

# **Bcl-x<sub>L</sub> deamidation in oncogenic tyrosine kinase signalling**

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## **ACKNOWLEDGEMENTS**

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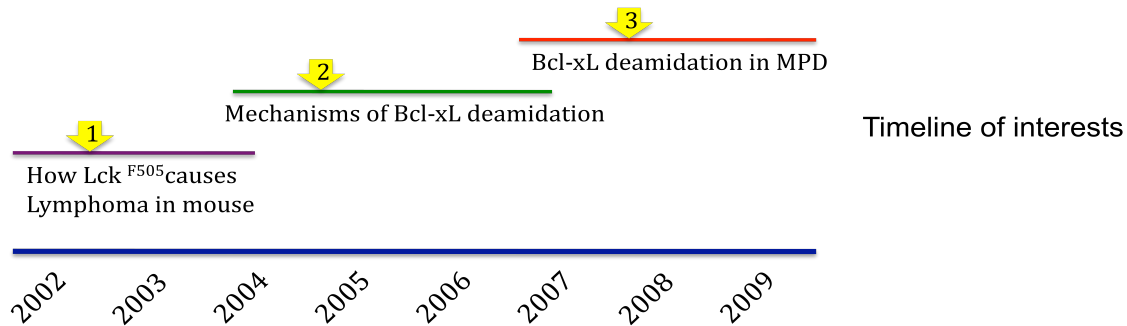
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## TIMELINE



### Fig 1. Key experiments in the progress of this project

The project encompassed three stages, which were represented in three time lines. A key experiment (represented as a yellow arrow symbol) in each stage, which has been critical in the development of this project, is also shown.

Key experiment 1. DNA damage-induced Bcl-x<sub>L</sub> deamidation is inhibited by oncogenic Lck<sup>F505</sup>, Fig 7A, Zhao R 2004

Key experiment 2. Bcl-x<sub>L</sub> deamidation induced by DNA damage involves up-regulation of the NHE-1 antiport, Fig 4, Zhao R 2007

Key experiment 3. Inhibition of the NHE-1/ Bcl-x<sub>L</sub> deamidation pathway induced by DNA damage in Chronic Myelogenous Leukaemia cells, Fig 1, Zhao R 2008

## SUMMARY

I have been interested in the molecular mechanisms of Haematopoietic malignant diseases such as leukaemia and lymphoma, especially those involving oncogenic tyrosine kinases. About 30 of the 90 tyrosine kinases in the human genome have been implicated in cancer (Blume-Jensen P, 2001). The oncogenic tyrosine kinases (OTKs), such as Bcr-Abl (product of chromosomal translocations of two genes bcr and abl) in Chronic Myelogenous Leukaemia, and Erythroblastic leukaemia viral oncogene homolog 2(Erb-B2) in mammary and other cancers, mediate their transforming effects via a diverse array of signalling pathways involved in DNA damage, cell survival and cell cycle regulation (Deutsch E, 2001; Skorski T, 2002; Kumar R, 1996)

My work has been centred around the analysis of a mouse cancer model that is driven by an oncogenic tyrosine kinase – p56<sup>Lck-F505</sup> expressed on CD45 knock- out background (Baker M, 2000). The investigation of this mouse model has revealed that oncogenic inhibition of deamidation of the Bcl-x<sub>L</sub> survival protein plays a critical role in protecting thymocytes from DNA-damage induced apoptosis. Cells that would normally be eliminated due to accumulating DNA damage are instead preserved with an increasing load of double-stranded breaks, leading to genomic instability, chromosomal abnormalities and transformation. This work was published in Cancer Cell (An oncogenic tyrosine kinase inhibits DNA repair and DNA-damage-induced Bcl-x<sub>L</sub> deamidation in T cell transformation. Zhao R, 2004). Following that I have tried to elucidate the different roles of the two deamidated species of Bcl-x<sub>L</sub> in apoptosis, and also the molecular mechanisms of DNA damage- induced Bcl-x<sub>L</sub> deamidation in order to understand the inhibition of Bcl-x<sub>L</sub> deamidation by oncogenic tyrosine kinases. Recently I have shown that Bcl-x<sub>L</sub> deamidation, whereby two critical Asn residues are converted to iso-Asp, cripples the ability of the protein to sequester pro-apoptotic BH3-only proteins such as Bim and p53- upregulated modulator of apoptosis (PUMA), thereby explaining its loss of pro-survival functionality. *In vivo*, DNA damage causes intracellular alkalinisation that is both necessary and sufficient to deamidate Bcl-x<sub>L</sub>, promoting apoptosis: no enzyme is necessary for this process. In pre-tumourigenic thymocytes alkalinisation is blocked, so preserving Bcl-x<sub>L</sub> in its pro-survival mode.

Furthermore murine tumours are protected from genotoxic attack by native Bcl-x<sub>L</sub>, but enforced alkalinisation and consequent Bcl-x<sub>L</sub> deamidation promotes apoptosis. This part of work was published in Plos Biology (DNA damage-induced Bcl-x<sub>L</sub> deamidation is mediated by NHE-1 antiport regulated intracellular pH. Zhao R, 2007).

Through collaboration with Prof AR Green's research group at the Department of Haematology of the University of Cambridge, I have also analysed the Bcl-x<sub>L</sub> deamidation pathway in human myeloproliferative disorders, e.g. Polycythemia vera(PV) and Chronic Myelogenous Leukaemia (CML). We found that the oncogenic tyrosine kinases involved in these disorders, i.e. Jak2<sup>V617F</sup> and Bcr-Abl also inhibit the Bcl-x<sub>L</sub> deamidation pathway in DNA damage responses. These findings shed light on potential therapeutic application of the Bcl-x<sub>L</sub> deamidation pathway in human malignancies. This piece of work was recently published in the New England Journal of Medicine (Inhibition of the Bcl-x<sub>L</sub> deamidation pathway in myeloproliferative disorders. Zhao R, 2008).

Overall the cited work has led to several important new insights into the molecular mechanisms involved in oncogenesis: first, that Bcl-x<sub>L</sub> deamidation is important in the cascade of events leading from DNA damage to apoptosis; second, that oncogenic tyrosine kinases inhibit these events in both the murine and human context; third, that up-regulation of the NHE-1 antiport and consequent intracellular alkalinisation are critical events in this DNA damage-induced cascade leading to apoptosis. In the process I have demonstrated the first in vivo mechanism for the deamidation of an internal protein Asn. Essentially, a completely new and unexpected signalling pathway has been uncovered that seems to pertain to all murine and human haematopoietic cell lineages that have been investigated so far.

## INTRODUCTION

Tyrosine kinases play important roles in cellular function. They normally behave as tightly regulated switches in the signal transduction network, however they also have the potential to induce oncogenic transformation.

Receptor tyrosine kinases (RTKs) such as EGFR and Erb-B2, are composed of an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic domain that contains a catalytic kinase core and regulatory sequences (Schlessinger *et al.*, 2000). RTKs have three general mechanisms to become oncogenic. Firstly, genomic rearrangements can generate fused proteins that maintain the kinase in a more stable manner; Secondly, gene amplification leads to spontaneous dimerisation that stabilizes the kinase; Finally, some RTKs acquire point mutations that allow them to dimerise and stabilize (Blume-Jensen and Hunter, 2001).

Non-receptor tyrosine kinases, such as Src, Lck and Abl, lack extracellular and transmembrane domains. Their mechanisms of oncogenic activation are varied. Some become constitutively active by fusion to a dimerising partner, while others are transformed to onco-proteins by acquiring mutations that disrupt autoinhibitory functions (Blume-Jensen and Hunter, 2001).

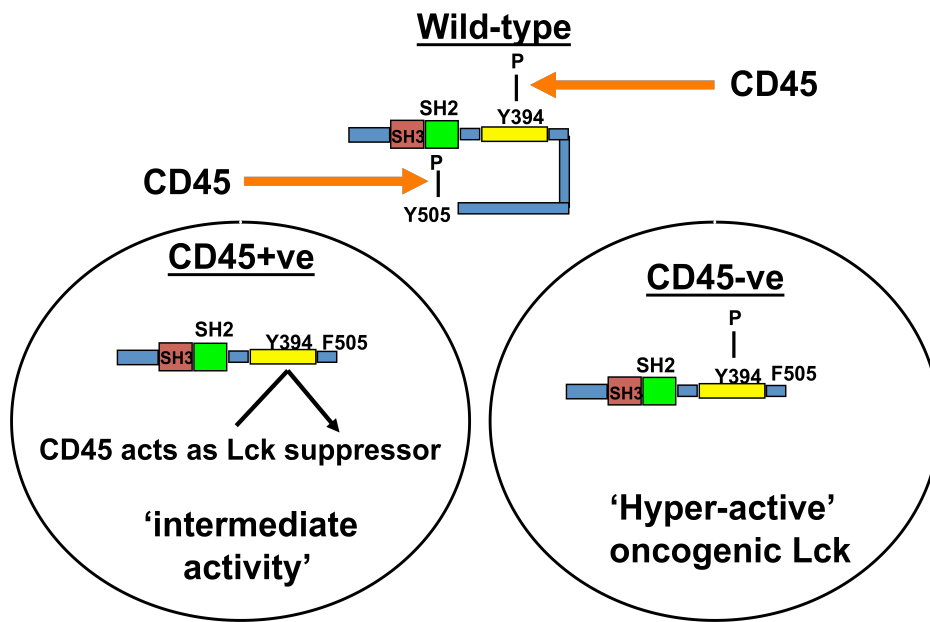
Oncogenic Tyrosine Kinases (OTKs) are extensively involved in cancer by promoting proliferation, invasion, and metastasis. Compared to other onco-proteins, OTKs are unique in that they tend to render cells extraordinarily resistant to DNA damage-induced apoptosis (Skorski *et al.*, 2002). This is demonstrated clinically that cancers that express OTKs are usually highly resistant to radio- and chemo- therapy. This clinical observation has attracted great interests from clinicians and researchers. The resistance, although likely contributed by several mechanisms, has been found consistently linked with markedly increased Bcl-x<sub>L</sub> expression (Kumar *et al.*, 1996; Amarante-Mendes *et al.*, 1998; Karni *et al.*, 1999; Zamo *et al.*, 2002).

Bcl-x<sub>L</sub> is an anti-apoptotic member of the Bcl-2 family. Like Bcl-2, Bcl-x<sub>L</sub> is believed to act by binding and sequestering BH3-only proteins such as Bim, thereby

preventing their pro-apoptotic interactions with Bax (Cheng EH, 2001; Kuwana T, 2002). Bcl-x<sub>L</sub> deamidation in response to DNA damage has been recently proposed as a critical switch to subvert the pro-survival function of Bcl-x<sub>L</sub> (Deverman BE, 2002).

P56<sup>Lck</sup> is a member of src family tyrosine kinases expressed predominantly in T thymocytes. Lck is constitutively located to membranes as a result of modification of amino acid residues close to the N-terminus that are sites of myristoylation/palmitoylation (Kabouridis PS, 1997). The N-terminal domain contains a di-cysteine motif that is required for association with the CD4 and CD8 coreceptors (Kim PW, 2003). As with other src-family kinases, the regulation of kinase activity/functionality is tightly controlled by conformational changes arising from binding of ligands to the SH3 and/or Sh2 domains of the kinases (Holdor AD, 1999; da Silva AJ, 1997) and by the phosphorylation and dephosphorylation of two critical tyrosine residues (Palacios EH, 2004).

Earlier studies (Koretzky GA, 1990; Shiroo M, 1992; Kishihara K, 1993; Byth KF, 1996; Mee PJ, 1999) have established both positive and negative roles for CD45 in controlling the signaling threshold of T-cell antigen receptor (TCR), thereby regulating T-cell development (Alexander DR, 2000). Mice expressing active Lck<sup>F505</sup> at non-oncogenic levels develop aggressive thymic lymphomas on a CD45 null background (Baker M, 2000). CD45 suppresses the tumorigenic potential of the kinase by dephosphorylation of the Tyr394 autophosphorylation site (Baker M, 2000; Alexander DR, 2000). In CD45<sup>-/-</sup> thymocytes the kinase is switched to hyperactive oncogenic state, resulting in increased resistance to apoptosis. Transformation occurs in early CD4-CD8<sup>-</sup> thymocytes during the process of TCR- $\beta$  chain rearrangement by a recombinase-independent mechanism (Baker M, 2000).



**Fig 2. A model explaining the interaction between CD45 and various phosphorylated sites of p56<sup>Lck</sup> in CD45+ve and CD45-ve cells. Overall CD45 acts as both a positive and negative regulator of immune cells function.**

Phosphorylation of Tyr 394 increases p56<sup>Lck</sup> kinase activity, while phosphorylation of Tyr 505 decreases p56<sup>Lck</sup> kinase activity.

## CHAPTER 1. CHRONOLOGY OF RESEARCH INTERESTS

### 1.1.1 Aim and strategy

The first part of my work (Zhao R, 2004) was aiming to elucidate the mechanisms involved in the tumourigenesis of CD45<sup>-/-</sup> Lck<sup>F505</sup> mice, progeny produced by crossing CD45<sup>-/-</sup> mice and Lck<sup>F505</sup> mice, instigated by the interesting phenomenon that thymic tumours develop in these mice with a 100% penetrance at a quite early age, i.e. 5-15 weeks. As a contrast, the parental CD45<sup>-/-</sup> and Lck<sup>F505</sup> mice do not develop tumours during their whole life span.

In an earlier published work from Dr DR Alexander's group (Baker M, 2000), CD45<sup>-/-</sup> Lck<sup>F505</sup> mice were phenotypically characterised, with comparisons to wild-type, CD45<sup>-/-</sup> and Lck<sup>F505</sup> mice in respective of the thymic development and differentiation. Higher kinase activity of Lck<sup>F505</sup> was proposed to be the origin of the tumourigenesis, however the mechanisms behind this were obscure.

Intrigued by the dramatic tumour development in CD45<sup>-/-</sup> Lck<sup>F505</sup> mice, I joined Dr Alexander's group in early 2002, with a medical background in Haemato-oncology, hoping to discover the mechanisms entailed by the oncogenic tyrosine kinase Lck<sup>F505</sup>. The strategy I used in the study was to analyse the signalling pathways that might be involved in the transformation in pre-tumourigenic double-negative (DN) thymocytes in relation to Lck<sup>F505</sup> activity and function.

### 1.1.2 Kinase activity

Initially, the phosphorylation status and kinase activity of Lck in CD45<sup>-/-</sup> Lck<sup>F505</sup> DN thymocytes were analysed in detail. For technical simplicity, I took the advantage of having the above mice on Rag<sup>-/-</sup> background in-house. Rag<sup>-/-</sup> and Rag<sup>-/-</sup>CD45<sup>-/-</sup> mice only produce DN thymocytes in their thymus. It is noteworthy that it has been shown in the previous study (Baker 2000) that deletion of Rag does not confer any changes to the tumour development of CD45<sup>-/-</sup> Lck<sup>F505</sup> mice.

There are two regulatory sites in wild- type Lck that are important for the kinase activity: pTyr- 394 and pTyr- 505, of which pTyr- 394 is a positive regulatory site, while pTyr-505 a negative one. CD45 tyrosine phosphatase dephosphorylates both sites, keeping Lck activity at an appropriate level and with normal function. However in Lck<sup>F505</sup> Tyr 505 is mutated to Phe, causing a non-regulatory site at the end of Lck. CD45 cannot dephosphorylate this site, thus the overall kinase activity in Lck<sup>F505</sup> is lower than in wild- type Lck. However in the absence of CD45, i.e. CD45<sup>-/-</sup>, Lck<sup>F505</sup> has an increased activity (Alexander DR, 2000).

Phosphorylation studies on the immunoprecipitated Lck protein from Rag<sup>-/-</sup>CD45<sup>-/-</sup> Lck<sup>F505</sup> DN thymocytes showed an increased phosphorylation of Tyr 394 and decreased phosphorylation of Tyr 505 respectively. In vitro kinase assay on the same material revealed a 2- 3 fold increase of the kinase activity. The results were consistent with previous study and current understandings of the CD45/Lck interaction. Kinase activities in DN thymocytes can be defined as basal (Rag<sup>-/-</sup>), intermediate (Rag<sup>-/-</sup>CD45<sup>-/-</sup> or Rag<sup>-/-</sup>Lck<sup>F505</sup>), or hyperactive (Rag<sup>-/-</sup>CD45<sup>-/-</sup> Lck<sup>F505</sup>).

### **1.1.3 Survival and cell- cycle progression**

I considered the commonest signalling pathways that might be involved in oncogenesis to start with, i.e. those relating to cellular survival and cell- cycle control. Both analyses were performed on gated DN3 and DN4 thymocytes by flow- cytometry (FACs). A pool of monoclonal antibodies conjugated with different fluorescent dyes allows analysis of DN3/ DN4 subsets with relatively small number of cells.

Not surprisingly, Rag<sup>-/-</sup> thymocytes had a high level of apoptosis and a significant growth arrest due to a complete failure of  $\beta$ - selection and lack of pre-TCR mediated mitogenic signals. This made Rag<sup>-/-</sup> mice and its CD45<sup>-/-</sup> and CD45<sup>-/-</sup> Lck<sup>F505</sup> crosses most useful in addressing these points. Compared with Rag<sup>-/-</sup>, Rag<sup>-/-</sup> CD45<sup>-/-</sup> thymocytes had much less apoptosis- 29.6% compared to 97.3%. While in Rag<sup>-/-</sup> Lck<sup>F505</sup> and Rag<sup>-/-</sup> CD45<sup>-/-</sup> Lck<sup>F505</sup>, the reduction of apoptosis was even more significant- both of them had less than 1% of apoptotic cell in their DN3



compartment of the thymocytes. Likewise, there were more cells in G2 phase in Rag<sup>-/-</sup> CD45<sup>-/-</sup> than in Rag<sup>-/-</sup> DN3- 7% compared to 2-4%, whilst in Rag<sup>-/-</sup> Lck<sup>F505</sup> and Rag<sup>-/-</sup> CD45<sup>-/-</sup> Lck<sup>F505</sup>, cells in cycle were increased to nearly 30%.

The results described imply that the “intermediate activity” Lck in Rag<sup>-/-</sup> Lck<sup>F505</sup> and the “hyperactive” Lck in Rag<sup>-/-</sup> CD45<sup>-/-</sup> Lck<sup>F505</sup> have similar effects on the survival and cell cycle progression of DN3 thymocytes, though the former is non- oncogenic and the latter is oncogenic. This suggests that “intermediate activity” Lck is sufficient for keeping the normal survival and cell cycle progression of thymocytes. Since “hyperactive” Lck has 2-3 fold increase of the kinase activity compared to “intermediate activity” Lck, this begs the question: what is the role of the increased kinase activity? There must be something extra conferred by the “hyperactive” Lck to transform the thymocytes into cancerous phenotype.

One point to argue here is that the link between kinase activity and apoptosis in DN 3 thymocytes might be casual. However, as we know Lck<sup>F505</sup> mice with a higher copy number of the transgene, and consequently a higher kinase activity, develop thymic lymphoma in a way similar to CD45<sup>-/-</sup> Lck<sup>F505</sup> mice. The evidence demonstrates an exquisitely sensitive link between Lck kinase activity and the cellular survival and cell cycle progression of DN thymocytes.

#### **1.1.4 DNA repair and genomic instability**

It is believed that accumulation of unrepaired double strand breaks (DSBs) can cause genomic instability and secondary mutations, which are an important source of transformation. Thymocytes are known to be sensitive to genotoxic drugs and irradiation. So the DNA repair pathway is another potential candidate to investigate (Khanna KK, 2001; Richardson C, 2000).

My first test of the DNA repair pathway was to give same gamma-irradiation to the cells, then leave them to repair and measure the repair efficiency at 6h and 24h. Irradiated cells were cast into agarose plugs, and proteins were digested with proteinase K. DNA containing DSBs were separated from intact DNA by pulse field

gel electrophoresis (PFGE) technique (Bassing CH, 2003). The results were striking- wild- type, CD45<sup>-/-</sup> and Lck<sup>F505</sup> DN thymocytes showed similar repair efficiency at multiple time points, whilst CD45<sup>-/-</sup>Lck<sup>F505</sup> expressed a much more reduced repair of DSBs over a 6h or 24h time course.

The results were encouraging in the way that difference between non-oncogenic and oncogenic “hyperactive” Lck was first revealed. To ensure that this is a true phenomenon, the finding was vigorously tested with various methods.

Phosphorylation of H2AX was measured by western blots and also FACs as phosphorylated H2AX ( $\gamma$ H2AX) was thought to be a sensitive marker of the DSBs in the chromosomes following DNA damage (Bassing CH, 2003; Rogakou EP, 1998). The results showed a dramatic increase of  $\gamma$ H2AX in untreated CD45<sup>-/-</sup>Lck<sup>F505</sup> DN thymocytes, suggesting that at basal conditions more DSBs were accumulating in the cells, possibly due to the DNA repair deficiency.

DSBs are a source of genomic instability, which can be assessed by karyotype analysis with chromosomal painting. This was achieved through collaboration with Dr F.T. Yong at the Veterinary School of the University of Cambridge (Yang FT, 1995). In pre- tumorigenic DN thymocytes, we detected multiple chromosomal abnormalities in 5 Rag<sup>-/-</sup> CD45<sup>-/-</sup>Lck<sup>F505</sup>, but no aberrations were found in 43 wild-type, CD45<sup>-/-</sup> or Lck<sup>F505</sup> DN thymocytes. In transformed cell lines from the CD45<sup>-/-</sup> Lck<sup>F505</sup> thymic tumours (Matt cell lines), chromosomal abnormalities were also detected. These results provided strong evidence of the genomic instability caused by the “hyperactive” Lck in CD45<sup>-/-</sup>Lck<sup>F505</sup> DN thymocytes.

The results together demonstrate a striking correlation between inhibition of DNA repair and genomic instability in CD45<sup>-/-</sup>Lck<sup>F505</sup> DN thymocytes expressing oncogenic Lck.

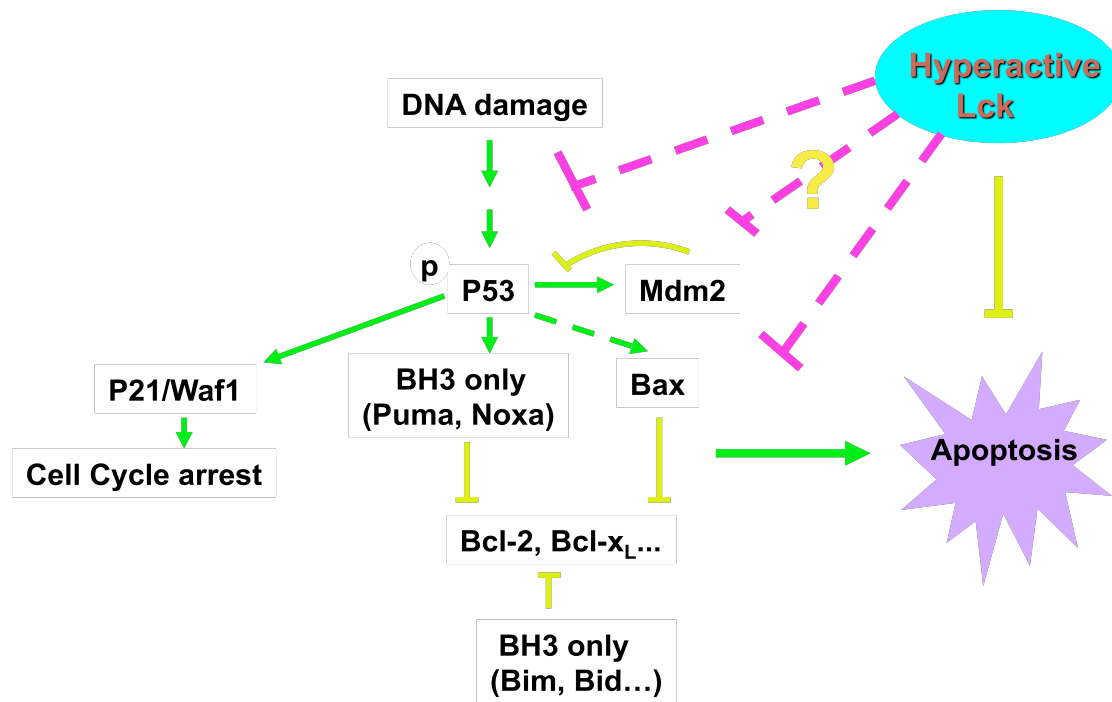
### **1.1.5 DNA damage- induced apoptosis**

Since DNA repair mechanisms were inhibited in CD45<sup>-/-</sup>Lck<sup>F505</sup> DN thymocytes, this begged the following question: what is keeping these cells with DSBs alive; and expanding? I hypothesised whether the apoptotic pathway is disrupted in these cells.

The survival profile was analysed in the cells that were exposed to ionising irradiation or etoposide (Fig 4, Zhao R, 2004). The results showed that only in the thymocytes expressing oncogenic Lck, was there a powerful survival signal protecting the cells from apoptosis. This was a very interesting finding. It seemed that a “double whammy” mechanism was functioning behind the transformation of these cells. On the one hand, cells were prone to accumulation of DSBs, which were possible source of further mutations; on the other hand, the cells were waived from the deadly consequence, so the cells with loads of DSBs could grow, proliferate and expand.

### **1.1.6 Examining the survival pathway in CD45<sup>-/-</sup>Lck<sup>F505</sup> DN thymocytes in DNA damage response**

In normal thymocytes, a hallmark of DNA damage is the induction and activation of p53 pathway. P53 transcription factor is up-regulated by ATM and ATR serine/threonine kinases, which are activated by DNA damage signals, probably through  $\gamma$ H2AX. P53, as a transcription factor, initiates apoptotic pathway mediated by Bax; and cell cycle arrest through p21<sup>WAF1</sup> (Sherr CJ, 2002; Vousden KH, 2002).

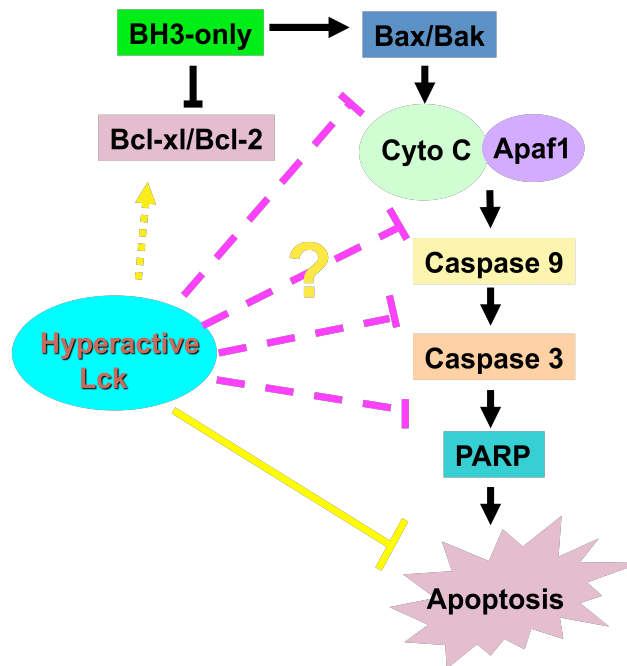


**Fig 3. A diagram to show the signalling network involving p53 and downstream effectors, and putative mechanisms how hyperactive lck affects the various pathways.** DNA damage induces phosphorylation of p53, which stabilises p53 and subsequently drives cells into apoptosis by promoting p21<sup>WAF1</sup> mediated cell cycle arrest, inducing up-regulation of BH3-only proteins Puma and Noxa, and increasing expression of pro-apoptotic protein Bax directly. Hyperactive Lck could exert its function by targeting at the various pathways or molecules.

I compared the phosphorylation, induction of p53, and the downstream effectors Bax and p21<sup>WAF1</sup> between non- oncogenic lck expressing and oncogenic lck expressing cells. On finding no significant difference in all the aspects, I concluded that the p53 machinery was functioning normally in CD45<sup>-/-</sup>Lck<sup>F505</sup> DN thymocytes in DNA damage response (Fig 2, Zhao R, 2004).

Moving down from p53 to the mitochondria apoptotic machinery, key molecules involved in the caspase execution cascade were also examined. In mammalian cells, cytochrome c initiates caspase activation following its release from mitochondria, it

also forms an active apoptosome complex with Apaf-1, which activates procaspase-9, which in turn activates caspase-3. Active caspase-3 can cleave poly(ADP-ribose)polymerase (PARP) to yield an inactive form of PARP.



**Fig 4. A diagram showing various molecules involved in the mitochondrial apoptotic pathway and the possible roles that hyperactive Lck plays in this signalling network.**

Starting from PARP I found that the cleavage of PARP was clearly blocked in CD45<sup>-/-</sup> Lck<sup>F505</sup> DN thymocytes that were exposed to DNA damage. This suggested that caspase-3 might not be activated properly. Next, I examined caspase-3 status in these cells under the same condition, and the results were again striking- caspase-3 was not cleaved hence not activated, suggesting that the active apoptosome complex comprised of cytochrome c and Apaf-1 was not formed. This pointed to the possibility of inhibition of cytochrome c release from mitochondria, which had been

reported as a critical switch in the initiation of apoptosis (Zou H, 1999; Wang X, 2001).

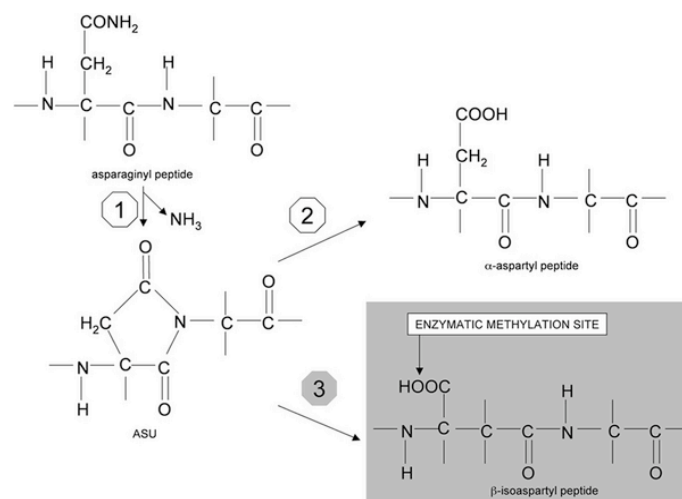
In order to measure the quantity of released cytochrome c from mitochondria, subcellular fractionation on the cells was carried out and mitochondria (M) and cytosol (C) fractions were purified. The quantities of cytochrome c in these two fractions were measured by immunoblotting with an anti- cytochrome c antibody. In CD45<sup>-/-</sup>Lck<sup>F505</sup> DN thymocytes, cytosolic fraction of cytochrome c was greatly reduced. By re-probing the same gel- membrane with a Bax antibody, I found that Bax was mostly located in cytosolic fraction instead of mitochondrial fraction in these cells.

Bax is a BH3- only protein that translocates from cytosol to mitochondria upon apoptotic signals, promoting release of cytochrome c from mitochondria (Gross A, 1998). A conformational change in Bax precedes its translocation to mitochondria. An antibody (6A7 mab) recognises an epitope in its N- terminus that becomes exposed during apoptotic signalling (Hsu YT, 1998; Nechushtan A, 1999). I therefore examined the conformational status of Bax by utilising this antibody. Naïve Bax was immunoprecipitated by 6A7 mab, the quantity of which was then measured by immunoblotting with a Bax antibody. While in CD45<sup>-/-</sup>Lck<sup>F505</sup> DN thymocytes, DNA damage induced no conformational change of Bax, it was clearly opposite in wild-type, CD45<sup>-/-</sup> and Lck<sup>F505</sup> DN thymocytes.

Previous study on mitochondrial apoptosis and Bcl-2 family molecules suggested that anti-apoptotic proteins such as Bcl-2 and Bcl-x<sub>L</sub> bind and sequester BH3-only proteins, e.g Bid and Bim, thereby preventing their pro-apoptotic interactions with Bax (Cheng EH, 2001; Kuwana T, 2002). As Bcl-2 levels were similar in the DN thymocytes from the four mouse lines (previous unpublished work from Dr Alexander's group), I focused my interest on Bcl-x<sub>L</sub>, which is known to be well-expressed in immune cells. In DN thymocytes, Bcl-x<sub>L</sub> expression was increased similarly in both Rag<sup>-/-</sup> Lck<sup>F505</sup> and Rag<sup>-/-</sup> CD45<sup>-/-</sup>Lck<sup>F505</sup> mice, compared to controls. This still does not explain why tumours develop in (Rag<sup>-/-</sup>) CD45<sup>-/-</sup>Lck<sup>F505</sup> mice, but not in (Rag<sup>-/-</sup>) Lck<sup>F505</sup> mice.

Interestingly Bcl-x<sub>L</sub> deamidation in response to DNA damage had been proposed as a critical switch to subvert the pro-survival function of Bcl-x<sub>L</sub> (Deverman BE, 2002). I therefore studied the Bcl-x<sub>L</sub> deamidation status in response to DNA damage in the above four mouse lines. I found that Bcl-x<sub>L</sub> deamidation was inhibited in CD45<sup>-/-</sup> Lck<sup>F505</sup> mice, while it clearly occurred in control mice. This result provided the most convincing evidence of how oncogenic Lck<sup>F505</sup> subverted the apoptotic pathway, although the mechanism of how Bcl-x<sub>L</sub> was deamidated *per se* was still not clear.

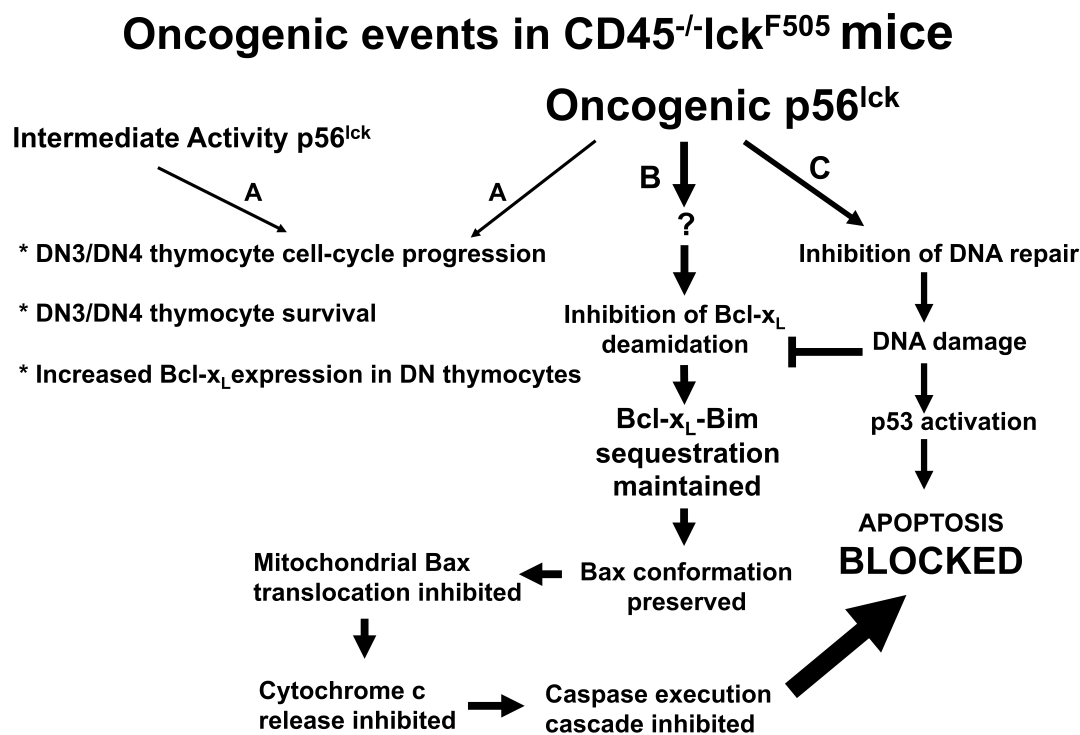
Protein deamidation occurs spontaneously at Asn residues, which are flanked, on the α-carboxyl side, by small non-bulky residues, such as Gly, Ala, Ser or Thr (Robinson NE, 2001). The nucleophilic attack of the peptidyl nitrogen of the Asn+1 residue onto the b-carbonyl carbon of the Asn, leads to the formation of an aspartyl succinimidyl intermediate, with the elimination of the ammonia moiety. The aspartyl succinimidyl intermediate itself is unstable and its ring can open on either side of the nitrogen atom, yielding either a normal peptide or an atypical isopeptide containing a β-linked isoaspartyl residue (isoAsp), the latter form being generally predominant (Asward DW, 2000).



**Fig 5. Mechanism for deamidation of asparaginyl residues in peptides. (Fig. from Climmino A, 2008)**

(Step 1): the nitrogen of the Asn+1 residue (a Gly in the example) attacks the β-

carbonyl carbon of the Asn, thus forming the succinimidyl derivative of the peptide (ASU) with the ammonia elimination. The ASU ring can open spontaneously on either side of the nitrogen atom. In one case the  $\alpha$ -aspartyl peptide is formed (Step 2). In the other case the  $\beta$ -isoaspartyl peptide does occur (Step 3).



**Fig 6. A model integrating the findings on the DNA damage response, cell survival and cell- cycle progression in cells with various Lck kinase activity.** Pathway A refers to signals that are common to both intermediate activity Lck (as in DN CD45<sup>-/-</sup>Lck<sup>F505</sup> thymocytes). Pathway B and C are unique to the oncogenic hyperactive Lck found in DN CD45<sup>-/-</sup>Lck<sup>F505</sup> thymocytes. (Zhao R, 2007)



## 1.2 How is Bcl-x<sub>L</sub> deamidated in DNA damage responses?

This is the main question to be answered in the next part of work (Zhao R, 2007). To answer this question would be the key to understanding how oncogenic tyrosine kinases interact with the Bcl-x<sub>L</sub> deamidation pathway, and potentially, uncovering new strategies in cancer treatment.

### 1.2.1 Elucidation of the roles of different species of Bcl-x<sub>L</sub> by *in vitro* and *in vivo* studies

Initial work from the Weintraub laboratory suggested that when Asn52 and Asn 66 were both mutated to Asp, Bcl-x<sub>L</sub> lost its ability to bind that BH3-only pro-apoptotic protein Bim, thereby providing a putative link between DNA damage and apoptosis (Deverman BE, 2002). However, a secondary mutation was later identified, which, when corrected, enabled the N52D/N66D Bcl-x<sub>L</sub> to bind Bim, casting doubt on this interpretation (Deverman BE, 2003). As the sequestration of BH3-only proteins by Bcl-x<sub>L</sub> was thought to explain its anti-apoptotic function (Cheng EH, 2001), resolution of this question was clearly important for establishing a molecular link between DNA damage and apoptosis.

To address this point, a series of cellular and biochemical experiments were carried out. First of all, whether deamidated forms of Bcl-x<sub>L</sub> bind to BH3-only proteins needed to be elucidated. In the previous work (Zhao R, 2004), Bim could only immuno-precipitate Bcl-x<sub>L</sub> protein from the lysates of CD45<sup>-/-</sup> Lck<sup>F505</sup> thymocytes which had been treated with etoposide, and also Bcl-x<sub>L</sub> pulled down much more Bim from the CD45<sup>-/-</sup> Lck<sup>F505</sup> thymocyte lysates, suggesting that deamidation crippled the ability of Bcl-x<sub>L</sub> to bind Bim. However, Weintraub's correction showed that mutated N52D/N66D Bcl-x<sub>L</sub> was still able to bind Bim. The discrepancy might have been caused by the following reasons: A) The amount of protein Bim may be different between CD45<sup>-/-</sup> Lck<sup>F505</sup> and other non-tumourigenic mice; B) DNA damage/etoposide treatment may induce different amount of Bim; and C) N52-iso D/N66-iso D Bcl-x<sub>L</sub> does not bind Bim, although N52D/N66D Bcl-x<sub>L</sub> does bind Bim. Points A and B were easily eliminated by thorough Bim/ Bcl-x<sub>L</sub> binding experiments

(Fig7B, Zhao R, 2004; Fig 2 A& B, Zhao R, 2007), in which the native and deamidated Bcl-x<sub>L</sub> species were also tested separately for their binding ability with Bim, and the results clearly indicated that deamidated Bcl-x<sub>L</sub>, including both the N52D/N66D and N52-iso D/N66-iso D Bcl-x<sub>L</sub> forms, lost most of its ability to bind Bim *in vivo*. Recombinant Bcl-x<sub>L</sub> also lost part of its binding to Bim when one site was deamidated, and lost most of its binding when two sites were deamidated. I also tested the binding of N52D/N66D Bcl-x<sub>L</sub> form to Bim in thymocytes, which confirmed what Weintraub's laboratory had claimed.

Collectively, the results supported the hypothesis C that N52-iso D/N66-iso D Bcl-x<sub>L</sub> does not bind Bim, although N52D/N66D Bcl-x<sub>L</sub> does bind Bim. If physiologically N52-iso D/N66-iso D Bcl-x<sub>L</sub> exists as the dominant form, it would not be surprising to see that deamidated Bcl-x<sub>L</sub> loses most of its ability to bind BH3-only proteins.

Consistently, previous literature on protein deamidation showed that when an Asn is converted to a mixture of Asp and iso-Asp, the ratio of Asp/iso-Asp is about 1:5 (Robinson NE, 2001). To confirm this was applicable to Bcl-x<sub>L</sub> deamidation, it was necessary to measure the ratio of Asp/iso-Asp at Asn52 and Asn66. This was achieved by Mass Spectrometric analysis. Respective peptides were designed and synthesised, which encompass Asn 52/ Asn 66 and their corresponding Asp/ iso-Asp forms as identifiers of these species in the same peptides digested from recombinant Bcl-x<sub>L</sub>. The results showed that the ratios of Asp/iso-Asp for Asn 52 and Asn66 were 10:1 and 5:1, respectively.

Taken together, my results showed that conversion of Bcl-x<sub>L</sub> Asn 52 and Asn 66 to iso-Asp forms, but not Asp counterparts, prevented sequestration of BH3-only proteins. In fact deamidation of Bcl-x<sub>L</sub> to iso-Asp causes greater perturbation of protein structure than conversion to Asp (Aritomi M, 1997), presumably explaining its loss of BH3-only protein binding ability.

### **1.2.2 Is Bcl-x<sub>L</sub> deamidation a consequence of mitochondrial apoptosis?**

Whether Bcl-x<sub>L</sub> deamidation is a cause or a consequence of apoptosis is an obvious and important point to elucidate. Clearly if Bcl-x<sub>L</sub> deamidation is a cause of

apoptosis, it will play a more critical role in thymic transformation than being a consequence of apoptosis.

This was addressed by using a caspase inhibitor Z-VAD-fmk, which can effectively block the occurrence of apoptosis in thymocytes (Fig 1a&b, Zhao R, 2007). In the cells treated with etoposide, and with or without Z-VAD-fmk, Bcl-x<sub>L</sub> deamidation occurred at similar levels, suggesting that Bcl-x<sub>L</sub> deamidation was not a consequence of apoptosis.

As Bax and Bak are required in apoptosis mediated by BH3-only proteins, I also tested whether depletion of Bax or Bak could block Bcl-x<sub>L</sub> deamidation. Thymocytes were transfected with shRNA for Bax or Bak prior to exposure to DNA damage. As shown in Fig 1c (Zhao R, 2007), neither the depletion of Bax or Bak affected the occurrence of Bcl-x<sub>L</sub> deamidation.

### **1.2.3 The DNA damage- NHE-1 up-regulation- intracellular alkalinisation- Bcl-x<sub>L</sub> deamidation axis**

How DNA damage caused Bcl-x<sub>L</sub> deamidation was a completely unexplored area when we started to think about it in early 2006. It was not surprising though as the link between DNA damage and Bcl-x<sub>L</sub> deamidation was just revealed in late 2002 (Deverman BE, 2002).

It has been known that protein Asn deamidation is accelerated by increased pH *in vitro*. Antiport Sodium-Hydrogen-Exchanger family member 1 (NHE-1) is responsible for maintaining the intracellular pH in thymocytes. It seemed to be the only clue to follow. The hypothesis would be that DNA damage induces NHE-1 mediated intracellular pH (pHi) change. A quick test of this hypothesis was to check whether DNA damage could induce any change in NHE-1, which could potentially cause intracellular alkalinisation.

Many types of stimuli induce phosphorylation of NHE-1 and thus increase its activity. I believed that it might be the same case in DNA damage response. Therefore I tried

to check the phosphorylation status of NHE-1 in DNA damaged thymocytes. Using a set of anti-phospho antibodies against known phosphorylation sites in NHE-1, I could not find altered phosphorylation of NHE-1 after DNA damage. However, I noticed on the western blots that the NHE-1 expression level was increased after DNA damage. It was an interesting finding although there was not much emphasis on the role of NHE-1 protein expression level in its function in previous literature.

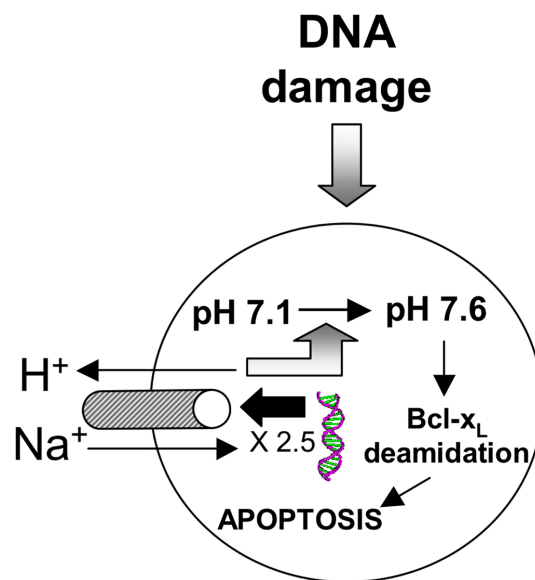
In parallel I tried to measure the intracellular pH (pHi) before and after DNA damage. Using a pH- sensitive fluorescent dye SNARF-1, pHi can be measured by FACS. It was encouraging to find that pHi consistently was increased in DNA damaged cells. This increase in pHi was about 0.4-0.5 units, which was both necessary and sufficient to cause Bcl-x<sub>L</sub> deamidation in the cells without exposure to DNA damage. These results were achieved by artificially changing the pHi in the cells and then examining the Bcl-x<sub>L</sub> deamidation status.

Whether it was just a casual link between up-regulation of NHE-1 protein level and intracellular alkalinisation or whether NHE-1 up-regulation caused intracellular alkalinisation needs to be clarified before drawing a conclusion on the mechanism of Bcl-x<sub>L</sub> deamidation. This entailed a series of experiments. In summary, I had shown (Fig 4&5, Zhao R, 2007):

- a) DNA damage- induced Bcl-x<sub>L</sub> deamidation requires de novo protein synthesis.
- b) DNA damage causes up-regulation of NHE-1 in wild- type but not in CD45<sup>-/-</sup> Lck<sup>F505</sup> thymocytes.
- c) NHE-1 over-expression by retroviral transfection causes intracellular alkalinisation and Bcl-x<sub>L</sub> deamidation.
- d) NHE-1 function blockage by specific inhibitor (DMA) blocks intracellular alkalinisation and Bcl-x<sub>L</sub> deamidation.
- e) NHE-1 gene knock- down by shRNA also blocks intracellular alkalinisation and Bcl-x<sub>L</sub> deamidation.

Collectively the combined evidence strongly supports a model whereby DNA damage- induced Bcl-x<sub>L</sub> deamidation is mediated by NHE-1 antiport regulated intracellular alkalinisation. The current model is equally applicable to fully-

transformed cells- i.e. CD45<sup>-/-</sup>Lck<sup>F505</sup> tumour cells, and human Chronic lymphoblastic leukaemia (CLL) cells, though the former express an OTK, the latter do not.



**Fig 7. A novel signaling pathway triggered by DNA damage leads to the up-regulation of the NHE-1 antiport, increased intracellular pH, Bcl-x<sub>L</sub> deamidation, and finally apoptosis. (Fig. from Zhao et al, 2007)**

## **1.3 Bcl-x<sub>L</sub> deamidation pathway in Myeloproliferative Disorders (MPDs)**

### **1.3.1 Why investigate Bcl-x<sub>L</sub> deamidation pathway in MPDs?**

Bcl-x<sub>L</sub> plays important roles in many tumour types (Amundson SA, 2000), including MPDs. My finding on deamidation of Bcl-x<sub>L</sub> in DNA damage responses therefore have potential relevance to cancer therapy, whereby enforced alkalinisation, perhaps by amplification of NHE-1 expression, would promote Bcl-x<sub>L</sub> deamidation, thereby triggering apoptosis.

Relevance of Bcl-x<sub>L</sub> deamidation for human cancers associated with OTKs remained unclear, although DNA damaged- induced Bcl-x<sub>L</sub> deamidation was intact in cell lines of osteosarcoma and cervical, bladder and ovarian cancers, and in primary human lymphoblastic leukaemia cells (Zhao R, 2007).

MPDs are good candidates for studying Bcl-x<sub>L</sub> deamidation pathway because, as a group of human haematopoietic malignant diseases, many of them express OTKs. Among MPDs, Chronic Myeloid Leukaemia (CML) and Polycythemia vera (PV) are the two seeing most significant breakthroughs in the understanding of their pathological mechanisms. They are associated with the BCR-ABL fusion tyrosine kinase and the Janus tyrosine kinase 2 (JAK2) mutation V617F, respectively. Both disorders initially usually present with chronic diseases, but carry a risk of progression to blastic phase resembling acute leukaemia that resists further therapy (Goldman JM, 2003; Campbell PJ, 2006).

Bcl-x<sub>L</sub> has been shown to be up-regulated in patients with CML and PV and is thought to inhibit apoptosis. BCR-ABL protein expression is associated with a reduced apoptotic response to genotoxic drugs. Moreover, quiescent CML stem cells, thought to be responsible for residual disease, are resistant to apoptosis that tyrosine kinase inhibitors induce. Collectively the combined results suggested there might be an undiscovered link between Tyrosine kinases BCR-ABL and Jak2<sup>V617F</sup> and the resistance to DNA damage- induced apoptosis in CML and PV patient myeloid cells.

### **1.3.2 Patients' sample collection and cell purification**

Access to human samples was made possible by collaboration with Prof. AR Green's research group at the Department of Haematology, Addenbrooks Hospital, University of Cambridge. The study was approved by the Cambridge and Eastern Region ethics committee. All patients involved provided written informed consent.

Cells of myeloid or lymphoid lineage were purified from peripheral blood samples. The process involves using density separation through centrifugation, followed by immunological targeting to cell surface markers combined with flow cytometry. Unlike using thymocytes from established mouse lines, human samples were precious in terms of how many cells were available and when they were available. This responsibility proved challenging but satisfying, as it constituted a particularly good model system upon which to work.

### **1.3.3 Bcl-x<sub>L</sub> deamidation pathway is inhibited in CML myeloid cells**

Purified myeloid/ granulocytes from normal subjects and CML patients were treated with etoposide or exposed to  $\gamma$ -irradiation. Then every step in the Bcl-x<sub>L</sub> deamidation pathway was examined, i.e. NHE-1 expression level change, intracellular pH alteration, Bcl-x<sub>L</sub> deamidation status and apoptosis of the cells. Results from 6 normal controls and 10 CML patients consistently showed that the Bcl-x<sub>L</sub> deamidation pathway in DNA damage responses was intact in normal cells while inhibited in CML cells.

T cells purified from the same CML patient samples, as good internal controls, showed no effect on the Bcl-x<sub>L</sub> deamidation pathway (Suppl Fig 3, Zhao R, 2008). The same CML myeloid cells were subjected to a series of 'forced alkalinisation' experiments, where cells were cultured in alkaline medium i.e. pH>8. I found that the resistance to Bcl-x<sub>L</sub> deamidation was overcome in these cells. It suggested that the inhibition by Bcr-Abl was at the step of up-regulation NHE-1, which was same as in p56Lck<sup>F505</sup>.

The dysfunction of Bcl-x<sub>L</sub> deamidation pathway in CML cells was further examined aiming to identify the critical steps responsible. CML cells were transfected with a retroviral vector carrying NHE-1 cDNA in order to over-express NHE-1 in CML cells. These cells not only became alkalinised intracellularly, but also had more Bcl-x<sub>L</sub> protein deamidated and subsequently apoptosed.

#### **1.3.4 BCR-ABL inhibitor Imatinib reverses the inhibition of Bcl-x<sub>L</sub> deamidation pathway in CML myeloid cells**

Imatinib is a very effective BCR-ABL inhibitor. Its application in treating CML patients has been so successful that most CML patients will achieve complete regression. However, a small proportion of CML patients develop resistance to Imatinib by point mutation of the BCR-ABL kinase domain. In my experiments Imatinib-sensitive CML cells were either pre-treated with Imatinib or not, then were exposed to etoposide/irradiation. NHE-1 expression level and Bcl-x<sub>L</sub> deamidation status were examined subsequently. The results were clear that Imatinib completely reversed the inhibition of Bcl-x<sub>L</sub> deamidation in these CML cells.

#### **1.3.5 Imatinib does not reverse the inhibition of Bcl-x<sub>L</sub> deamidation pathway in Imatinib-resistant CML cells that carry an E255V mutation in the BCR-ABL kinase domain**

Although Imatinib is known to be a very specific BCR-ABL kinase inhibitor, it also inhibits a few other kinases. Strictly speaking, the above reversion of the inhibition of Bcl-x<sub>L</sub> deamidation pathway might possibly be due to the other effects of Imatinib.

At this point I managed to obtain an imatinib-resistant CML sample, which carried an E255V mutation in the BCR-ABL kinase domain. The same experiments were performed with this sample. Strikingly, the reversion of the Bcl-x<sub>L</sub> deamidation pathway did not occur. Furthermore, the same cells were transfected with a NHE-1 vector using a Nucleofector kit. NHE-1 was over-expressed in these cells, which subsequently caused intracellular alkalinisation and Bcl-x<sub>L</sub> deamidation.



This series of experiments involving Imatinib sensitive and resistant CML cells strongly support the critical role of BCR-ABL in the inhibition of Bcl-x<sub>L</sub> deamidation pathway in CML.

### **1.3.6 Bcl-x<sub>L</sub> deamidation pathway is inhibited by Jak2<sup>V617F</sup> in PV myeloid cells**

Likewise, in a series of similar experiments, Jak2<sup>V617F</sup> in PV myeloid cells exhibited its role in blocking the DNA damage- induced NHE-1/Bcl-x<sub>L</sub> deamidation pathway, i.e. NHE-1 up-regulation, intracellular alkalinisation, Bcl-x<sub>L</sub> deamidation and apoptosis. T cells purified from the same PV patient samples, as good internal controls, showed no effect on the Bcl-x<sub>L</sub> deamidation pathway (suppl Fig 3, Zhao R, 2008).

### **1.3.7 Jak 2 inhibitor reverses the inhibition of Bcl-x<sub>L</sub> deamidation pathway in PV myeloid cells**

Whilst there were no established specific Jak2 inhibitors available, three inhibitors with different sensitivity and specificity were used. Jak inhibitor 1 (Calbiochem) is a pan- inhibitor of Jak, TG101209 (Targagen) and AT9283 (Astex) are inhibitors currently in clinical trials, which have been shown in cellular experiments inhibiting Jak2 (Prof AR Green unpublished data).

Surprisingly all three Jak2 inhibitors all showed significant effects in inhibiting the Bcl-x<sub>L</sub> deamidation pathway, although one of them was apparently more potent than others. Similarly the PV myeloid cells were subjected to 'forced alkalinisation' experiments. The resistance to Bcl-x<sub>L</sub> deamidation was also overcome in these cells suggesting that the inhibition by Jak2<sup>V617F</sup> is at the step of up-regulation NHE-1, which is same as p56Lck<sup>F505Y</sup> and Bcr-Abl.

### **1.3.8 Bcl-x<sub>L</sub> deamidation pathway in Jak2<sup>V617F</sup>-positive Idiopathic Myelofibrosis (IMF)**

IMF represents a sub- group of diseases in myeloproliferative disorders. Some IMF patients carry Jak2<sup>V617F</sup> mutations, whereas others do not. Luckily I was able to obtain two Jak2<sup>V617F</sup>-positive and two Jak2<sup>V617F</sup>-negative IMF patient samples to study the Bcl-x<sub>L</sub> deamidation pathway. The results were very encouraging- the Bcl-x<sub>L</sub> deamidation pathway was inhibited in Jak2<sup>V617F</sup>-positive IMF patient cells. This again supports the correlation of Jak2<sup>V617F</sup> and the blockage of DNA damage- induced Bcl-x<sub>L</sub> deamidation pathway in myeloproliferative disorders.

#### **1.4 Tyrosine kinases in other haematological malignancies- potential research interests and therapeutic targets?**

Eight cancer cell lines were also studied, representing different haematologic cancers associated with distinct molecular mechanisms. Among these, K562 expresses Bcr-Abl, HEL expresses Jak2<sup>V617F</sup> in Jak2<sup>V617F</sup> and Karpas 299 expresses NPM-ALK, a tyrosine kinase; Daudi expresses c-myc, DU528 expresses Tal1, JVM2 expresses cyclin D1, OPM2 expresses FGFR3 and DOHH2 expresses Bcl-2.

While demonstrated again that Bcr-Abl and Jak2<sup>V617F</sup> inhibit the NHE-1/Bcl-x<sub>L</sub> deamidation pathway, none of the other onco-proteins do, including tyrosine kinase NPM-ALK. So the inhibition of Bcl-x<sub>L</sub> deamidation pathway is not a general feature of haematologic cancers and is only mediated by a sub- group of tyrosine kinases or is dependent on a particular cellular context.

## CHAPTER 2. CRITIQUES/REFLECTION

### 2.1. Asn 52/66 issues

Original work from Weintraub laboratory suggested that deamidated Bcl-x<sub>L</sub> does not bind to BH3- only protein Bim. However, this result was later withdrawn due to the identification of a secondary mutation in the original DNA construct used in the experiments. When the secondary mutation was corrected deamidated Bcl-x<sub>L</sub> did bind to Bim (Deverman BE, 2003).

Interestingly, I found that whereas the ability of Bcl-x<sub>L</sub> to bind Bim was ablated in control thymocytes exposed to DNA damage, it was strikingly retained in CD45<sup>-/-</sup> Lck<sup>F505</sup> thymocytes, tightly correlating with the resistance to Bcl-x<sub>L</sub> deamidation noted in these cells.

These findings cast doubt on the model that Bcl-x<sub>L</sub> triggers apoptosis because the sequestration of BH3-only proteins by Bcl-x<sub>L</sub> is thought to explain its anti-apoptotic function. Resolution of this question is clearly important for establishing a molecular link between DNA damage and apoptosis. So at an early stage of my project I carried out a series of cellular and biochemical experiments to address this key point, such as, immunoprecipitation of Bim from cell lysates with a Bcl-x<sub>L</sub> antibody or the other way around, and measuring the precipitated proteins by western blots. Recombinant His-tagged Bcl-x<sub>L</sub> was exposed to alkaline conditions to cause partial deamidation and separated by anion exchange chromatography into purified native, singly deamidated and doubly deamidated Bcl-x<sub>L</sub> proteins, and these different species of Bcl-x<sub>L</sub> bound with endogenous Bim in a completely different manner, i.e. native Bcl-x<sub>L</sub> has a maximum binding ability, doubly deamidated Bcl-x<sub>L</sub> does not bind with Bim at all, while singly deamidated Bcl-x<sub>L</sub> lies in between. And interestingly, both the Bcl-x<sub>L</sub> N52A/N66A and N52D/N66D mutants maintain the complete binding ability with BH3-only proteins as the native Bcl-x<sub>L</sub>. Given that most asparagines deamidate to iso-aspartate forms at physiological conditions in cells, it is reasonable to suggest that the change from asparagine to aspartate does not affect the binding with BH3-only proteins, but the conversion from asparagine to iso-aspartate does.

This speculation was also validated by a definitive experiment, which nevertheless, was to prove an established view, was however, critical in the interpretation of the mechanism of Bcl-x<sub>L</sub> deamidation. Aspartate and iso-aspartate species of Bcl-x<sub>L</sub> were separated from naturally occurred Bcl-x<sub>L</sub> mixture by LC-MS chromatography respectively, and their quantities were compared, which confirm that more than 90% of asparagines in Bcl-x<sub>L</sub> convert to iso-aspartates, but not aspartates.

## **2.2. Global effects of alkalinisation**

One argument about the NHE-1/ Bcl-x<sub>L</sub> deamidation pathway is that intracellular alkalinisation can possibly cause deamidation of other proteins. What roles do they play in apoptosis and transformation? This is a fair argument. I have been trying to address this question in different ways. The most critical evidence is that when Bcl-x<sub>L</sub> is mutated to N52A/N66A mutant, a constitutively native form, enforced intracellular alkalinisation does not increase apoptosis. So although the global effect of alkalinisation is inevitably present, its role in apoptosis might be negligible.

The possibility of pH manipulation as a means to cancer therapy has in the past attracted intermittent interest. The pioneering work of Warberg established that tumours display acidic extracellular pH (Warberg O, 1930), although half a century later it was established that the intracellular pH of tumours is comparable with normal cells (Griffiths JR, 1991). My findings suggest that strategies for pH manipulation in anti-neoplastic therapy should continue to receive attention, albeit for reasons different from those envisaged by Warberg.

## **2.3. Protein modification in signal transduction**

Proteins are frequently modified in signal transduction. Modification of proteins confers new function to the molecules and often the proteins with altered function play critical roles in signalling networks. Phosphorylation, methylation, sumoylation etc. have attracted more research attention and indeed demonstrated their importance in protein and cellular function. Deamidation, though universally

occurring in all proteins, has only recently been shown being involved in rapid physiological events, i.e. signal transduction of Bcl-x<sub>L</sub>. Is it just an individual phenomenon, or revealing a completely new type of protein modification with physiological significance, needs to be elucidated.

A number of cell stress conditions have recently been linked to protein deamidation. Oxidative conditions have been considered as a way through which protein deamidation is facilitated. Protein Isoaspartate Methyltransferase (PIMT) may be able to mediate protection from apoptosis induced by Bax in a neuronal cell line by catalyzing protein methyl esterification (Cimmino A, 2008). PIMT has been shown to be able to prevent isoaspartate accumulation in the Eukaryotic Initiator Factor Binding Protein 2 (4E-BP2), an important factor in learning and memory, in the brain (Bidinosti M, 2010). Deamidation has also been involved in ubiquitination and ubiquitin- dependent degradation of peptides (Cui J, 2010)

## **CHAPTER 3. CONCLUSION AND FUTURE WORK**

Protein deamidation is a naturally occurring process, which increases protein turnover, and has been proposed as a molecular timer of biological events (Robinson NE, 2001). However, the significance of protein deamidation to the cell has never been firmly established. It has been suggested, in respect to the regulation of DNA damage-induced apoptosis, Bcl-x<sub>L</sub> deamidation may serve as a chronometric buffer, affording the cell time to reverse low-level genotoxic stress-induced events (Deverman BE, 2002). Rapid deamidation of Bcl-x<sub>L</sub> induced by DNA damage indicates that the deamidation "clock" is a dynamic process that can be regulated in vivo by biological events (Zhao R, 2007).

Bcl-x<sub>L</sub> is an important pro-survival protein whose potency is emphasised by its protection of a wide range of tumour cells from genotoxic attack (Amarante-Mendes et al., 1998; Amundson et al., 2000; Brumatti et al., 2003). The role of Bcl-x<sub>L</sub> deamidation in the transformation of OTK- expressing cells is particularly critical, as it explains how these cells gain their survival advantage under extreme conditions- i.e. cytotoxic drugs or gamma-radiation. It also explains why these cells, once they become cancerous, are notoriously resistant to chemo- or radiotherapy.

It is interesting that a few OTKs inhibit the NHE-1/Bcl-x<sub>L</sub> deamidation pathway, whereas others do not. Further study is needed given the vast number of OTKs involved in human cancer. Finally whether it is applicable to target at the NHE-1/Bcl-x<sub>L</sub> deamidation pathway warrants attention, although variances are predictable due to biological/cellular complexity.

### **Future work**

There are a number of lines of work that need to be done in order to understand the relationship between OTKs and the NHE-1/Bcl-x<sub>L</sub> deamidation pathway, and hopefully, to be able to manipulate the components involved in oncogeneis to generate potential new cancer therapy.

The hypothesis that inhibition of DNA-damage induced alkalisation and Bcl-x<sub>L</sub> deamidation causes transformation needs to be vigorously tested.

Firstly, what is the role of NHE-1 in tumourigenesis? This can be achieved by generating new transgenic/ knock-in mouse models based on the CD45<sup>-/-</sup>p56<sup>lck-Y505F</sup> mouse model. If we can express NHE-1 in the CD45<sup>-/-</sup>p56<sup>lck-Y505F</sup> mouse in a regulatory manner, i.e. tetracycline- controlled expression, then we can observe whether NHE-1 up-regulation can prevent tumour development, and, whether NHE-down- regulation can induce tumour relapse. Tumour growth monitoring can be achieved by using the immunofluorescence/illuminescence technique, which itself will require careful design of the molecular tags.

Secondly, is prevention of Bcl-x<sub>L</sub> deamidation sufficient for transformation? Blockade of the Bcl-x<sub>L</sub> deamidation pathway plays an important role in the transformation driven by three OTKs - Lck<sup>Y505F</sup>, Bcr-abl and Jak2<sup>V617F</sup>. Whether Bcl-x<sub>L</sub> deamidation is directly involved in the transformation process needs to be validated *in vivo*. One way of doing this is to generate a mouse model with the two asparagines in Bcl-x<sub>L</sub> replaced by Alanines so that they are not able to deamidate, i.e. a mouse model with a constitutively “native” Bcl-x<sub>L</sub>. If this mouse is crossed with an OTK model, i.e. CD45<sup>-/-</sup>p56<sup>lck-Y505F</sup>, then the role of Bcl-x<sub>L</sub> deamidation can be tested by subjecting the live mice to DNA damage and monitor tumour occurrence etc.

How NHE-1 expression is regulated following DNA damage also needs to be studied. This is still a mystery so far. NHE-1 expression and modification have been studied in the context of various stimuli, such as growth factors, cytokines, homeostasis, etc. But the link between DNA damage and NHE-1 has not been paid much attention, probably due to the fact that NHE-1, as an antiport, was mainly investigated in cardiovascular diseases (Fliegel L, 2001)

Lacking this important information, it is impossible to understand how the oncogenic tyrosine kinases (e.g. Lck<sup>Y505F</sup>, Bcr-abl and Jak2<sup>V617F</sup>) carry out their abnormal function and drive the transformation of the cells. The increased expression level of NHE-1 could be caused at various stages: 1) messenger RNA level - transcriptional

or mRNA stability, and 2) protein level – translational or protein stability. My preliminary data show that DNA damage triggers increased NHE-1 mRNA levels in wild-type thymocytes, but not in the thymocytes transformed by hyper- active Lck<sup>F505</sup> (Appendix Fig 7). This suggests that the up-regulation of NHE-1 in response to DNA damage is caused at least in part by increased mRNA. NHE-1 antiport is a well- characterised protein, and regulation of NHE-1 expression in response to multiple stimuli other than DNA- damage has been extensively investigated (Dyck JR, 1995; Yang W, 1996). These investigations have established that NHE-1 expression is mainly regulated by transcription, and a number of transcription binding sites for these other stimuli have been identified in the promoter region of the NHE-1. So it is not premature to hypothesise that in DNA damaged cells NHE-1 increases expression level by transcriptional mechanism.

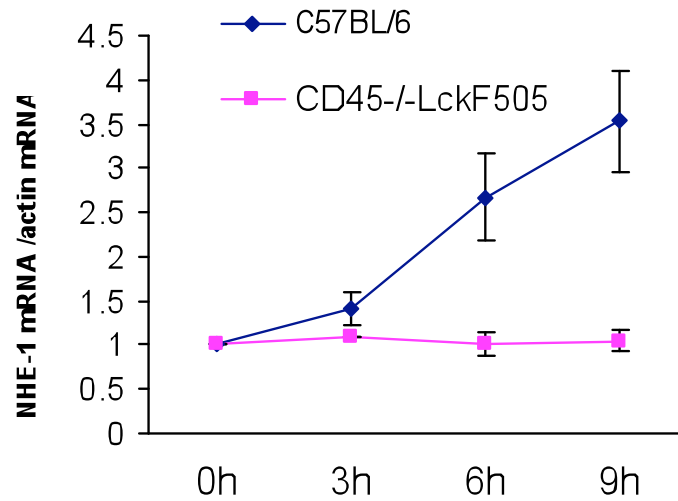
There is plenty to discover in this completely untouched area- such as, the responsive elements in NHE- promoter and the transcription factors that are critical in initiating the transcription in response to DNA damage signals; the inhibitory mechanisms employed by the OTKs to block the transcription, etc.

Thirdly, but not lastly, any other proteins that could be deamidated by the DNA damage induced intracellular alkalinisation?

A large percentage of proteins deamidate to a substantial extent during their biological life times (Nordhoff E, 1999). Among these spontaneous protein deamidation processes, Bcl-x<sub>L</sub> deamidation is the first example of rapid protein deamidation triggered by genotoxic stress, and playing a central role in the regulation of biological process. Other protein deamidation processes might also be promoted following DNA damage. There could be a plenary of molecules with various roles in cellular physiology that are regulated by deamidation.



## APPENDIX



**Fig 8. Quantitative RT-PCR results** showing the NHE-1 mRNA level (mean values  $\pm$  SD, n=3) in C57BL/6 and CD45<sup>-/-</sup> Lck<sup>F505</sup> thymocytes at time 0, 3, 6, 9 h of etoposide treatment. The RT-PCR were run on Bio-Rad Chromo4, and NHE-1 mRNA is normalised for actin mRNA.

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