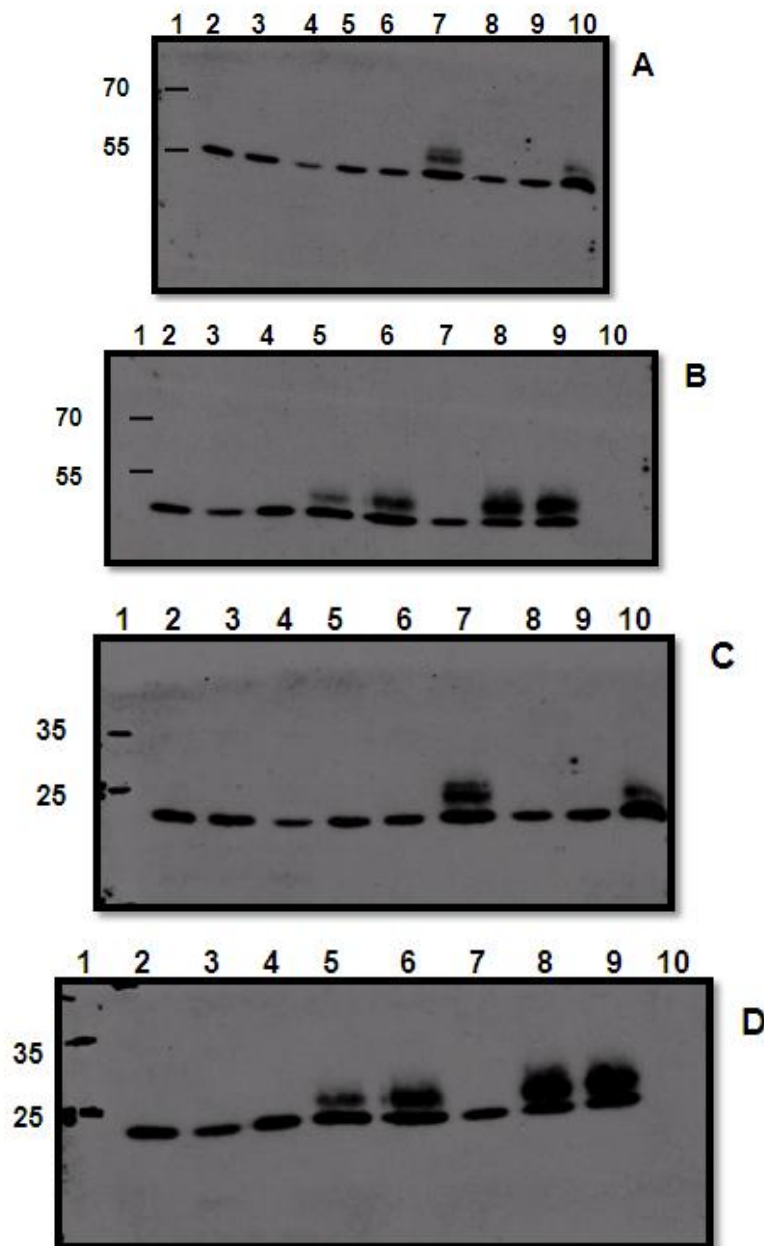
**Figure 33 : Marker used for Western blot. PageRuler Plus prest. Prot Ladder, SM1811, Fermentas [27]**

## Appendix 6: Western blots from protein extracts

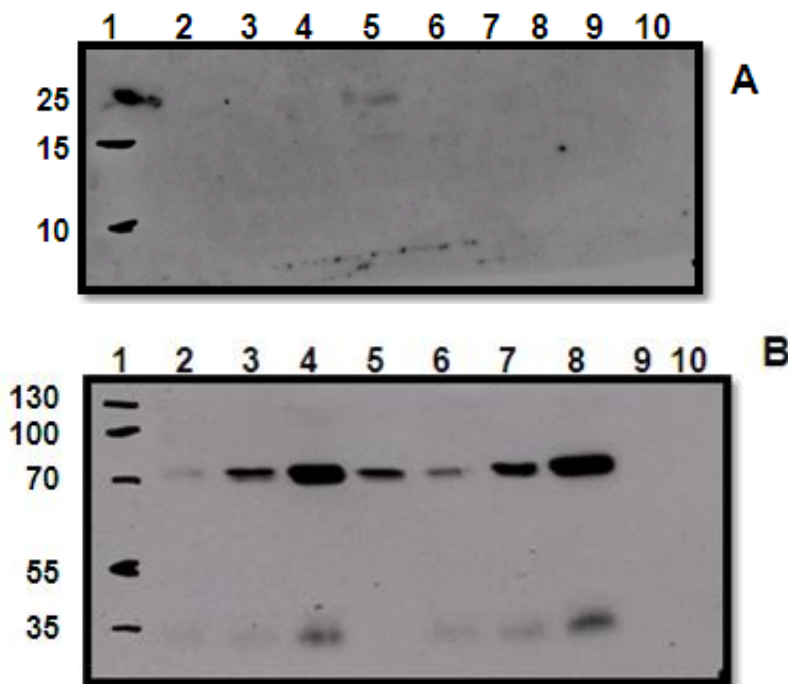


**Figure 34 : (A) Western blot from B and mast cell protein extracts revealed with anti-tubulin during 15 sec exposition, 08.07.2011. PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - Mast cells WT5.1 extract from 17.06.2011 (2) - Mast cells WT5.2 extract from 17.06.2011 (3) - Mast cells  $bok^{-/-}$  extract from 17.06.2011 (4) - Mast cells WT5.1 extract from 27.06.2011 (5) - Mast cells  $bok^{-/-}$  extract from 27.06.2011 (6) - B cells WT extract T0h from 28.06.2011 (7) - Mast cells  $bok^{-/-}$  extract T24h from 28.06.2011 (8) - Mast cells WT extract T24h from 28.06.2011 (9) B cells  $bok^{-/-}$  extract T0h from 28.06.2011 (10) (B) Western blot from B and mast cell protein extracts revealed with anti-tubulin during 15 sec exposition, 08.07.2011. PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - Mast cells WT extract T72h from 30.06.2011 (2) - Mast cells  $bok^{-/-}$  extract**

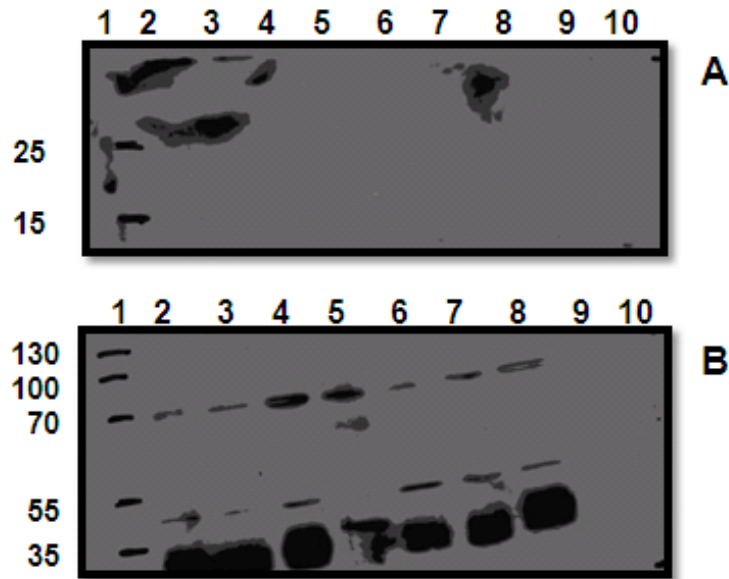
T72h from 30.06.2011 (3) – Mast cells WT extract T48h from 29.06.2011 (4) – B cells WT extract T24h from 29.06.2011 (5) – B cells *bok*<sup>-/-</sup> extract T24h from 29.06.2011 (6) – Mast cells *bok*<sup>-/-</sup> extract T48h from 29.06.2011 (7) – B cells WT extract T48h from 30.06.2011 (8) – B cells *bok*<sup>-/-</sup> extract T48h from 30.06.2011 (9) *ddH<sub>2</sub>O* (10)  
**(C) Western blot from B and mast cell protein extracts revealed with anti-bok during 5 min exposition, 08.07.2011.** PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) – Mast cells WT5.1 extract from 17.06.2011 (2) – Mast cells WT5.2 extract from 17.06.2011 (3) – Mast cells *bok*<sup>-/-</sup> extract from 17.06.2011 (4) – Mast cells WT5.1 extract from 27.06.2011 (5) – Mast cells *bok*<sup>-/-</sup> extract from 27.06.2011 (6) – B cells WT extract T0h from 28.06.2011 (7) – Mast cells *bok*<sup>-/-</sup> extract T24h from 28.06.2011 (8) – Mast cells WT extract T24h from 28.06.2011 (9) B cells *bok*<sup>-/-</sup> extract T0h from 28.06.2011 (10) **(D) Western blot from B and mast cell protein extracts revealed with anti-bok during 5 min exposition, 08.07.2011.** PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) – Mast cells WT extract T72h from 30.06.2011 (2) – Mast cells *bok*<sup>-/-</sup> extract T72h from 30.06.2011 (3) – Mast cells WT extract T48h from 29.06.2011 (4) – B cells WT extract T24h from 29.06.2011 (5) – B cells *bok*<sup>-/-</sup> extract T24h from 29.06.2011 (6) – Mast cells *bok*<sup>-/-</sup> extract T48h from 29.06.2011 (7) – B cells WT extract T48h from 30.06.2011 (8) – B cells *bok*<sup>-/-</sup> extract T48h from 30.06.2011 (9) *ddH<sub>2</sub>O* (10)



**Figure 35 : (A) Western blot from B and mast cells protein extract revealed with anti-tubulin during 2 sec exposition, 14.07.2011.** PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - Mast cells WT5.1 extract from 17.06.2011 (2) - Mast cells WT5.2 extract from 17.06.2011 (3) - Mast cells bok<sup>-/-</sup> extract from 17.06.2011 (4) - Mast cells WT5.1 extract from 27.06.2011 (5) - Mast cells bok<sup>-/-</sup> extract from 27.06.2011 (6) - B cells WT extract T0h from 28.06.2011 (7) - Mast cells bok<sup>-/-</sup> extract T24h from 28.06.2011 (8) - Mast cells WT extract T24h from 28.06.2011 (9) B cells bok<sup>-/-</sup> extract T0h from 28.06.2011 (10) **(B) Western blot from B and mast cells protein extract revealed with anti-tubulin during 2 sec exposition, 14.07.2011** PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - Mast cells WT extract T72h from 30.06.2011 (2) - Mast cells bok<sup>-/-</sup> extract T72h from 30.06.2011 (3) - Mast cells WT extract T48h from 29.06.2011 (4) - B cells WT extract T24h from 29.06.2011 (5) - B cells bok<sup>-/-</sup> extract T24h from 29.06.2011 (6) - Mast cells bok<sup>-/-</sup> extract T48h from 29.06.2011 (7) - B cells WT extract T48h from 30.06.2011 (8) - B cells bok<sup>-/-</sup> extract T48h from 30.06.2011 (9) ddH<sub>2</sub>O (10) **(C) Western blot from B and Mast cells protein extract revealed with anti-Bcl-2 during 4 min exposition,, anti-Bcl-2, 14.07.2011** PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - Mast cells WT5.1 extract from 17.06.2011 (2) - Mast cells WT5.2 extract from 17.06.2011 (3) - Mast cells bok<sup>-/-</sup> extract from 17.06.2011 (4) - Mast cells WT5.1 extract from 27.06.2011 (5) - Mast cells bok<sup>-/-</sup> extract from 27.06.2011 (6) - B cells WT extract T0h from 28.06.2011 (7) - Mast cells bok<sup>-/-</sup> extract T24h from 28.06.2011 (8) - Mast cells WT extract T24h from 28.06.2011 (9) B cells bok<sup>-/-</sup> extract T0h from 28.06.2011 (10) **(D) Western blot from B and Mast cells protein extract revealed with anti-Bcl-2 during 4 min exposition, 14.07.2011** PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - Mast cells WT extract T72h from 30.06.2011 (2) - Mast cells bok<sup>-/-</sup> extract T72h from 30.06.2011 (3) - Mast cells WT extract T48h from 29.06.2011 (4) - B cells WT extract T24h from 29.06.2011 (5) - B cells bok<sup>-/-</sup> extract T24h from 29.06.2011 (6) - Mast cells bok<sup>-/-</sup> extract T48h from 29.06.2011 (7) - B cells WT extract T48h from 30.06.2011 (8) - B cells bok<sup>-/-</sup> extract T48h from 30.06.2011 (9) ddH<sub>2</sub>O (10)



**Figure 36 : (A) Western blot from B cells protein extract revealed with anti-bok during 5 min exposition, 19.07.2011** PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - B cells WT extract T0h from 28.06.2011 (2) - B cells bok<sup>-/-</sup> extract T0h from 28.06.2011 (3) - B cells WT extract T24h from 29.06.2011 (4) - Hepatocyte extract (5) - B cells bok<sup>-/-</sup> extract T24h from 29.06.2011 (6) - B cells WT extract T48h from 30.06.2011 (7) - B cells bok<sup>-/-</sup> extract T48h from 30.06.2011 (8) - ddH<sub>2</sub>O (9) - ddH<sub>2</sub>O (10) **(B) Western blot from B cells protein extract revealed with anti-tubulin during 5 sec exposition, 19.07.2011** PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - B cells WT extract T0h from 28.06.2011 (2) - B cells bok<sup>-/-</sup> extract T0h from 28.06.2011 (3) - B cells WT extract T24h from 29.06.2011 (4) - Hepatocyte extract (5) - B cells bok<sup>-/-</sup> extract T24h from 29.06.2011 (6) - B cells WT extract T48h from 30.06.2011 (7) - B cells bok<sup>-/-</sup> extract T48h from 30.06.2011 (8) - ddH<sub>2</sub>O (9) - ddH<sub>2</sub>O (10)



**Figure 37 : (A) Western blot from B cells protein extract revealed with anti-Bim during 5 min exposition, 20.07.2011. PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - B cells WT extract T0h from 28.06.2011 (2) - B cells *bok<sup>-/-</sup>* extract T0h from 28.06.2011 (3) - B cells WT extract T24h from 29.06.2011 (4) - Hepatocyte extract (5) - B cells *bok<sup>-/-</sup>* extract T24h from 29.06.2011 (6) - B cells WT extract T48h from 30.06.2011 (7) - B cells *bok<sup>-/-</sup>* extract T48h from 30.06.2011 (8) - *ddH<sub>2</sub>O* (9) - *ddH<sub>2</sub>O* (10) (B) Western blot from B cells protein extract revealed with anti-tubulin during 5 min exposition, 20.07.2011 PageRuler Plus prest. Prot Ladder, SM1811, Fermentas (1) - B cells WT extract T0h from 28.06.2011 (2) - B cells *bok<sup>-/-</sup>* extract T0h from 28.06.2011 (3) - B cells WT extract T24h from 29.06.2011 (4) - Hepatocyte extract (5) - B cells *bok<sup>-/-</sup>* extract T24h from 29.06.2011 (6) - B cells WT extract T48h from 30.06.2011 (7) - B cells *bok<sup>-/-</sup>* extract T48h from 30.06.2011 (8) - *ddH<sub>2</sub>O* (9) - *ddH<sub>2</sub>O* (10)**

## Appendix 7: Fluorochromes

**Table 4: List of the most common used fluorochromes for flow cytometry [3]**

Fluorochrome	Fluorescence Emission Color	Ex-Max (nm)	Excitation Laser Line (nm)*	Em-Max (nm)
Hoechst 33342	Blue	350	355, 375	461
BD Horizon™ V450	Blue	404	405	448
Pacific Blue™	Blue	401	405	452
BD Horizon™ V500	Green	415	405	500
AmCyan	Green	457	405	491
Alexa Fluor® 488	Green	495	488	519
FITC	Green	494	488	519
PE	Yellow	496, 564	488, 532, 561	578
PE-Texas Red®	Orange	496, 564	488, 532, 561	615
PI	Orange	351	488, 532, 561	617
7-AAD	Red	543	488, 532, 561	647
APC†	Red	650	633, 635, 640	660
Alexa Fluor® 647	Red	650	633, 635, 640	668
PE-Cy™5†	Red	496, 564	488, 532, 561	667
PerCP	Red	482	488, 532	678
PerCP-Cy™5.5	Far Red	482	488, 532	695
Alexa Fluor® 700	Far Red	696	633, 635, 640	719
PE-Cy™7	Infrared	496, 564	488, 532, 561	785
APC-Cy7	Infrared	650	633, 635, 640	785
BD APC-H7	Infrared	650	633, 635, 640	785