# Dissertation

## Generation and Analysis of a Mouse Model Conditionally Expressing AKT1-E17K in T-Cells

**Stefan Wanninger**

Technische Universität München
2014

# Technische Universität München

**Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt**

**Lehrstuhl Proteomik und Bioanalytik**

## Generation and Analysis of a Mouse Model Conditionally Expressing AKT1-E17K in T-Cells

Stefan Wanninger

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

**Doktors der Naturwissenschaften (Dr. rer. nat)**

genehmigten Dissertation.

| | |
|---|---|
| Vorsitzender: | Univ.-Prof. Dr. Michael Schemann |
| Prüfer der Dissertation: | 1. Univ.-Prof. Dr. Bernhard Küster<br>2. Univ.-Prof. Dr. Jürgen Ruland |

Die Dissertation wurde am 30.04.2014 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 25.09.2014 angenommen.

# Index

# Abbreviations

| | |
|---|---|
| ALCL | anaplastic large-cell lymphoma |
| BAFF | B-cell activating factor |
| B-cell | B lymphocyte |
| BCR | B-cell receptor |
| CBM | Carma1-Bcl10-Malt1 |
| cc | cell count |
| CD | cluster of differentiation |
| CLL | chronic lymphocytic leukemia |
| CML | chronic myelogenous leukemia |
| CTL | cytotoxic T lymphocyte |
| DAG | diacylglycerol |
| DLBCL | diffuse large B-cell lymphoma |
| DN | double negative |
| DNA | deoxyribonucleic acid |
| DP | double positive |
| DTT | dithiothreitol |
| E | glutamic acid |
| EGFR | epidermal growth factor receptor |
| EMSA | electromobility shift assay |
| FAM | fluorescein amidite |
| FCS | fetal calf serum |
| FTOC | fetal thymic organ culture |
| GDP | guanosin diphosphate |
| GFP | green fluorescent protein |
| GPCR | G protein-coupled receptor |
| GTP | guanosine triphosphate |
| h | hour |
| HM | hydrophobic motif |
| IκB | inhibitor of NFκB |
| IFN | interferon |

| | |
|---|---|
| IKK | inhibitor of NFκB kinase |
| IL | interleukin |
| IP3 | inositol 1,4,5-trisphosphate |
| IRES | internal ribosome entry site |
| ITAM | immunreceptor tyrosine-based activation motif |
| K | lysine |
| kD | kilo Dalton (molecular mass) |
| MFI | mean fluorescence intensity |
| min | minute |
| MSCV | murine stem cell virus |
| MZ | marginal zone |
| NFAT | nuclear factor of activated T-cells |
| NFκB | nuclear factor kappa-B |
| NK cells | natural killer cells |
| NOS | not otherwise specified |
| PCR | polymerase chain reaction |
| PDGFR | platelet-derived growth factor receptor |
| PFA | paraformaldehyde |
| PH | pleckstrin homology |
| PI | phosphatidylinositol |
| PI3K | phosphatidylinositide 3-kinase |
| PIP2 | phosphatidylinositol 4, 5-bisphophate |
| PIP3 | phosphatidylinositol 3, 4, 5-trisphophate |
| PMA | phorbol 12-myristate 13-acetate |
| PTCL | peripheral T-cell lymphoma |
| RTK | receptor tyrosine kinase |
| SEM | standard error of the mean |
| Ser | serine |
| SH2 | Src homology 2 |
| SP | single positive |
| T-ALL | T-lineage acute lymphoblastic leukemia |
| T-cell | T lymphocyte |
| TCR | T-cell receptor |

| | |
|---|---|
| Thr | threonine |
| TNF | tumor necrosis factor |
| T-PLL | T-cell prolymphocytic leukemia |
| Tyr | tyrosine |
| WHO | World Health Organization |
| Y | tyrosine |

# Abstract

The phosphatidylinositol-3-kinase (PI3K)/AKT pathway is involved in regulating various fundamental cellular functions such as transcription, translation, proliferation, survival, and cell growth. Especially the serine/threonine kinase AKT has emerged as a central signalling node in controlling these processes. However, deregulations of PI3K/AKT pathway components are linked to diseases such as diabetes, autoimmunity, and most notably cancer. Recently, a new mutation (E17K) in the pleckstrin homology domain of AKT1 has been identified in human tumor samples. To investigate the oncogenic potential of this clinically relevant AKT1-E17K mutation *in vivo*, a novel transgenic mouse model was generated. By gene targeting, the AKT1-E17K sequence was inserted into the mouse genome under the control of the endogenous Rosa26 promotor allowing celltype-specific expression of the transgene together with eGFP.

T-cell specific expression of the transgene resulted in a fatal and aggressive lymphoproliferative disease. Pronounced splenomegaly and lymphadenopathy caused by an accumulation of T-cells, which showed an activated phenotype and increased proliferative potential, were observed. Furthermore, clonal expansion of T-cells and their infiltration into organs were detected. In combination with the histopathological analysis the disease was characterized as a Peripheral T-Cell Lymphoma. On a molecular level, constitutive downstream signalling of the AKT1-E17K kinase was noticed in transgenic T-cells. Several components of other crucial signalling pathway were also found to be affected. Additionally, an increased activation of the transcription factor NFκB was detected in T-cells in part of the mice. Deletion of Bcl10, a crucial component of antigen receptor mediated NFκB signalling, did not rescue the mice, but led to a small survival benefit, an increased incidence of thymic lymphoma and an absence of secondary B-cell activation compared to the situation on a normal background.

Taken together, these results shed new light onto the oncogenic potential of AKT, as well as its impact on other crucial signalling pathways. Therefore, the generated mouse model can serve as an important tool for studies of PI3K/AKT driven malignancies and for the development of novel therapeutic approaches.

# 1 Introduction

In order to accomplish a multitude of different tasks, eukaryotic cells are able to adapt to changing environmental influences, to integrate information into their cellular program and to communicate with each other. Especially immune cells have to sense, process and respond to both external and internal signals in order to successfully coordinate the defence against various pathogens. The majority of extracellular signals are received by specific surface receptors, leading to the engagement of intracellular signalling cascades with varying complexity. The signals from the activated receptors are transduced, amplified and modulated to finally result in an appropriate response, such as changes in gene expression profiles. Over the last decades much effort has been made to identify and unravel divers signalling pathways and their components which regulate critical aspects of cellular function. Remarkable new insights were gained in many fields of biology and medicine that deepened the understanding of normal and also aberrant cellular function. It is clear that deregulated signalling pathways play a crucial role in the development of multiple diseases including various forms of cancer. One central pathway involved in many cellular processes is the evolutionary highly conserved Phosphoinositide-3-Kinase (PI3K)/AKT pathway, in which the serine/threonine kinase AKT acts as an important signalling node.

## 1.1 PI3K/AKT Signalling

### 1.1.1 The Serine/Threonine Kinase AKT

AKT, also called protein kinase B (PKB), was discovered in 1977, when a transforming murine retrovirus (AKT8) was isolated from a spontaneous thymoma of the high leukemia AKR mouse strain (Staal et al., 1977). The virus contained an oncogenic sequence, termed v-akt, whose cellular human homologues AKT1 and AKT2 were later described (Staal, 1987). AKT belongs to the family of AGC kinases which consists of a large group of serine/threonine protein kinases in which the kinase domain sequence shows homologies to the protein kinases A, G and C. The identified AKT gene products also exhibited *in vitro* kinase activity (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991). Today, it is known that the AKT kinase family is evolutionary conserved in all eukaryotes and three isoforms, AKT1, AKT2 and AKT3, have been identified in mammals up so far.

Although, the isoforms are encoded by separate genes and localized on different chromosomes, they share approximately 80% amino acid identity (Vanhaesebroeck and Alessi, 2000). In addition, only marginal differences of the sequence between mice and humans are observed. All three isoforms share a protein structure that is composed of an N-terminal pleckstrin homology (PH) domain, a central kinase domain and a C-terminal regulatory domain containing a hydrophobic motif (HM) (Figure 1). The kinase domain and the HM show a high degree of similarity with other AGC kinases, characterizing the protein as a family member. The PH domain is also a crucial element for AKT activation and regulation. It was first identified within the protein pleckstrin, the major phosphorylation substrate of PKC (protein kinase C) in platelets, but can be found in numerous proteins involved in signalling. In general, the PH domain mediates protein-lipid interactions by binding to membrane phosphoinositides. In the case of the AKT PH domain, it specifically mediates interaction with phosphatidylinositol-3,4,5-trisphosphate (PIP3) and phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2) (Song et al., 2005).

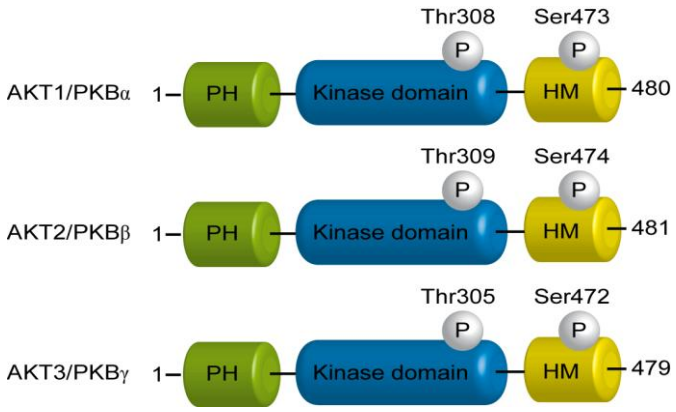


**Figure 1: Schematic representation of the human AKT isoforms.**
Protein length, domain structure and phosphorylation sites, which are crucial for kinase activation, are depicted for all three AKT isoforms. PH (pleckstrin homology), HM (hydrophobic motif).

The AKT isoforms seem to have overlapping as well as specific roles in cellular signalling, indicated by their differential gene expression pattern. AKT1 is ubiquitously expressed, whereas AKT2 is predominantly expressed in insulin target tissues like fat cells, liver and skeletal muscle. AKT3 expression was found to be high in brain and in 3T3-L1 adipocyte cells (Sale and Sale, 2008). Each of the three isoforms has been knocked out in mice and resulted in viable, but phenotypically different offspring. AKT1-deficient mice have an increased postnatal lethality and are smaller in size compared to their wildtype counterparts.

AKT1 knock out (k.o.) cells display a higher rate of apoptosis, indicating a crucial role in cell survival. AKT2 k.o. mice suffer from hyperglycemia, hyperinsulinemia, insulin resistance and age-dependent loss of adipocytes, which are signs for a Type II diabetes-like disease. Also, a mild growth retardation was observed, suggesting a central role of AKT2 in maintenance of glucose homeostasis. AKT3 k.o. mice have a reduced brain size, hinting at a brain specific function. Although these results implicate independent roles for the AKT isoforms, the analysis of double k.o. animals reveals some overlap or compensation between the isoforms. The AKT1/AKT2 double k.o. mice die at birth with abnormal skin, bone and muscle development and defects in adipogenesis. AKT1/AKT3 double k.o. results in embryonic lethality, whereas AKT2/AKT3 double k.o. mice are viable but show reduced body weight and insulin and glucose intolerance. It remains unclear how intense compensatory effects between the particular isoforms are and to what extent each of them participates in specific signal transduction processes. The celltype-specific expression pattern of the isoforms, differential activation and cell intrinsic factors that affect activity or substrate specificity certainly play a role in answering these questions (Yang et al., 2004).

### 1.1.2 Engagement of the PI3K/AKT Pathway

Activation of AKT takes place in response to numerous stimuli as a result of complex upstream signalling events. These are initialized by the recognition of an external signal by a cellular receptor leading to the activation of very crucial kinases in this pathway, phosphatidylinositol-3-kinases (PI3K). The function of the conserved PI3K family is to phosphorylate phosphatidylinositols and phosphoinositides on the 3´ position of the inositol ring. Eight different isoforms of PI3Ks have been identified and can be divided into three classes, depending on their structure and substrate preference: Class I, II and III (Figure 2).
The Class I PI3Ks are the most studied class and can be divided into Class IA and Class IB, depending on how they are activated. Both classes are heterodimeric enzymes, which consist of a regulatory and a catalytic subunit. The Class IA is activated by receptor tyrosine kinases (RTKs), such as the Epidermal Growth Factor Receptor (EGFR) or the Platelet-Derived Growth Factor Receptor (PDGFR) and also by tyrosine-kinase-associated receptors which include antigen-, costimulatory- and interleukin receptors. In mammals, three genes encode for the catalytic subunits p110α, p110β and p110δ, which are structurally

characterized by an N-terminal p85-binding domain, a Ras-binding domain, a C2 domain, a phosphatidylinositol kinase homology (PIK) domain and a catalytic domain. p110α and p110β are expressed in all tissues, whereas p110δ is preferentially expressed in leukocytes (Chantry et al., 1997; Vanhaesebroeck et al., 1997). Each of the catalytic subunits joins with one of the five regulatory subunits p85α, p85β, p55α, p55γ and p50α to form a heterodimer. p85α, p55α and p50α are derived from the same gene by alternate splicing and all regulatory subunits keep the catalytic subunits in an inactive state. The regulatory subunits harbour two SH2 domains that bind specific phosphorylated tyrosine residues in receptor proteins as well as other signalling (adaptor) proteins. Upon RTK engagement, the intracellular tyrosine residues of the receptor or adaptor proteins are phosphorylated and the regulatory domain of Class IA PI3Ks binds to these phosphosites via their SH2 domains. This results in the recruitment of cytosolic PI3K to the plasma membrane, where its lipid substrates reside. Binding of the regulatory subunit also relieves its inhibitory effect on the catalytic subunit and its kinase activity (Figure 3).

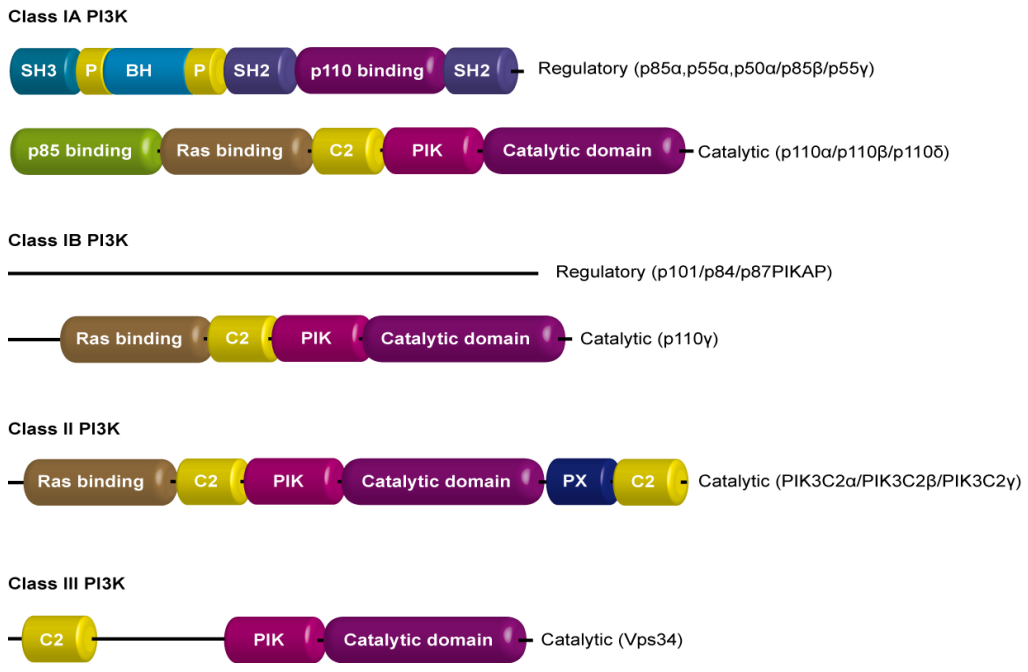


**Figure 2: The PI3K family members.**
The PI3K family can be divided three classes, depending on their structure, activation mode and substrate prevalence. Class I PI3Ks can be further subdivided into Class IA and Class IB. Both form heterodimers consisting of a regulatory and a catalytic subunit. Class II and III are monomers. For each class the different isoforms and the characteristic domains are indicated (adapted from Engelman et al., 2006).

The Class IB PI3Ks are also heterodimers consisting of one of the regulatory subunits p101, p84 or p87PIKAP, and the catalytic p110γ protein. Class IB PI3Ks are activated by the large family of G protein-coupled receptors (GPCRs), which include many hormone and chemokine receptors. Engagement of GPCRs leads to activation of the heterotrimeric Gαβγ proteins by exchanging the molecule GDP, bound to the Gα subunit, for GTP. This, in turn, results in dissociation of the GTP-bound Gα subunit from the Gβγ subunits. The p101 regulatory subunit then facilitates the interaction of p110γ with the Gβγ subunits and subsequent activation of the kinase (Figure 3).

The catalytic subunits of Class IA and IB are also able to bind to activated Ras (Ras-GTP) and seem to be important for Ras-dependent PI3K activation (Engelman et al., 2006). *In vivo*, both subclasses seem to have a substrate prevalence for the lipid phosphatidylinositol-4,5-bisphosphate (PIP2), although *in vitro* they are also able to phosphorylate phosphatidylinositol (PI) and phosphatidylinositol-4-phosphate (PI-4-P). Recently, the differentiation of the two subclasses has been questioned by findings that indicate that the p110β catalytic subunit of PI3K Class IA can be activated by Gβγ subunits downstream of GPCRs (Dbouk et al., 2010).

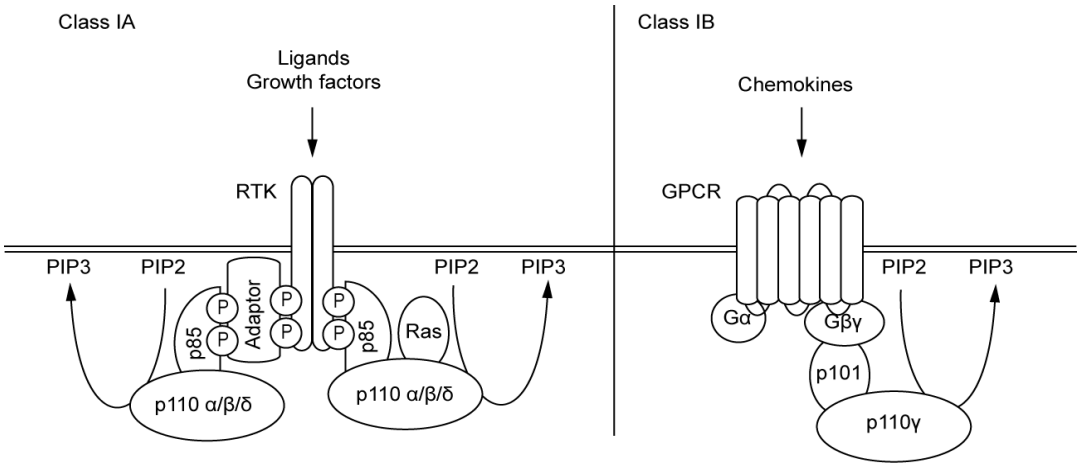


**Figure 3: Mechanism of Class I PI3K activation and PIP3 generation.**

Engagement of receptor tyrosine kinases (RTKs) or tyrosine kinase associated receptors results in binding of the regulatory subunits of Class IA PI3K to phosphorylated tyrosine residues of receptor or adaptor proteins via their SH2 domains. Thereby the catalytic subunit is recruited to the membrane, the inhibitory effect of the regulatory domain is relieved and PIP3 is produced. Stimulation of G protein-coupled receptors (GPCR) leads to the dissociation of GTP-bound Gα subunits from the Gβγ subunits, which allows binding of PI3K Class IB regulatory subunits and subsequent activation of the catalytic subunit (adapted from Chalhoub and Baker, 2009).

Class II and Class III PI3Ks have not been as extensively characterized as Class I. The monomeric Class II PI3K consists of three isoforms, PI3K-C2α, PI3K-C2β and PI3K-C2γ which are distinguished by the presence of an additional C-terminal C2 and a Phox homology (Px) domain. PI3K-C2α and PI3K-C2β seem to be ubiquitously expressed, but with higher levels present in heart, placenta and ovary or placenta and thymus, respectively. In contrast to PI3K-C2γ, which shows a more restricted expression pattern for liver, prostate, breast and salivary gland, they can be activated by cellular stimulation. The activating mechanisms for Class II PI3K are not completely defined yet. Most studies observe that phosphatidylinositol-3-phosphate (PI-3-P) is the main product of Class II PI3Ks. Recent studies on Class II PI3K isoforms also describe a role in endocytosis, insulin and neuroactive substance secretion, migration and vascular smooth muscle cell contraction (Falasca and Maffucci, 2012).

There is only one Class III PI3K monomer, the hVps (human vacuolar protein sorting) 34 protein, which associates with the serine kinase p150 and specifically catalyzes the synthesis of PI-3-P. This subgroup seems to be implicated in vesicle transporting and protein sorting, but also a role in the regulation of autophagy, protein synthesis and nutrient sensing pathway has been reported (Backer, 2008).

### 1.1.3 AKT Activation

Class I PI3Ks are the best-characterized members of the whole family because their product, the phospholipid PIP3, acts as a potent second messenger at the plasma membrane. PIP3 transduces the signal from the receptors to pathway components further downstream. In the presence of this phospholipid, proteins containing a PIP3 specific PH domain can interact with it and are thereby recruited to the membrane. Several proteins have been identified to contain such a PIP3-specific PH domain, including Bruton's tyrosin kinase (BTK), dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP1), Gab1, centaurin α, Grp1 and, most importantly, PDK1 and AKT (Lemmon and Ferguson, 2000).

The binding of AKT to PIP3 does not directly activate the kinase but rather recruits it to the membrane. This event is thought to initialize a conformational change within the protein that enables the kinase PDK1, which is also recruited to the membrane via its PH domain, to phosphorylate AKT at the threonine position 308 (Song et al., 2005). Hence, the binding of

PDK1 to PIP3 is also necessary for its membrane localization. Phosphorylation of AKT at Thr308 leads to stabilization in an active conformation, but for full kinase activation also phosphorylation of Ser473 (or an isoform corresponding residue) in the regulatory tail is needed. This event might even precede Thr308 phosphorylation and the responsible kinase was elusive for many years. In 2005 the mTOR complex 2 (mTORc2), containing the kinase mTOR (mammalian target of rapamycin) and the adaptors GβL and Rictor, was identified to mediate this event (Sarbassov et al., 2005) (Figure 4). Earlier studies also implicated several additional kinases including ILK-1, PDK1, DNA-PK and even AKT itself in phosphorylation of Ser473 and still are not excluded to function this way in some settings (Song et al., 2005).

Furthermore, AKT was found to be constitutively phosphorylated at Ser124 and Thr450, independently of cell stimulation or PI3K activation. These sites do not seem to be required for kinase function but seem to contribute to protein activation. They are thought to act as markers for proper protein folding or render AKT competent to undergo activation (Chan et al., 1999). Other reports indicate that tyrosine phosphorylation on Tyr315, Tyr326 and Tyr474 could also play a role in regulation of the kinase (Song et al., 2005). Following activation, AKT mediates a multitude of cellular effects by phosphorylation of numerous downstream targets in the cytoplasm and in the nucleus.

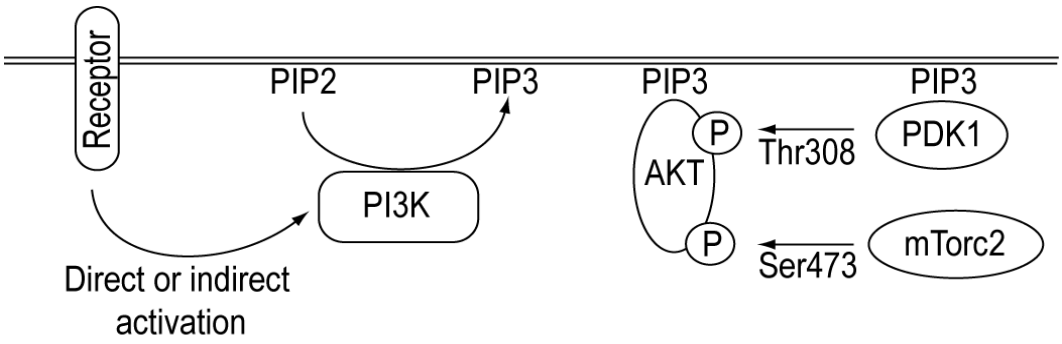


**Figure 4: Mechanism of AKT activation.**
After ligand induced PI3K activation and PIP3 generation, AKT can bind PIP3 and is recruited to the membrane. This induces a conformational change, enabling PDK1 to phosphorylate AKT in the T-loop at Thr308. Full AKT activation needs a second phosphorylation event on Ser473, which is mediated by the mTORc2 (adapted from Teitell, 2005).

### 1.1.4 Regulation of the PI3K/AKT Pathway

Since the PI3K/AKT pathway contributes to numerous cellular functions, there are several mechanisms that regulate or modulate AKT activation and deactivation. First of all, dephosphorylation of receptors, or their associated molecules, by tyrosine phosphatases can disable PI3K function (Chan et al., 1999). Secondly, regulation of the PIP3 levels at the membrane is a very important mechanism by which PI3K/AKT signalling can be deactivated. One antagonist of PI3K is the Phosphatase and tensin homologue (PTEN) which removes the D3 phosphate from the inositol ring of PIP3 and PI-3,4-P2 (Chalhoub and Baker, 2009). Thereby PTEN terminates PIP3-dependent signalling and especially AKT activation. Thirdly, influences on AKT kinase function by other proteins further modulate the pathway:

The phosphatase PP2A is directly targeting and inactivating AKT by dephosphorylation of Thr308 and to a lesser extent Ser473 (Liao and Hung, 2010). Additionally, the phosphatase family PHLPP was shown to dephosphorylate AKT in an isoform-specific way. PHLPP2 only regulates AKT1 Ser473 phosphorylation, whereas PHLPP1 dephosphorylates AKT2 Ser474 and both influence the AKT3 Ser472 phosphorylation status. In addition, a protein called PHLDA3 was also shown to inhibit AKT activity, most likely through competitive binding of PIP3. Also proteasomal degradation and caspase-mediated cleavage of AKT were reported to contribute to negative regulation of AKT signalling (Liao and Hung, 2010). Furthermore, several interaction partners of AKT were identified, which also play a role in regulation of the kinase. For instance, CTMP (carboxy-terminal modulator protein) was described as a binding partner that can inhibit AKT signalling by reducing Ser473 phosphorylation (Song et al., 2005). TRB3 binds to the central region of the kinase domain and is likely to be a negative regulator of AKT activation, since it inhibits phosphorylation at both activation sites induced by growth factors and insulin. Keratin 10 is proposed to bind and sequester AKT to the cytoskeleton, thereby inhibiting intracellular translocation and subsequent target phosphorylation (Song et al., 2005).

Also binding partners which positively regulate AKT function have been described. Tcl1 (T-cell leukemia/lymphoma protein 1) was shown to interact with AKT and increases activation possibly by oligomerization of AKT-Tcl1 heterodimers and stabilization at the plasma membrane. In addition, it seems to facilitate nuclear translocation of AKT (Du and Tsichlis, 2005). Also the constitutive interaction of Grb10 with AKT has been reported to

positively contribute to AKT activation induced by growth factors. Finally, the heat shock proteins Hsp27 and Hsp90 were implicated in stabilization of the kinase (Du and Tsichlis, 2005; Song et al., 2005).

### 1.1.5 AKT Kinase Substrates and Functions

Upon activation, AKT phosphorylates numerous downstream targets within the cytoplasm or the nucleus. Over seventy substrates carrying the minimal AKT consensus sequence RXRXXS/T (R standing for arginine, X for any amino acid, S/T for serine/threonine) have been reported, but many of them were only identified by *in vitro* kinase assays and remain to be further characterized (Manning and Cantley, 2007). The vast amount of substrates explains why AKT is involved in many varied cellular processes like cell survival, growth, proliferation, migration, metabolism and angiogenesis. The precise role of AKT in each of these processes is not mediated by one specific target and a corresponding function. Rather, the engagement of multiple targets in concert leads to a coherent response. Furthermore, some substrates themselves control several cellular functions. Additionally, isoform-, celltype- and stimulus-dependent variations complicate the signalling network.

An example of a direct AKT target, thought to be important in cell survival, is the proapoptotic protein BAD (Figure 5). BAD promotes apoptosis through binding and neutralization of antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-XL, Mcl1). Phosphorylation of BAD on Ser136 (human Ser99) by AKT induces its sequestration in the cytoplasm by binding to 14-3-3 proteins. Thus BAD is inactivated and the Bcl-2 proteins can fulfil their proapoptotic function (Datta et al., 1997; Zha et al., 1996). Furthermore, there is evidence that AKT can influence apoptotic mechanisms by inhibiting conformational change and mitochondrial membrane translocation of Bax (Tsuruta et al., 2002; Yamaguchi and Wang, 2001), necessary for Bax and/or Bak mediated cytochrome c release by a still highly controversial mechanism. In neutrophils, AKT was even found to directly phosphorylate proapoptotic Bax on Ser184 (human and murine) and to regulate its activity (Gardai et al., 2004).

Other proposed AKT targets that regulate apoptotic cell death are procaspase-9 and XIAP (X-linked inhibitor of apoptosis protein). Procaspase 9 is activated upon cytochrome c release and initiates effector caspases 3 and 7 activation, resulting in apoptosis. The human

form was shown to be phosphorylated on Ser196 by AKT, leading to an attenuation of protease activity (Cardone et al., 1998). This finding does not appear to be a major AKT function, because the phosphorylation site is not conserved in other mammalian species. AKT has also been reported to prevent degradation of the antiapoptotic protein XIAP by direct phosphorylation on Ser87 (Dan et al., 2004). XIAP is a crucial inhibitor of the caspases 3, 7, and 9. An alternative mechanism to stabilize XIAP was proposed, where AKT phosphorylates and inactivates HtrA2/Omi, a mitochondrial protease that cleaves XIAP when released into the cytoplasm by apoptotic stimuli (Yang et al., 2007).

Indirectly, AKT can also influence the stability of Mcl-1, another antiapoptotic protein of the Bcl-2 family, by regulating the kinase GSK3 (Figure 5). Being the first AKT target identified, GSK3 (glycogen synthase kinase 3) itself controls diverse cellular functions. Both isoforms (GSK3α/β) are directly phosphorylated by AKT at Ser21/Ser9 leading to inactivation of the kinase (Cross et al., 1995). Since GSK3 mediated phosphorylation at Ser159 targets Mcl-1 for ubiquitination and subsequent degradation, the inactivation of GSK3 by AKT enhances the stability of Mcl-1 (Maurer et al., 2006). Another effect of GSK3 inactivation was shown to be the upregulation of glycogen synthase (Cross et al., 1995).

Since GSK3 is involved in cell cycle regulation and proliferation, AKT is also able to influence these processes. GSK3 mediates the phosphorylation of the cyclins D and E and the transcription factors c-jun and c-myc, which all play a central role in the G1 to S phase cell cycle transition, and targets them for nuclear export and proteasomal degradation (Manning and Cantley, 2007). Phosphorylation and inhibition of GSK3 by AKT enhances the stability of these proteins and promotes cell cycle progression.

Recent reports point out that GSK3 is able to phosphorylate $p21^{Cip1}$, a cyclin dependent kinase (Cdk) inhibitor, at Thr57 and thereby triggers its degradation (Rossig et al., 2002). AKT itself also phosphorylates $p21^{CIP1}$ (Thr145, Ser146) promoting nuclear export, cytoplasmic accumulation and stabilization (Xu et al., 2012). Phosphorylation of another Cdk inhibitor by AKT, $p27^{KIP1}$, again functions via promoting its nuclear export, cytoplasmic accumulation but also degradation (Xu et al., 2012). Additionally, AKT can influence cell cycle regulation by inducing transcription of cyclin D and c-myc (Liang and Slingerland, 2003).

One more prominent protein, which is influenced by AKT signalling, is the tumor suppressor p53, a major regulator of cell cycle, DNA repair and apoptosis in response to

stresses. AKT phosphorylates the E3 ubiquitin ligase Mdm2 at Ser166 and Ser186, which leads to nuclear translocation and p53 ubiquitination and degradation. MdmX, a close homologue to Mdm2, has also been shown to be an AKT target, which stabilizes Mdm2 and increases p53 ubiquitination efficiency (Xu et al., 2012).

Another important branch of the AKT signalling network includes the FoxO transcription factors (Figure 5). They belong to the large family of Forkhead proteins which are characterized by their conserved DNA binding domain, the forkhead box. FoxO proteins act as transcriptional activators and repressors of genes that promote cell cycle arrest, apoptosis and metabolic processes. AKT can phosphorylate up to three different sites of the isoforms FoxO1 (Thr24, Ser256, Ser319), FoxO3A (Thr32, Ser253, Ser315) and FoxO4 (Thr32, Ser197, Ser262) in the nucleus, leading to 14-3-3 protein binding, nuclear export and cytoplasmic sequestration, thereby inhibiting the function of the transcription factors (Greer and Brunet, 2005; Manning and Cantley, 2007). The FoxO proteins strongly influence G1 phase arrest by upregulating cell cycle inhibitors ($p27^{Kip1}$, $p21^{CIP}$) and by repressing cell cycle activators (cyclin D1/2). There are also hints that FoxOs play a role in regulating the G2/M transition (Greer and Brunet, 2005).

Additionally, FoxO transcription factors can influence cell survival by inducing the expression of Bcl-2 family member Bim and FasL, both proapoptotic proteins (Downward, 2004). Lately, one more interesting task of the AKT/FoxO axis has been uncovered. The axis has been shown to regulate the migration of CD8+ T-cells in mice. The FoxO proteins control the expression of Klf2, another transcription factor, which in turn mediates expression of the adhesion molecule CD62L and the chemokine receptors CCR7 and S1P1. All three factors play a role in migration and homing of mouse lymphocytes (Finlay and Cantrell, 2011; Macintyre et al., 2011).

Another crucial function of AKT is the activation of mTORc1 (Figure 5). mTOR is a serine/threonine kinase that plays an important role in mRNA translation, in complex with Raptor, GβL, Deptor and the Tti/Tel2 complex. This multiprotein complex controls protein synthesis by phosphorylation of 4eBP1 (eukaryotic initiation factor 4E binding protein) and prevents its binding to the cap-binding protein eIF4E (eukaryotic initiation factor 4E). This enables eIF4E to participate in the formation of the eIF4F complex, which is required for the initiation of cap-dependent translation. Also S6K1, a kinase targeting the 40S ribosomal protein S6, is phosphorylated and activated via AKT and mTORC1, leading to an increase in mRNA biogenesis, as well as translational initiation and elongation. mTORc1 has also

been implicated in cellular processes such as ribosome and lipid (membrane) biogenesis, autophagy and general cell growth (Laplante and Sabatini, 2012). Activation of the mTORc1 by AKT is mediated indirectly by phosphorylation of TSC2 (tuberin), which functions in complex with TSC1 (hamartin) as a GTPase activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain). Rheb can only activate mTORc1 in its GTP bound form, but due to its GTPase activity Rheb is mostly present in its GDP bound form. AKT phosphorylation abrogates the GAP function of the TSC complex and Rheb GTPase activity, resulting in more GTP-bound Rheb and consequently activation of mTORc1 (Wullschleger et al., 2006).

Together, the substrates and their cellular functions discussed above are only a fraction of the many known AKT targets and hopefully give an impression of the complexity of this signalling network.

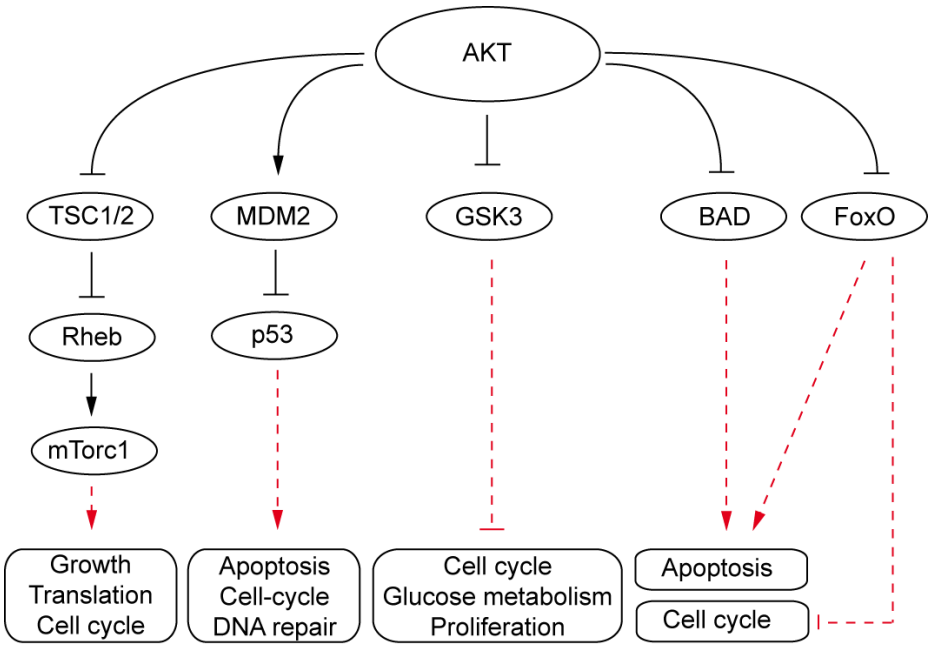


**Figure 5: Selected AKT downstream targets and functions.**
The serine/threonine kinase AKT is able to activate or inactivate numerous substrates by phosphorylation, e.g. TSC1/2, MDM2, FOXO, BAD or GSK3. The targets are implicated in regulation of important cellular processes as growth, proliferation, translation, cell cycle, apoptosis and glucose metabolism (adapted from Chalhoub and Baker, 2009).

### 1.1.6 PI3K/AKT Signalling in Lymphocytes

The PI3K/AKT pathway is also involved in coordinating immune cell function in adaptive and innate immunity. It is engaged in lymphocytes, mast cells, macrophages, NK cells, neutrophils, and dendritic cells by diverse stimuli. For example, many cytokines activate Class I PI3Ks through activation of the Janus kinase. Pharmacological inhibition experiments have shown that PI3K positively regulates the cytotoxic activity in NK cells. The Class IB PI3K p110γ mediates chemoattractant-induced neutrophil migration to the site of infection. Additionally, it was observed that Class IA PI3Ks are involved in phagosomal cup formation (Koyasu, 2003).

Especially in lymphocytes, PI3K/AKT signalling has been extensively studied and was reported to be activated by antigen-, Toll-like-, chemokine-, cytokine receptors, as well as costimulatory- and adhesion molecules. Further, the small GTPase Ras was implicated in PI3K activation. Genetic and pharmacological approaches have shown that PI3K regulates many steps in the development, activation and differentiation of both T- and B-cells. Especially the p110δ isoform of Class IA PI3K has emerged as a central driver of lymphocyte activation, clonal selection, differentiation and trafficking. But also Class IB PI3K isoform p110γ is involved in lymphocyte signalling. The activation of AKT and its numerous substrates, especially the FoxO transcription factors and mTORc1, play a key role in mediating the above described effects (So and Fruman, 2012). But the pathway appears to be not only a simple switch to promote cellular activation, but rather is integrated in a complex web of interactions that must be properly balanced to ensure appropriate cellular responses and to maintain immune homeostasis (Fruman and Bismuth, 2009).

### 1.1.7 PI3K/AKT Signalling in Response to Antigen Receptor Ligation

Lymphocytes are the key mediators of the adaptive immune system and are indispensable for mounting an effective immune response against pathogens. The recognition of a foreign molecule by their antigen receptors initializes a complex signalling cascade which finally results in an appropriate change in gene expression. Antigen receptor signalling resulting in an activation of crucial transcription factors, especially NFκB, was extensively studied and led to the identification of numerous proteins involved.

A short summary of antigen receptor signalling and of some of the major players in this signalling cascade, resulting in the activation of NFκB as only one outcome, are presented in the following:

After binding of an antigen to the antigen receptor, phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic region of the receptor by Src kinases is induced. Syk kinase family members are then able to bind to the phosphorylated ITAMs via their SH2 domains and phosphorylate diverse adaptor proteins, which in turn recruit and activate Tec kinases and PLCγ. PLCγ hydrolyzes PIP2 to generate IP3 (inositol trisphosphate), initiating $Ca^{2+}$ mobilization and activation of the transcription factor NFAT, and DAG (diacylglycerol), which activates PKC isoforms. PKC mediated phosphorylation induces the association of the CARMA1-Bcl10-Malt1 (CBM) complex, which activates the IKK (inhibitor of NFκB kinase) complex. This complex, consisting of the 3 subunits IKKα, β, γ, phosphorylates Iκbα (inhibitor of NFκB), which targets the protein for ubiquitination and subsequent degradation. Thereby the transcription factor NFκB is set free and can translocate to the nucleus to mediate its function in immune response (Ruland and Mak, 2003; Hayden and Ghosh, 2008).

The PI3K/AKT pathway also plays an important role in antigen receptor signalling, since rapid accumulation of PIP3 at the membrane, preferentially at the site of antigen contact, was observed after BCR as well as TCR engagement. Inhibition of PI3K was shown to block antigen receptor mediated processes. But the exact mechanisms coupling PI3K activation to the antigen receptor remain to be fully established.

After antigen receptor stimulation a multiprotein complex, called the signalosome, is formed at the membrane. It consists of numerous receptor-proximal proteins, building a platform that transduces the signal downstream towards PLCγ, PKCβ and finally NFκB activation. B-cells, deficient for components of this complex, such as Syk, BTK, BLNK, Vav or PLCγ2, show reduced intracellular $Ca^{2+}$ levels. The same is true for B-cells deficient in the PI3K subunits p85α or p110δ or treated with PI3K inhibitors. These similar defects indicate a role of PIP3 as a membrane targeting signal for proteins involved in signalosome assembly and immune synapse formation. Consequently, the downstream signalling in B-cells of these mice, including PKCβ activation, Iκbα degradation and NFκB accumulation in the nucleus, is impaired and their proliferation is defective (Fruman and Bismuth, 2009). This shows that B-cell function is highly dependent on Class IA PI3K.

Further studies revealed that crosslinking of the BCR leads to tyrosine phosphorylation of the coreceptor CD19 and B-cell adaptor protein (BCAP). Both possess characteristic Y-X-X-M motifs which are phosphorylated by the kinase Syk. Subsequent recruitment of Class IA PI3Ks to these motifs through SH2 domain interactions seems to be the major contributor of PI3K activation in mouse B-cells. Similar to CD19-deficient mice, B1 and marginal zone (MZ) B-cells are nearly absent in p85α, p110δ, and AKT1/2 k.o. mice, implicating a crucial role of the PI3K/AKT pathway in commitment to B1 and MZ B-cell lineage and a function at key stages of B-cell development (So and Fruman, 2012). Additionally, p110δ inhibition also suppresses chemotaxis, proliferation and antibody production by B1 and MZ B-cells (Durand et al., 2009). The PI3K subunits p110α and p110δ were also shown to be essential at the pre-BCR checkpoint for developmental progression from the pro-B to pre-B-cell transition (Ramadani et al., 2010).

The mechanisms by which PI3Ks are recruited to the TCR signalosome are different and remain also largely unknown. Physiological engagement of the TCR triggers rapid recruitment of p85α to the contact site and sustained production of PIP3, concentrated at the site of antibody contact (Fabre et al., 2005; Harriague and Bismuth, 2002). Inhibition of PI3K after T-cell stimulation shows rapid disappearance of PIP3, indicating the participation of phosphatases, and marked suppression of CD4+ T-cell proliferation, with a major role for p110δ (So and Fruman, 2012).

One clear mechanism of PI3K activation in T-cells is via the phosphorylated Y-X-X-M motifs in the cytoplasmic tails of the costimulatory receptors CD28 and ICOS. CD28 is the primary costimulatory molecule on resting T-cells, and its ligation results in rapid activation of PI3K and AKT. ICOS functions more in response to already activated T-cells and its engagement leads to stronger PI3K activation than by CD28 (Rudd et al., 2009). But there is also PI3K activation independent of costimulatory molecules. Possible mechanisms include binding of p85 via its Rho-GAP domains to SH3 domains of Src kinases or to Rac-GTP, and also association of the SH2 domains in p85 or p50 with the pTyr residues in other proteins associated with the TCR signalosome (So and Fruman, 2012).

To the various functions of the PI3K/AKT pathway in T-cells after TCR induction belong the blockade of Fas mediated apoptosis by preventing assembly of the DISC complex, but also the increase of glucose transport, metabolism and glycogen synthesis to finally be able to promote proliferation and survival (Jones et al., 2002; Frauwirth et al., 2002). In thymocytes, pre-TCR signalling was found to be attenuated in the absence of p85α or p110δ,

even if overall number of thymocytes was not significantly reduced (Janas et al., 2010; Shiroki et al., 2007). The combined deletion of AKT1 and AKT2 profoundly impairs thymocyte development and survival, with loss of AKT1 alone showing a partial phenotype (Fayard et al., 2007; Juntilla et al., 2007).

Additionally, numerous studies indicate that T-cell differentiation is highly regulated by PI3K signalling and its downstream effectors, particularly the AKT/FoxO axis and mTOR (So and Fruman, 2012). For example, T-cells from a p110δ kinase-inactive knock-in mouse have increased thymic regulatory T-cell numbers, but also a defect in both Th1 and Th2 differentiation and cytokine production (Okkenhaug et al., 2006). Moreover, T-cells expressing a myristoylated form of AKT are not able to differentiate into $T_H17$ cells *in vitro* (Pierau et al., 2009). In CD8+ T-cells, PI3K/AKT signalling after TCR stimulation is required to initiate the transcriptional program for granzymes, perforin, and IFNγ, all important for CTL effector function. AKT mediated inactivation of the FoxO transcription factors also coordinates the expression of homing and trafficking receptors that allow effector CTL to leave the lymph node and traffic to sites of infection (Sinclair et al., 2008; Macintyre et al., 2011).

### 1.1.8 Influences of PI3K/AKT Signalling on the Transcription Factor NFκB

Since NFκB is one of the most important transcription factors induced after antigen recognition, a possible connection between PI3K/AKT and NFκB has been investigated. NFκB is involved in the control of normal cellular processes, such as development, growth, and survival, but also in immune and inflammatory responses. The five eukaryotic family members of NFκB can be divided into two classes. The Rel proteins (RelA, RelB and c-Rel) possess a highly conserved Rel homology domain, which is responsible for DNA binding, localization and binding to other proteins, and in addition a C-terminal transcription activation domain. The NFκB proteins (p105 and p100) consist of a Rel domain and several ankyrin repeats, which act to inhibit these proteins by not allowing protein-protein interactions and hence have to be cleaved off for functionality. All members of the family can form homo- and heterodimers with distinct DNA binding specificities and thereby contribute to the regulation of distinct genes. NFκB transcription factors are kept in an inactive state by association with inhibitory proteins, called IκB, which retain the NFκB dimers in the cytoplasm and block their ability to bind DNA. In immune cells, canonical

NFκB signalling can be initiated by signals such as proinflammatory cytokines, pathogen associated molecules or antigens.

The non-canonical NFκB pathway is only engaged in response to a restricted subset of TNF receptor superfamily members such as Lymphotoxin β, CD40 and BAFF receptors and involves IKKα activation by NIK (NFκB-inducing kinase). IKKα phosphorylates p100/RelB heterodimers and induces cleavage of p100 to p52, forming the active p52/RelB heterodimer (Hayden and Ghosh, 2008; Ruland and Mak, 2003).

Some studies claim that PI3K/AKT signalling can directly influence NFκB activation. The group of David Donner showed that TNFα-induced NFκB activation in HEK, Hela and 3T3 cells is dependent on AKT, which constitutively associates with IKKα and increases its phosphorylation on Thr23 (Ozes et al., 1999). Subsequent studies also state that the noncoordinate expression of IKKs is involved in determining the celltype-specific role of AKT in NFκB activation (Gustin et al., 2004). Further AKT seems to be involved in basal and induced processing of p100 to p52 (Gustin et al., 2006). AKT-dependent NFκB activation was also described in fibroblasts after PDGF receptor stimulation. Again, the kinase was found to be associated with IKKα/β, but only a temporal interaction was observed in this study (Romashkova and Makarov, 1999). However, the initial findings were questioned because other groups were not able to reproduce or validate the results in similar systems (Delhase et al., 2000; Madge and Pober, 2000; Rauch et al., 2000).

Other studies in HepG2 cell, fibroblasts and PC3 prostate cancer cells argue, that AKT is not directly involved in NFκB activation, but in enhancing the transactivation potential of p65, maybe through mTORc1 and IKKs (Dan et al., 2008; Madrid et al., 2001; Madrid et al., 2000; Sizemore et al., 1999). In Jurkat T-cells overexpression of AKT potentiated NFκB reporter gene activation, especially in conjugation with PMA (Kane et al., 1999). Also a role of AKT in modulation of NFκB induction and subsequent gene expression was suggested, perhaps involving the proteins Carma1 and Bcl10 of the CBM complex (Cheng et al., 2011; Narayan et al., 2006). T-cells, expressing constitutively active AKT (myrAKT) in a mouse model, seemed to have a survival benefit after various apoptosis-inducing challenges. There AKT was found to be insufficient to directly induce NFκB, but it enhanced the intensity of the response after stimulation (Jones et al., 2000; Jones et al., 2005). In contrast to that, stimulated T-cells from another mouse model expressing myrAKT showed less NFκB and NFAT nuclear localization (Patra et al., 2004). In summary, the role of AKT in NFκB signalling is still controversial and has to be further elucidated.

## 1.2 The PI3K/AKT Pathway and its Role in Cancer

The term "cancer" stands for a group of various diseases, all involving uncontrolled cell growth and invasion of cells into other tissue. For the year 2008, the WHO statistics shows that cancer accounts for 7.6 million death worldwide. The main causes are considered to be alterations in the cellular genome affecting the expression, function or regulation of genes in signalling pathways controlling cell growth, survival, proliferation, migration and differentiation. Hanahan and Weinberg tried to organize the complexity of factors driving cancerogenesis and proposed several properties, which normal cells have to gain on their way to become cancer cells. These "hallmarks of cancer" include self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, unlimited replicative potential, tissue invasion and metastasis (Hanahan and Weinberg, 2011). The PI3K/AKT pathway is implicated in all of the cellular functions underlying these hallmarks and was indeed found to be one of the most frequently deregulated pathways in human cancers.

### 1.2.1 Aberrations within the PI3K/AKT Pathway Involved in Oncogenesis

Unique to the PI3K/AKT pathway is, that mutations and amplifications were found at every major signalling node in a wide variety of solid tumors and haematological malignancies (Figure 6) (Yuan and Cantley, 2008). Common alterations upstream of PI3K, subsequently leading to an activation of downstream signalling, can already be found at the level of cell surface receptors. Examples are the overexpression of the EGFR (epidermal growth factor receptor) family member Her2/neu in 25-30% of invasive breast- and ovarian cancer or the activating mutations of Kit and PDGFRα in gastrointestinal tumors (Yuan and Cantley, 2008).

Receptor proximal tyrosine kinases can be activated constitutively by chromosomal translocation or fusion and can turn on pathological downstream signalling, as described for BCR-ABL in hematological malignancies as CML (Chronic Myelogenous Leukemia) (Altomare and Testa, 2005). Furthermore, PI3K gain-of-function mutations have frequently been observed in many human cancers. The PIK3CA gene, encoding for the p110α catalytic subunit of PI3K, was shown to be amplified or overexpressed in head and neck- (nearly

40%), ovarian- (~ 40%), gastric- (35%), cervical-, breast- and lung cancer. Additionally PIK3CA is somatically mutated in 32% of colorectal-, 25% of gastric-, 36% of hepatocellular-, 18-40% of breast-, 4-12% of ovarian-, 4% of lung cancer, 27% of glioblastoma and several other cancer types as thyroid and endometrial cancer. These frequencies make PIK3CA to one of the two most commonly mutated genes identified in human cancer, the other one being K-Ras (Samuels and Ericson, 2006). The small G protein K-Ras is also activated by RTK and GPCR and plays an important role in several cellular processes by stimulation of the Raf-MEK-MAPK and the PI3K pathway. Interestingly, more than 80% of PIK3CA mutations cluster in two conserved regions, which encode the kinase and the helical domains. Three hotspot mutations (H1047R, E545K, E542K) have been identified and were found to induce constitutive kinase activity and oncogenic transformation with a high efficiency. In mouse models, a mutational activated p110α expressed in lung was shown to produce lung adenocarcinomas and p110α membrane targeting resulted in spontaneous neoplasia (Carnero, 2010).

Evidence of PI3K-signaling deregulation in human malignancies

| Cancer type | PI3K | PTEN | AKT |
|---|---|---|---|
| Glioblastoma | PIK3CA (p110α) mutation | PTEN mutation | |
| Ovarian cancer | PIK3CA (p110α) amplification<br>p85α mutation | Allelic imbalance and mutations of PTEN gene | Elevated AKT1 kinase activity<br>AKT2 amplification and overexpression |
| Breast cancer | PI3K overactivation<br>PIK3CA (p110α) mutation | Loss of heterozygosity at PTEN locus | Elevated AKT1 kinase activity<br>AKT2 amplification and overexpression |
| Endometrial cancer | | PTEN mutation, PTEN silencing | |
| Hepatocellular carcinoma | PIK3CA (p110α) mutation | PTEN mutation | AKT amplification, overexpression, activation |
| Melanoma | | PTEN mutation, PTEN silencing | |
| Digestive tract | p85α mutation; PIK3CA (p110α) amplification, overexpression, mutation | Abberant PTEN transcripts | AKT amplification, overexpression, activation |
| Lung cancer | PIK3CA (p110α) mutation | PTEN inactivation | |
| Renal-cell carcinoma | | PTEN mutations | |
| Thyroid cancer | | PTEN mutations | AKT overexpression and overactivation |
| Lymphoid cancer | | PTEN mutations | |
| Head and neck cancer | PIK3CA (p110α) amplification, overexpression | | AKT2 amplification, overexpression, activation |
| Prostate cancer | | PTEN mutations | |
| Pancreatic cancer | | | AKT2 amplification, overexpression, activation |

**Figure 6: Deregulation of the PI3K/AKT pathway in human malignancies.**
Findings of PI3K/AKT pathway alterations in several human cancer types are depicted. PTEN and PIK3CA gene aberrations can be observed frequently (adopted from Vivanco and Sawyers, 2002).

A real hotspot for alterations in cancer seems to be the PTEN gene. It is deleted, transcriptionally silenced or mutated in a way that abrogates its function as a tumor suppressor in a wide spectrum of cancers (Figure 6). The highest numbers for somatic PTEN mutations are found in endometrium (39%), central nervous system (20%), skin (17%) and prostate cancers (14%) (Chalhoub and Baker, 2009). Moreover, some PTEN mutations were also identified in melanoma, colorectal, breast and lung cancer. Germline mutations of PTEN are known to cause autosomal dominant hamartoma tumor syndromes

(Cowden´s disease, Proteus-like syndrome, Bannayan-Riley-Ruvalcaba syndrome), which are all familial cancer predisposition syndromes (Carnero, 2010; Yuan and Cantley, 2008). The PTEN k.o. turned out to be embryonically lethal, but mice with loss of just one PTEN allele suffer from neoplasia of multiple epithelia (intestine, prostate, endometrium, and mammary gland).

Also AKT, the central kinase in the pathway, was found to be amplified, overexpressed and activated in several cancer types (Figure 7). Especially the AKT2 gene was demonstrated to be amplified in 30% of head and neck squamous cell carcinoma, 20% of pancreatic-, 12% of ovarian-, and 3% of breast cancer (Chalhoub and Baker, 2009). Elevated expression was also observed in approximately 40% of hepatocellular carcinomas and 57% of colorectal cancers (Altomare and Testa, 2005). AKT3 gene amplification has not been described, but overexpression of AKT3 mRNA has been demonstrated in breast and prostate cancer (Osaki et al., 2004). Infrequent AKT1 amplification in single cases of gastric carcinoma, glioblastomas and gliosarcomas were identified, thus seem to be rarer. Nevertheless, AKT1 protein levels were elevated in some cancer types. Additionally AKT activation was found to correlate with resistance to chemo- and radiotherapy, advanced disease status and poor prognosis, even more turning the kinase into an interesting target for drug discovery and therapeutic strategies (Altomare and Testa, 2005; Jiang and Liu, 2008). In summary, numerous mutations in the PI3K/AKT pathway were found, but not so many affecting AKT itself. Nevertheless, a high percentage of activated AKT can be observed in many tumor types, presumably due to upstream mutations (Figure7).

AKT activation in human cancers

| Tumor type | % tumors with active AKT |
|---|---|
| Glioma | ~ 55 |
| Thyroid carcinoma | 80 - 100 |
| Breast carcinoma | 22 - 55 |
| Small-cell lung carcinoma | ~ 60 |
| Non-small- lung carcinoma | 30 - 75 |
| Gastric carcinoma | ~ 80 |
| Gastrointestinal stromal tumors | ~ 30 |
| Pancreatic carcinoma | 30 - 70 |
| Bile duct carcinoma | ~ 85 |
| Ovarian carcinoma | 40 - 70 |
| Endometrial carcinoma | > 35 |
| Prostate carcinoma | 45 - 55 |
| Renal cell carcinoma | ~ 40 |
| Anaplastic large-cell lymphoma | 100 |
| Acute myeloid leukemia | ~ 70 |
| Multiple myeloma | ~ 90 |
| Malignant mesothelioma | ~ 65 |
| Malignant melanoma | 43 - 67 |

**Figure 7: AKT activation in human malignancies.**
Several human tumor types and the corresponding percentage of activated AKT (adapted from Altomare and Testa, 2005).

### 1.2.2 The PI3K/AKT Pathway in Lymphoma

Considering the crucial role of PI3K/AKT signalling in lymphocytes, it is not surprising that the pathway is also implicated in the development of lymphoma. Aberrations within the whole pathway were identified in several forms of lymphoma. For example, PDGFRα, a growth factor receptor upstream of PI3K, was shown to be the most differentially expressed gene in a study analyzing 17 cases of Peripheral T-Cell Lymphoma (Not Otherwise Specified) (PTCL NOS) (Piccaluga et al., 2007). The NPM-ALK fusion protein, resulting from a chromosomal translocation and found in approximately 50% of all anaplastic large cell lymphoma (ALCL), can trigger constitutive activation of the PI3K pathway (Drexler et al., 2000; Altomare and Testa, 2005). Furthermore, some PIK3CA (p110α) gene mutations were identified in samples of human diffuse large B-cell lymphomas (DLBCL) (Abubaker et al., 2007; Baohua et al., 2008) and T-cell acute lymphoblastic leukemia (T-ALL) (Gutierrez et al., 2009). Also the PI3K regulatory subunit PIK3R1 (p85α) contained mutations, which were found in T-ALL samples and in a cell line derived from a Hodgkin's lymphoma (Gutierrez et al., 2009; Jucker et al., 2002). Besides, a mouse model expressing a constitutive active p85 allele ($p65^{PI3K}$) develops a lymphoproliferative disorder, which further progresses to a lymphoma when crossed with Trp53 k.o. mice (Borlado et al., 2000). No mutations, amplifications or overexpression of the p110δ subunit, which is very important in immune cell function, were detected in human cancer up to now. But targeting of this subunit with the selective inhibitor CAL-101, which is still in clinical trials, showed promising effects in patients with CLL, indolent Non-Hodgkin's lymphoma or mantle cell lymphoma, indicating a role for p110δ in lymphoma cells (So and Fruman, 2012).

Screening of primary tumor samples of B-cell origin (DLBCL, lymphoblastic leukemia/lymphoma, large B-cell lymphoma) resulted in the identification of mutations or (homo- and heterozygous) deletions of the PTEN gene (Sakai et al., 1998; Gronbaek et al., 1998; Butler et al., 1999; Abubaker et al., 2007). Loss of PTEN expression, leading to constitutive activation of AKT, was also shown to contribute to the pathogenesis and survival of mantle cell lymphoma (Rudelius et al., 2006). Moreover, PTEN mutations were found in samples of T-ALL and may play a role in the progression of this disease (Gutierrez et al., 2009; De Keersmaecker et al., 2005). Downregulation of the phosphatase is reported to be essential for the formation of typical nuclear lobules in T-cell leukemia/lymphoma (Fukuda et al., 2005). Additionally, partial or complete loss of PTEN was observed in 66.7%

of ALCL and in 12.5% of the few other mature T/NK-cell lymphomas analyzed (Uner et al., 2005). Heterozygous deletion of PTEN in mice led amongst other tumors to a higher incidence of T-cell lymphoma (Suzuki et al., 1998). The T-cell specific deletion consequently resulted in the development of a T-cell lymphoma, too (Suzuki et al., 2001). Also to mention is the fact that many hematopoietic cell lines lack or have low PTEN protein expression (Chang et al., 2003).

Further aberrations, identified in lymphoma and influencing PI3K/AKT signalling, affect the Tcl1 gene locus. The Tcl1 protein can directly bind to AKT and is thought to serve as a coactivator by increasing AKT activation. Chromosomal rearrangement between a Tcl1 family gene and the TCR locus, placing the gene under the control of elements that regulate TCR expression, is frequently found T-cell prolymphocytic leukemia (T-PLL). Dysregulation of Tcl1 expression has also been observed in mature B-cell tumors, as in follicular lymphoma, Burkitt lymphoma, DLBCL, B-cell chronic lymphocytic leukemia, and mantle cell lymphoma. Mouse models, which express Tcl1 in the T- or B-cell lineage, develop corresponding lymphoid malignancies, which are comparable to the human disease (Teitell, 2005).

Only one aberration for the AKT gene itself, an amplification of AKT2 in Non-Hodgkin's lymphoma, was described (Arranz et al., 1996). Nevertheless, the murine lymphoma virus AKT8, isolated from spontaneous thymoma of AKR mice and carrying a viral form of AKT, is able to induce the formation of a thymic lymphoma in mice (Staal, 1987). The development of a lymphoma could be observed in two out of four transgenic mouse lines, expressing myrAKT in T-cells (Malstrom et al., 2001; Jones et al., 2000; Na et al., 2003; Rathmell et al., 2003). The different outcome of these approaches might be due to the use of different promotors.

But, even if there are no frequent alterations within the AKT gene itself in human cancer, the numerous others aberrations in the pathway lead to an activation of AKT as the central kinase. Without knowing the exact cause, high levels of activated AKT were found in patient samples of DLBCL, DLBCL cell lines, other Non-Hodgkin's Lymphoma samples and PTCL (Cai et al., 2012; Fillmore et al., 2005; Uddin et al., 2006). Taken together, the frequent activation of the PI3K/AKT pathway in lymphoma is a recurring phenomenon.

### 1.2.3 The AKT1-E17K Mutation

In 2007, Carpten et al. screened AKT family members for mutations in clinical tumor specimen of breast, colorectal and ovarian cancer. No genetic alterations were found in the catalytic domains of AKT1, 2 and 3, but a mutation in the PH domain of AKT1 was identified. The point mutation at nucleotide position 49 (G→A) resulted in an amino acid substitution from glutamic acid to lysine at position 17 (E17K). It occurred only at a low frequency (in 8% of breast, 6% of colorectal, 2% of ovarian cancer, respectively) but appeared to be mutually exclusive with respect to mutations in PIK3CA and PTEN genes, indicating that the AKT1 mutation is sufficient for pathological activation of the PI3K/AKT pathway (Carpten et al., 2007).

The E17K mutation resulted in an altered PIP specificity of the PH domain due to the modified side chain charge. The affinity for the abundant plasma membrane lipid PIP2, which is not bound by the AKT PH domain under normal circumstances, was found to be increased at least a hundredfold. In addition, the affinity for PIP3, the actual binding partner, was increased sevenfold. Detailed analysis showed that the mutation has little effect on PIP lipid association rates, but altered the dissociation kinetics, thus forming a stronger protein-PIP lipid complex (Landgraf et al., 2008). This led to a permanent membrane association of the mutant protein, increased phosphorylation of the two activation sites Thr308 and Ser473 and an approximately fourfold increase in kinase activity compared to wildtype AKT. Stronger phosphorylation of the downstream target FoxO1 under serum starved conditions confirmed the enhanced cellular activity of AKT1-E17K. The mutant AKT was able to transform cells in culture and to induce leukemia in mice in cooperation with myc in an adoptive transfer model. Furthermore, AKT-E17K showed diminished sensitivity to an inhibitor, which does not compete with ATP or peptide but targets the PH domain. Hence, the response to a potential therapy of cancer patients using AKT inhibitors depends on how exactly AKT and other pathway components are activated.

### 1.2.4 The AKT1-E17K Mutation in Cancer

After its initial identification, multiple screens for the E17K mutation were performed in various tumor specimens. The mutation was discovered in a low frequency in samples of

melanoma, breast-, colorectal-, lung-, squamous cell-, urothelial-, endometrial- and prostate cancer, and also in Proteus syndrome and T-ALL (Askham et al., 2010; Bleeker et al., 2008; Davies et al., 2008; Gutierrez et al., 2009; Kim et al., 2008; Lindhurst et al., 2011; Malanga et al., 2008; Shoji et al., 2009; Zilberman et al., 2009; Zuurbier et al., 2012). However, in some studies the E17K mutation was not detected at all (Bleeker et al., 2009; Cao et al., 2008; Cohen et al., 2009; Eom et al., 2009; Ismail et al., 2010; Mahmoud et al., 2008; Mohamedali et al., 2008; Riener et al., 2008; Zenz et al., 2008). This indicates that the serine/threonine kinase AKT is not a hotspot for activating mutations in the PI3K/AKT pathway. In contrast, genetic alterations in the PI3K genes and especially in the PTEN gene seem to be a more frequent event in cancer (Altomare and Testa, 2005). Nevertheless, all disturbances found in the pathway lead, amongst other things, to an activation of the central kinase AKT.

### 1.3 Aims of the Project

The central importance of the PI3K/AKT pathway in disease and especially in the development of cancer is clearly demonstrated by the findings listed above. To deepen the general understanding of how alterations in this pathway drive oncogenesis, further investigations of deregulated signalling events are needed. The aim of this study was to gain new insights into the potential oncogenic function of the newly identified mutant AKT1-E17K. For that purpose a novel and defined *in vivo* mouse model was generated by gene targeting, in which AKT1-E17K can be expressed in a celltype-specific manner. This model enables close investigation of the impact of the constitutive active kinase and additionally represents a useful tool to study effects of an activated PI3K/AKT pathway in various settings.

Since activation of AKT is often found in T-cell malignancies, the mutant AKT1 was expressed in T-lymphocytes to evaluate the consequences in a clinically relevant *in vivo* mouse model. The potential of AKT1-E17K to induce a T-cell lymphoma and the characteristics of the resulting disease were investigated. Additionally, its influence on crucial signalling pathways which are connected to oncogenesis was addressed. In this context, also the role of Bcl10, a protein important for antigen receptor mediated NFκB activation, in the development of a PI3K/AKT driven lymphoma was analyzed.

# 2 Materials and Methods

## 2.1 Materials

If not otherwise stated, the following reagents were purchased from the indicated companies. All chemicals were obtained from Sigma-Aldrich, antibodies were from Cell Signalling Technology (Western Blot) and eBiosciences or Becton Dickinson (Flow cytometry), respectively. Restriction enzymes and ligases for cloning were ordered from New England Biolabs. PCR reagents were purchased from Thermo Fisher Scientific and all primers (Table 1) were synthesized by MWG. The basic flag tagged and codon-optimized AKT1-E17K construct was synthesized by Geneart. Plasticware (dishes, flasks, plates, falcon tubes, etc) was purchased from TPP or Sarstedt. Analysis of flow cytometry data was done with FACS Diva (Becton Dickinson) and FlowJo (Treestar, Inc.) software. Graphs and statistical analysis was made with GraphPad Prism software.

| Primer | Sequence 5' - 3' | Usage |
|---|---|---|
| Rosa26 wt fwd | GTAGTAAGGATCTCAAGCAGG | Confirmation of germline transmission |
| Rosa26 wt rev | AGTCGCTCTGAGTTGTTATCAG | Confirmation of germline transmission |
| tgAKT fwd | TACAAGGACGACGACGACAAG | Genotyping, confirmation of germline transmission |
| tgAKT rev | CTGCCGCTTCTGAAGTCCA | Genotyping, confirmation of germline transmission |
| Rosa26 screen fwd | TCGCCTTCTATCGCCTTCTTG | Confirmation of germline transmission |
| Rosa26 screen rev | AGGTAGGGGATCGGGACTC | Confirmation of germline transmission |
| AKT K17E fwd | GCTGGCTGCACAAGAGGGGCGAGTACATCAAGAC | Site directed mutagenesis |
| AKT K17E rev | GTCTTGATGTACTCGCCCCTCTTGTGCAGCCAGC | Site directed mutagenesis |
| AKT K179M fwd | CAGGTACTACGCCATGATGATCCTGAAGGAAG | Site directed mutagenesis |
| AKT K179M rev | CTTCTTTCTTCAGGATCATCATGGCGTAGTACCTG | Site directed mutagenesis |
| CD4Cre fwd | ACCAGCCAGCTATCAACTCG | Genotyping |
| CD4Cre rev | TTACATTGGTCCAGCCACC | Genotyping |
| Bcl10 wt | TTGGCTCTCTGCTCTCCTCACT | Genotyping |
| Bcl10 com | CGCTCTGAGGACTGTGGGACTG | Genotyping |
| Bcl10 neo | GGGTGGGATTAGATAAATGCCTGCTC | Genotyping |
| TCRβ-Vβ1 | AAATGAGACGGTGCCCAGTCGTT | Spectratyping |
| TCRβ-Vβ2 | TCCTGGGGACAAAGAGGTCAAATC | Spectratyping |
| TCRβ-Vβ3 | GAAAAACGATTCTCTGCTGAGTGTCC | Spectratyping |
| TCRβ-Vβ4 | AGCTATCAAAAACTTATGGACAATCAG | Spectratyping |
| TCRβ-Vβ5 | CAGCAGATTCTCAGTCCAACAGTTT | Spectratyping |
| TCRβ-Vβ6 | AAGGCGATCTATCTGAAGGCTATGA | Spectratyping |
| TCRβ-Vβ7 | AGCTGATTTATATCTCATACGATGTTG | Spectratyping |
| TCRβ-Vβ8 | TATATGTACTGGTATCGGCAGGACA | Spectratyping |
| TCRβ-Vβ9 | TTCCAATCCAGTCGGCCTAACAAT | Spectratyping |
| TCRβ-Vβ10 | GCGCTTCTCACCTCAGTCTTCAG | Spectratyping |
| TCRβ-Vβ11 | TTCTCAGCTCAGATGCCCAATCAG | Spectratyping |
| TCRβ-Vβ12 | AGCTGAGATGCTAAATTCATCCTTC | Spectratyping |
| TCRβ-Vβ13 | CTGCTGTGAGGCCTAAAGGAACTAA | Spectratyping |
| TCRβ-Vβ14 | AGAGTCGGTGGTGCAACTGAACCT | Spectratyping |
| TCRβ-Vβ15 | CCCATCAGTCATCCCAACTTATCC | Spectratyping |
| TCRβ-Vβ16 | GATTTTAGGACAGCAGATGGAGTTTC | Spectratyping |
| TCRβ-Vβ17 | TCGAAATGAAGAAATTATGGAACAAAC | Spectratyping |
| TCRβ-Vβ18 | CCGGCCAAACCTAACATTCTCAAC | Spectratyping |
| TCRβ-Vβ19 | CTACAAGAAACCGGGAGAAGAACTC | Spectratyping |
| TCRβ-Vβ20 | CTGGTATCAACAAAAGCAGAGCAAA | Spectratyping |
| TCRβ-Dβ1 | GAGGAGCAGCTTATCTGGTGGTTT | Spectratyping |
| TCRβ-Dβ2 | GTAGGCACCTGTGGGGAAGAAACT | Spectratyping |
| TCRβ-Jβ1* | CACAACCCCTCCAGTCAGAAATG | Spectratyping |
| TCRβ-Jβ2* | TGAGAGCTGTCTCCTACTATCGATT | Spectratyping |
| TCRγ-Vγ4 | AGTGTTCAGAAGCCCGATGCA | Spectratyping |
| TCRγ-Jγ1* | AGAGGGAATTACTATGAGCT | Spectratyping |

**Table 1:** List of primers including sequence from 5' to 3' end and utilization purpose. Spectratyping primers marked with * were 5' FAM (Carboxyfluorescein) labelled for detection of the generated PCR products.

## 2.2 Methods

### 2.2.1 Generation of Rosa26-AKT1-E17K Transgenic Mice

The cDNA sequence of AKT1-E17K was cloned into the previously described Rosa26 targeting vector (Pechloff et al., 2010; Sasaki et al., 2006). Electroporation of 129J/Ola embryonic stem cells and generation of chimeric mice were performed by Polygene, Switzerland. Successful recombination of embryonic stem cell clones was evaluated by Southern Blot analysis of genomic DNA digested with XbaI. Germline transmission was confirmed by PCRs specific for the targeted locus. Rosa26-AKT1-E17K transgenic mice were breed with CD4Cre transgenic mice (Lee et al., 2001) to induce T-cell specific deletion of the Stop cassette and to initiate expression of AKT1-E17K and eGFP. Both mouse lines were also crossed onto a Bcl10-deficient background by mating them with Bcl10 deficient mice (Ruland et al., 2001). All animals were housed at the facilities of the Klinikum rechts der Isar in accordance to standard protocols and German and European laws and regulations.

### 2.2.2 Isolation and Purification of T- and B-Cells

Single cell suspensions of mouse organs were obtained using cell strainers (Becton Dickinson) of 100 and 70 micron pore size. Erythrocytes were lysed using G-DEX II RBC Lysis Buffer (Intron Biotechnology). For liver, lung and kidney the lymphocyte fraction was further enriched by centrifugation in a Percoll gradient (Easycoll Separating Solution, Density 1.124g/ml, BiochromeAG). CD4+ T-cells or B-cells were isolated from single cell suspensions using magnetic beads (MACS Cell Separation, Miltenyi Biotec) according to the manufacturer's protocols. The resulting purity was determined by flow cytometry and always reached 90% or better.

### 2.2.3 Flow Cytometric Analysis

The expression of surface markers on freshly isolated cells was evaluated by staining with fluorescently labelled antibodies (eBiosciences) according to standard protocols. Measurements were performed on a flow cytometer (FACS CantoII, Becton Dickinson) and the obtained data was analyzed with FACS Diva and FlowJo software.

For Phosflow analysis, the cells were previously stained with Live/Dead fixable dead cell stain (Invitrogen), followed by surface marker staining as described above. After cells were fixed (2% PFA, 15 min, room temperature) and permeabilized (70% methanol, 20 min, 4°C), staining for 1h with the phosphosite-specific antibodies (Becton Dickinson) was conducted. For statistical analysis of the Phosflow experiments the mean fluorescence intensity (MFI) of the Live/Dead stain negative population was set into relation with MFI from the phosphosite-specific antibodies, to compensate changes in cell size and protein expression. The relative change of the phosphosite-specific signal (phosphorylation) from transgenic to control cells was calculated for each experiment.

### 2.2.4 Proliferation Assay

Purified CD4+ T-cells were labelled with Cell Proliferation Dye eFluor 670 (eBioscience) according to the manufacturer's protocol and seeded out in 96 well plates. Cells were stimulated with plate-bound anti-CD3 antibody, plate-bound anti-CD3 antibody and additional soluble anti-CD28 antibody, or left unstimulated. Proliferation status was analyzed by flow cytometry at the timepoints 24h, 48h, and 72h after stimulation.

### 2.2.5 Genescan Analysis (Spectratyping)

To evaluate clonal rearrangements in the T-cell population of mice, genomic DNA was extracted from splenocytes of diseased AKT transgenic and control mice using GenElute™ Mammalian Genomic DNA Miniprep Kit, (Sigma Aldrich). V-D-J and incomplete D-J rearrangements of the TCRβ locus, and TCRγ locus rearrangements were analyzed by six PCR reactions with primer combinations previously described (Pechloff et al., 2010). The resulting 5'- FAM labelled PCR products were separated by capillary POP-7-Polymer electrophoresis and visualized by automated scanning using a 3700 Genetic Analyzer (Applied Biosystems). All reagents were obtained from Applied Biosystems. Fragment size distribution was determined by GeneMapper software (Applied Biosystems) and analyzed as previously described (Kneba et al., 1995; Pechloff et al., 2010; van Dongen et al., 2003).

### 2.2.6 Immunohistochemistry

Formalin-fixed and paraffin-embedded specimens of transgenic and control mice were sectioned in 5µm slides and stained with hematoxylin and eosin. For immunohistochemistry the slides were deparaffinized and rehydrated, followed by heating for 7 min in 0.01 M citrate buffer, pH 6.0 in a pressure cooker. Antibodies (CD3 rabbit monoclonal (DCS Innovative Diagnostic-System), Ki-67 rabbit monoclonal (Thermo Scientific) and TdT rabbit polyclonal (Abcam)) were incubated overnight at 4°C and detected using a peroxidase-based detection system (Envision+ System; DakoCytomation) following the manufacturer's protocol.

### 2.2.7 Western Blot Analysis

Purified cells were rested in RPMI 1640 (Gibco, Invitrogen) with 1% FCS/BSA for 1h at 37°C and stimulated with 100 nM PMA/Ionomycin for 20 min where indicated. Western Blot extracts were prepared by lysing the cells in CHAPS lysis buffer (10 mM Tris pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% Glycerol, 0,5% CHAPS, 1 mM β-Glycerophosphate, 1mM $Na_3VO_4$, 1mM DTT and protease inhibitors(Calbiochem)) for 30 min, followed by centrifugation at 14000 g for 10 min and collection of the supernatant. Protein content was quantified using BCA Protein Assay Kit (Pierce, Thermo Scientific). Western Blot analysis was carried out as previously described (Ruland et al., 2001) using equipment from Bio-RAD.

### 2.2.8 Coimmunoprecipitation

Purified CD4+ T-cells (unstimulated or stimulated with 100 nM PMA/Ionomycin) of transgenic AKT1-E17K and control mice were lysed with coimmunoprecipitation buffer (0.2% Nonident P40, 150 nM NaCl, 50 mM HEPES pH 7.5, 1 mM Glycerol, 10 mM NaF, 8 mM β-Glycerophosphate, 1 mM $Na_3VO_4$ and protease inhibitors (Calbiochem)). After preclearing with Protein G sepharose (Amersham), anti-Bcl10 antibody (SantaCruz) was incubated with the lysate overnight while gently shaking. Pulldown of the antibody with Protein G sepharose lasted 1 hour followed by washing, detaching and subjection to Western Blot.

### 2.2.9 Electrophoretic Mobility Shift Assay (EMSA)

Purified CD4+ T-cells were rested (see 2.2.7) and stimulated with 100 nM PMA/Ionomycin (each) for 20 min where indicated. Nuclear extracts of 2 x $10^7$ cells were prepared according to previously described protocols (Sun et al., 2000). Extracts (4 µg) were incubated with NFκB IRDye 700 infrared dye labelled oligonucleotides for 30 min in EMSA reaction buffer according to manufacturer's instructions (LI-COR Biosciences). Samples were electrophoretically separated on a 5% polyacrylamide gel and documented using the Odyssey Infrared Imaging System (LI-COR Biosciences).

### 2.2.10 Soft Agar Assay

AKT-K17E (wildtype) and AKT-E17K-KD (Kinase Dead) constructs were generated by site-directed mutagenesis (Stratagene) using the primers indicated in Table 1. The sequences of AKT-K17E, AKT-E17K, AKT-E17K-KD and BCR-ABL were cloned into the pMSCV-IRES-GFP (pMIG) vector using standard cloning techniques. The vectors were introduced into the retrovirus producer cell line Phoenix-E by $CaCl_2$ transfection. Viral supernatant was collected and used for overnight infection of Rat1 cells. Both cell lines were cultured in DMEM (Gibco, Invitrogen) with 10% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine and 0.5% β-Mercaptoethanol. The infection efficiency was determined by GFP expression of Rat1 cells using flow cytometry. 10,000 GFP+ cells/well were subsequently seeded out in IMDM with 0.26% agarose and 20% FCS in 6 well plates, which already contained a bottom layer of IMDM with 0.6% agarose. On top 1 ml of DMEM (10% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine and 0.5% β-Mercaptoethanol) was added and changed every 3 days. After 50 days of incubation, colony growth was documented using a stereomicroscope with 0.63x and 4x magnification.

### 2.2.11 Transplantations

For transplantation experiments, CD4+ T-cells were purified from diseased AKT1-E17K/CD4Cre mice. 1 x $10^7$ cells were intravenously injected into littermates/non-littermates of the donor. The transplanted mice were monitored and analysed upon signs for disease.

# 3 Results

## 3.1 The Oncogenic Potential of AKT1-E17K Is Dependent on its Kinase Activity

In order to verify the oncogenic potential of AKT1-E17K (Carpten et al., 2007), *in vitro* soft agar assays were performed with retrovirally infected Rat1 fibroblasts. Usually, immortalized fibroblast cell lines are not able to proliferate in viscous media, such as gel or soft agar, because they need attachment to a solid surface to undergo cell division (anchorage dependence). Further, they do not grow over one another (contact inhibition) and therefore cannot form colonies in cell culture. Upon oncogenic transformation, which describes the process of change a normal cell undergoes to become malignant, fibroblast often get anchorage independent and lose their contact inhibition. This leads to colony formation when embedded into soft agar and is assumed to correlate with carcinogenesis. This technique can be used to evaluate the transforming potential of chemicals or genes *in vitro*.

For that purpose a point mutation was introduced into the cDNA of human AKT1, resulting in an exchange of lysine to glutamic acid at position 17 (E17K) in the PH domain. The AKT1-E17K-encoding sequence was codon-optimized for expression in mouse and an N-terminal flag-tag was added (Figure 8). The construct was cloned into a pMSCV-based retroviral vector, carrying an additional IRES-GFP sequence. As a control the E17K mutation was reversed in another vector (AKT1-K17E), corresponding to the wildtype sequence. Further, a second point mutation (K179M) was inserted into the catalytic domain of the AKT1-E17K sequence, to generate a kinase-deficient mutant (AKT1-E17K-KD).

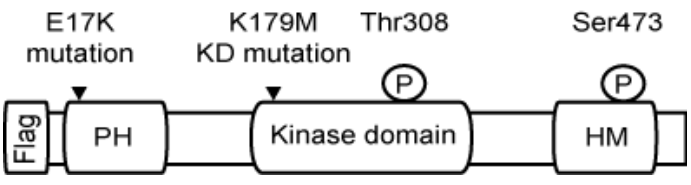


**Figure 8: Schematic representation of the AKT1-E17K protein**.
The N-terminal flag-tag followed by the human AKT1 sequence with its pleckstrin homology (PH) domain, central kinase domain and hydrophobic motif (HM) are depicted. The positions of the E17K mutation in the PH domain, the K179M mutation in the central kinase domain and two phosphorylation sites (threonine 308 and serine 473), relevant for activation, are depicted.

For infection, Rat1 fibroblasts were incubated overnight with viral supernatants. These were produced by transfection of the Phoenix helper cell line with the respective retroviral construct pMIG, pMIG-BCR-ABL, pMIG-AKT1-E17K, pMIG-AKT1-K17E, or pMIG-AKT1-E17K-KD. The infection rate was determined by flow cytometric detection of GFP expression. The infected cells (10,000/well) were then embedded into a soft agar layer and seeded out into 6-well plates. After 50 days, no colony formation was observed for non-infected (mock) or pMIG-infected cells, which both served as negative controls (Figure 9). As expected, Rat1 cells infected with a BCR-ABL expressing retrovirus developed several colonies. The BCR-ABL oncogene represents a well-known driver of Rat1 fibroblast transformation and was used as positive control for the experiment (Tao et al., 2008).The wildtype form of AKT1 was not able to induce colony growth and therefore did not transform the cells in culture. This is in line with previous reports and implicates that overexpression of wildtype AKT alone is not sufficient to transform the cells (Carpten et al., 2007; Sakoda et al., 2003). In contrast to that, AKT1-E17K-infected fibroblasts were able to generate multiple colonies in the soft agar. Compared to the positive control, even more colonies were detected, proving that the mutant AKT1 indeed has oncogenic potential. Further, the kinase-deficient mutant induced no colony formation, indicating that the transforming ability of AKT1-E17K is dependent on the functionality of its kinase domain.

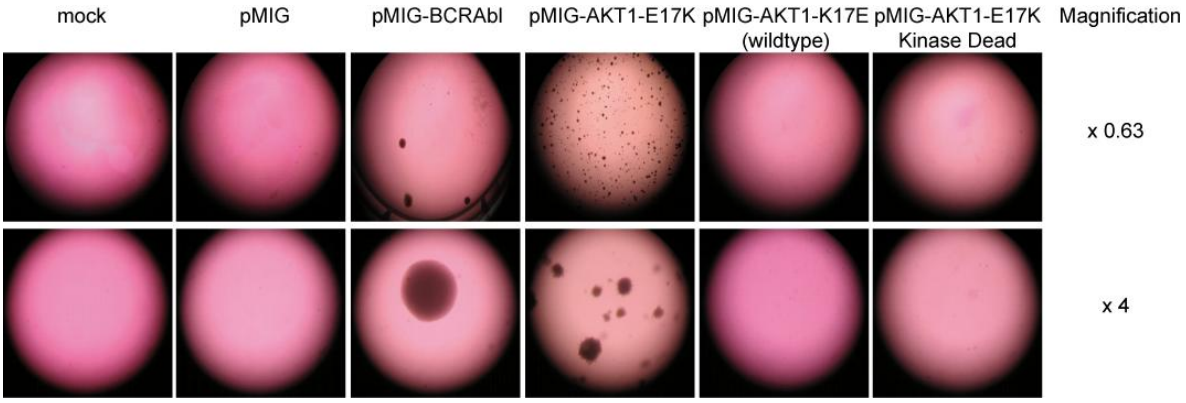


**Figure 9: AKT1-E17K has a transforming capacity in retrovirally infected Rat1 fibroblasts.**
Retroviral constructs (pMIG, pMIG-BCR-ABL, pMIG-AKT1-E17K, pMIG-AKT1-K17E, or pMIG-AKT1-E17K-KD) were transfected into the virus producer cell line Phoenix using $CaCl_2$ transfection. Viral supernatants were collected and used to infect Rat1 fibroblast with the indicated retroviruses or left uninfected (mock). Afterwards the cells (10.000 infected cells/well) were embedded in a soft agar layer and seeded out into 6-well plates. The agar was covered with a layer of medium (including all supplements) that was changed every three days. The pictures were taken 50 days after infection using a stereomicroscope with 0.63x and 4x magnification. No colonies were detected when Rat1 cells were left uninfected or were infected with pMIG, as well as when fibroblasts were infected with AKT1-K17E (wildtype) or AKT1-E17K-KD (kinase dead). In contrast, the expression of BCR-ABL and AKT1-E17K resulted in the formation of multiple colonies within the soft agar.

### 3.2 Generation of Transgenic Mice Conditionally Expressing AKT1-E17K

In order to study the role of constitutively active AKT1 in an *in vivo* model the AKT1-E17K construct was cloned into a Rosa26 targeting vector to generate a transgenic mouse line using previously established gene targeting (Pechloff et al., 2010; Sasaki et al., 2006). The Rosa26 locus is frequently used for this purpose and was first identified in the group of P. Soriano by gene trapping (Friedrich and Soriano, 1991). It is easily accessible by gene targeting, ensuring a high recombination rate, and its promotor was found to be ubiquitously expressed during embryonic development and in all hematopoietic cells (Zambrowicz et al., 1997). Further, there is no known function of the three transcripts derived from this locus, which therefore represents an ideal targeting site. The targeting vector contained an appropriate short and a long arm, required for homologous recombination, an internal splice acceptor site, a Neomycin-STOP cassette flanked by loxP sites followed by the cDNA of human AKT1-E17K, an IRES-eGFP cassette and a polyadenylation signal. The neomycin resistance allows selection of recombined ES cells, whereas the transcriptional and translational STOP signal terminates expression of the transgene from this locus. This strategy allows conditional expression of AKT1-E17K from the endogenous Rosa26 promotor by Cre-mediated excision of the Neomycin-STOP cassette. Additionally, Rosa26 promotor activity can be monitored by the green fluorescence of bicistronically expressed eGFP (Figure 10).

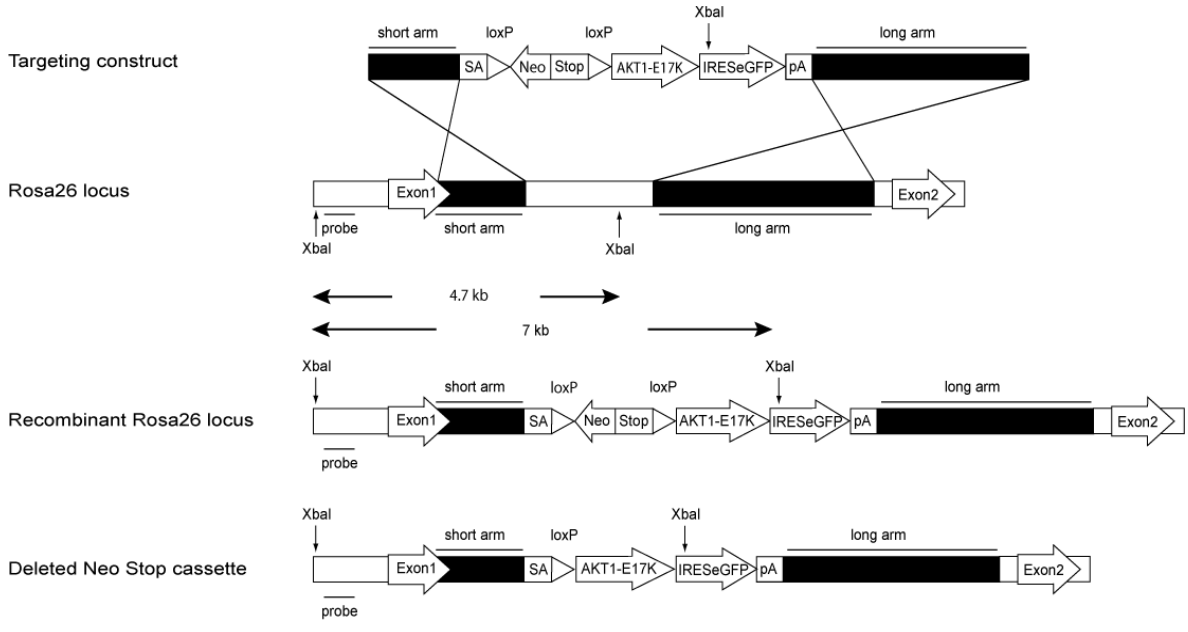

**Figure 10: Targeting strategy for the generation of Rosa26-AKT1-E17K transgenic mice.**
The targeting construct consists of a short and a long arm homologous to the murine Rosa26 locus, a splice acceptor site (SA), two loxP sites flanking the Neomycin (Neo)-Stop cassette, the human AKT1-E17K cDNA sequence (codon-optimized for expression in mouse), and the IRES-eGFP sequence followed by a polyadenylation signal (pA). Homologous recombination takes place between Exon 1 and 2 of the Rosa26 locus. The XbaI restriction sites, the resulting fragment sizes for the wildtype Rosa26 (4.7 kb) and the recombinant locus (7 kb), and the binding site for the Southern probe are indicated. Cre expression mediates deletion of the Neomycin-Stop cassette and subsequent expression of AKT1-E17K and eGFP.

Successful recombination of the targeting vector into the genome of murine embryonic stem (ES) cells was verified by Southern Blot analysis (Figure 11A). Genomic DNA from ES cells, digested with the restriction enzyme XbaI, led to a 4.7 kb fragment, representing the wildtype Rosa26 locus. Correct insertion into the locus resulted in an additional 7 kb fragment. The positive tested ES cell clones were afterwards used for blastocyst injection. The offspring of the resulting chimeras was analyzed for germline transmission by PCRs on genomic DNA, specific for the wildtype and the targeted Rosa26 locus, and specific for the transgenic AKT (Figure 11B). The same PCRs were also used for routine genotyping of the mouse breeding.

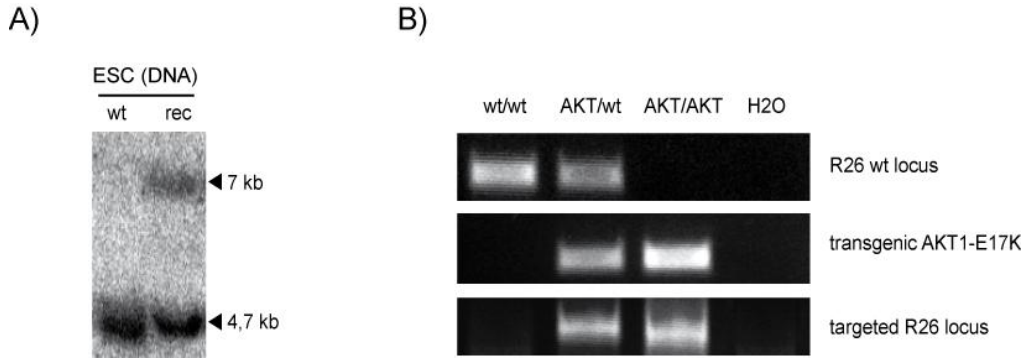


**Figure 11: Verification of correct ES cell targeting and of mouse germline transmission.**
A) Murine embryonic stem cells were electroporated with the linearized targeting construct, plated on feeder cells and selected with Neomycin. Appropriate colonies were picked, expanded, genomic DNA was prepared and analyzed by Southern Blot after digestion with XbaI. As expected, a band at 4.7 kb represented the wildtype Rosa26 locus. Successfully targeted clones showed an additional band at 7 kb. Representatively, one wildtype and recombinant clone are depicted. B) PCRs, specific for the Rosa26 wildtype locus, the transgenic (not endogenous) AKT1-E17K and the targeted Rosa26 locus, performed on genomic tail DNA were used to identify germline transmission in the offspring of chimeric mice.

To analyze the impact of AKT1-E17K expression on T-cells, these AKT transgenic mice were bred with CD4Cre mice, in which the Cre recombinase is under the control of the CD4 enhancer/promotor (Lee et al., 2001). To prove celltype-specific expression of the transgene, T- and B-cells of the resulting double transgenic mice (AKT/CD4Cre) and control animals were purified and AKT1-E17K expression was evaluated by Western Blot (Figure 12A). In

T- and B-cells of both transgenic and control mice, endogenous AKT was detected at the expected size of approximately 60 kD. But only T-cells from AKT/CD4Cre mice expressed AKT1-E17K, which was discriminable from the endogenous protein due to its flag-tag. Another result of the additional flag-tag was an increase in the size of the transgenic protein, visible as a strong and slightly larger band than seen for the endogenous AKT. No expression of AKT1-E17K in purified B-cells of AKT/CD4Cre transgenic mice was observed. Flow cytometric analysis of splenocytes also confirmed restricted expression of the transgenic locus, since most of the T-cells, but not the B-cells, derived from AKT/CD4Cre mice expressed eGFP (Figure 12B). These results proved the functionality of the conditional *in vivo* mouse model.

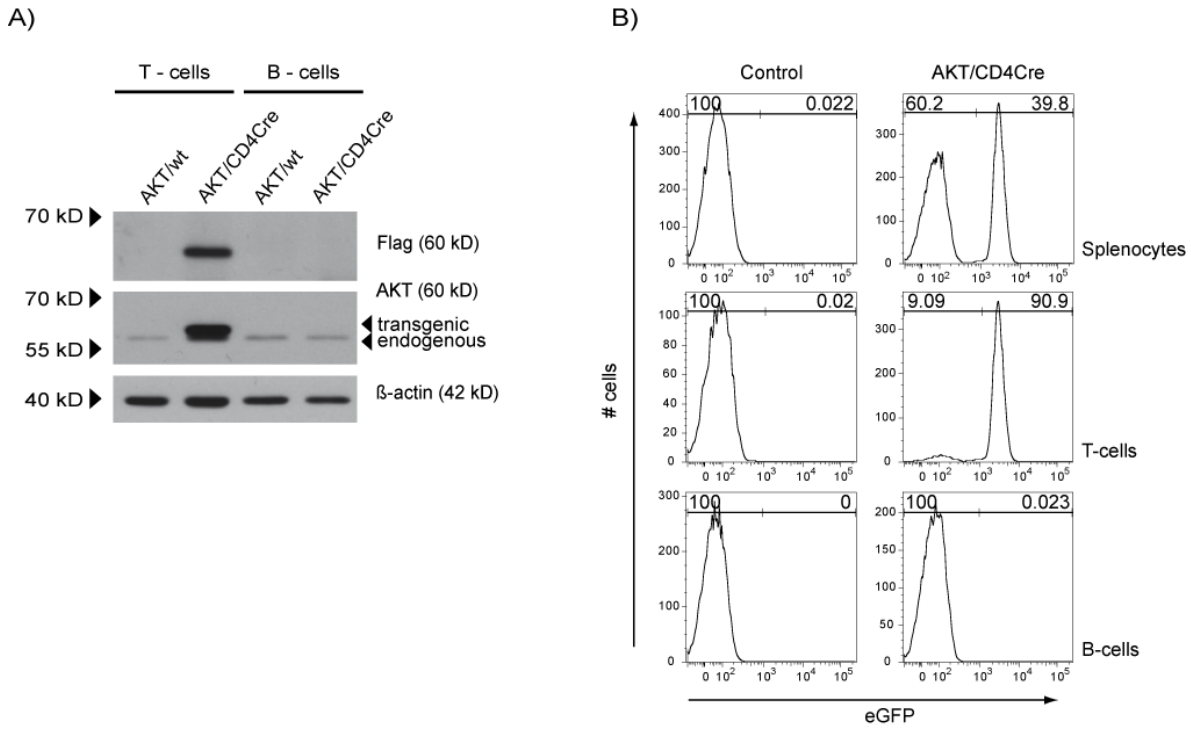


**Figure 12: The expression of AKT1-E17K and eGFP is restricted to the T-cell lineage.**
A) Lysates of purified T- and B-cells from 6 weeks old control and AKT/CD4Cre double transgenic mice were analyzed by Western Blot. Endogenous AKT was detected at the expected size of ~60 kD in T- and B-cells of both genotypes. As intended, AKT1-E17K expression was only observed in T-cells derived from double transgenic mice, visible as a prominent and slightly larger band than the endogenous AKT, because of its additional flag-tag. With a specific antibody only the flag-tagged AKT1-E17K was detected. This Western Blot is representative for three independent experiments. B) Single cell suspensions of total splenocytes from control and AKT/CD4Cre mice (5 weeks old) were analyzed by flow cytometry. In splenocytes of double transgenic mice expression of eGFP was observed. Staining with fluorescently labelled antibodies against TCRβ and B220 and subsequent gating on these populations revealed that most of the T-cells in double transgenic mice expressed eGFP, whereas the B-cells did not. Histograms are representative for at least 30 mice analyzed per genotype.

### 3.3 AKT1-E17K Expression in T-Cells Results in Premature Death

After providing evidence for a functional and conditional *in vivo* mouse model, the phenotype was analyzed in more detail. Breeding of transgenic AKT1-E17K with CD4Cre mice (both heterozygous) resulted in normal Mendelian distribution of sex and genotypes within the offspring (Figure 13A). However, soon after birth AKT/CD4Cre mice exhibited signs of disease like ruffled fur, slitted eyes, shortness of breath, lethargy, abdominal swelling, and kyphosis. In contrast to control mice, all AKT/CD4Cre mice died or had to be sacrificed due to severe sickness and ethical reasons around 8 weeks after birth. The median survival was 52 days after birth (Figure 13B). AKT/CD4Cre and appropriate control mice were mainly analyzed at the age of 5 to 10 weeks or when they displayed the above mentioned signs of disease. Documentation of body weights revealed a general decrease in weight for the transgenic mouse line (control versus AKT/CD4Cre, mean ± SEM, 19.60g ± 0.48 vs. 16.08g ± 0.53, n = 50, $p < 0.0001$ by Student's t test) (Figure 13C).

A)

| total mice | male | female | AKT/wt | AKT/ CD4Cre | wt/ CD4Cre | wt/wt |
|---|---|---|---|---|---|---|
| 222 | 108 | 114 | 53 | 52 | 61 | 56 |
| 100% | 48.6% | 51.4% | 23.9% | 23.4% | 27.5% | 25.2% |

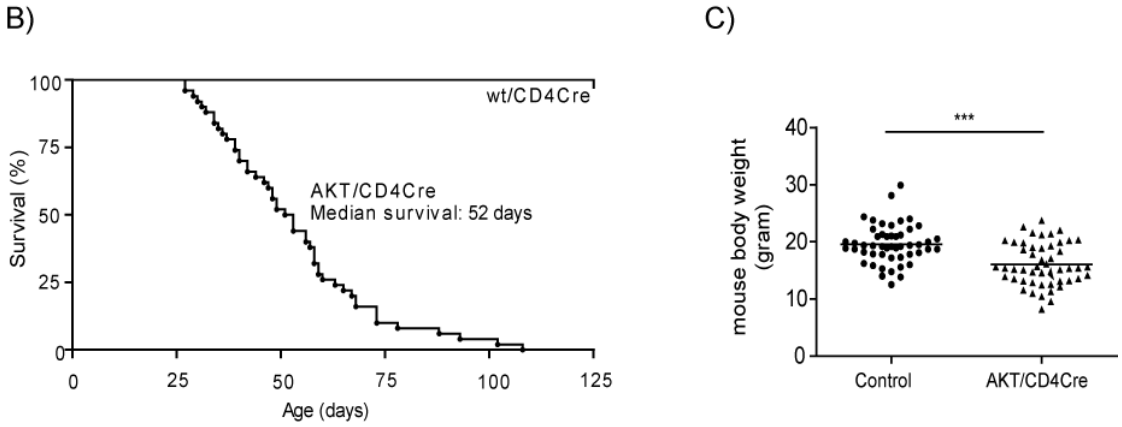


**Figure 13: Impaired survival of AKT/CD4Cre mice.**
A) Analysis of the offspring from heterozygous breeding of AKT transgenic and CD4Cre mice showed normal Mendelian distribution of sex and genotypes. B) Kaplan-Meier survival curve of AKT/CD4Cre mice (n = 50) and control mice (n = 25). Median survival of AKT/CD4Cre mice was 52 days. Analysis includes mice that died and mice with fatal illness that had to be sacrificed due to ethical reasons. C) Body weight of AKT/CD4Cre and healthy control mice (n = 50 mice per genotype) were documented. Animals were mostly analyzed upon signs of disease (5 to 10 weeks).

### 3.4 AKT/CD4Cre Mice Show an Altered Thymic Population Distribution

The development of T-cells in the thymus can be divided into four stages, characterized by the expression of CD4 and/or CD8 surface molecules. Early T-cell progenitors enter the thymus without expressing these two markers (double negative, DN) and progress through four DN (DN1 – DN4) substages, defined by the markers c-kit, CD44 and CD25. During the DN stages, thymocytes have to successfully install a pre-TCR on their surface and pass the β-checkpoint. Afterwards they start to express both, CD4 and CD8 (double positive, DP) costimulatory receptors, and undergo their last proliferative burst in the thymus. At this timepoint, Cre expression is turned on within the CD4Cre mouse strain. Accordingly, also AKT1-E17K expression starts in the AKT/CD4Cre transgenic mouse model. The DP thymocytes further have to undergo positive and negative selection to finally develop into mature CD4 or CD8 single positive (SP) cells, which then can exit the thymus (Carpenter and Bosselut, 2010).

Since AKT1-E17K expression in T-cells coincides with CD4 expression, thymi of 5 weeks old AKT transgenic mice, which showed no obvious signs of disease until then, were analyzed for changes in the thymic subpopulations by flow cytometry. Compared to control animals, the thymic subpopulations were altered in AKT transgenic mice, differing in magnitude between the individuals (Figure 14A). In general, the percentage of DP thymocytes in AKT transgenic thymi was decreased by about half compared to thymi of control mice (control versus AKT/CD4Cre, mean ± SEM, n = 17, 86.47% ± 0.56 vs. 42.01% ± 6.69, $p < 0.0001$ by Student's t test), whereas the other subpopulations were increased (DN: 2.68% ± 0.15 vs. 17.70% ± 2.84, $p < 0.0001$; CD4 SP: 7.75% ± 0.38 vs. 27.54% ± 4.46, $p < 0.0001$; CD8 SP: 3.10% ± 0.12 vs. 12.72% ± 1.88, $p < 0.0001$). Furthermore, a strong reduction in overall thymic cellularity was observed in AKT/CD4Cre mice (99.90 ± 7.88 $x10^6$ vs. 49.11 ± 6.78 $x10^6$, $p < 0.0001$) (Figure 14C). To find out at which developmental stage thymocytes were reduced, the cell number for each subpopulation was determined. The loss of thymic cellularity in AKT transgenic mice was due to a strong and significant reduction of cells in the DP population (86.30 ± 6.81 $x10^6$ vs. 24.20 ± 6.75 $x10^6$, $p < 0.0001$) (Figure 14C), interestingly at the timepoint when AKT1-E17K expression starts. In contrast, total cell numbers of the CD4 SP and CD8 SP populations were slightly, but not significantly, increased (CD4 SP: 7.82 ± 0.78 $x10^6$ vs. 11.72 ± 2.74 $x10^6$, $p = 0.1525$; CD8 SP: 3.12 ± 0.28 $x10^6$ vs. 4.75 ± 0.83 $x10^6$, $p = 0.0555$). Unexpectedly, also in the DN population significantly higher cell counts were observed (2.63 ± 0.23 $x10^6$ vs. 6.93 ± 0.94

x$10^6$, $p < 0.0001$). Gating on the different thymic subsets showed, that not only most of the SP (AKT/CD4Cre, mean ± SEM, CD4: 94.77% ± 0.64, CD8: 94.64% ± 0.64) and DP cells (91.86% ± 3.08) expressed eGFP and consequently AKT-E17K, but also a remarkable percentage of DN cells (78.68% ± 5.75), even though at this stage CD4 and Cre expression should not have been turned on yet (Figure 14D). The population of DN cells partially expressed TCRβ, indicating a more mature T-cell stage than the one that usually would be found in this compartment (9.94% ± 0.46 vs. 54.24% ± 7.2, $p < 0.0001$) (Figure 14B). The eGFP and TCRβ positive cells in the DN compartment must have expressed CD4 and/or Cre in the past, but somehow lost the CD4 and CD8 coreceptors on their surface. Moreover, the CD4 SP and DP thymocytes of AKT/CD4Cre mice showed, in contrast to CD8 SP thymocytes, an increase in cellular size (Figure 14D). Therefore, the expression of AKT1-E17K during T-cell maturation is able to alter the thymic population distribution, cellularity and cellular size.

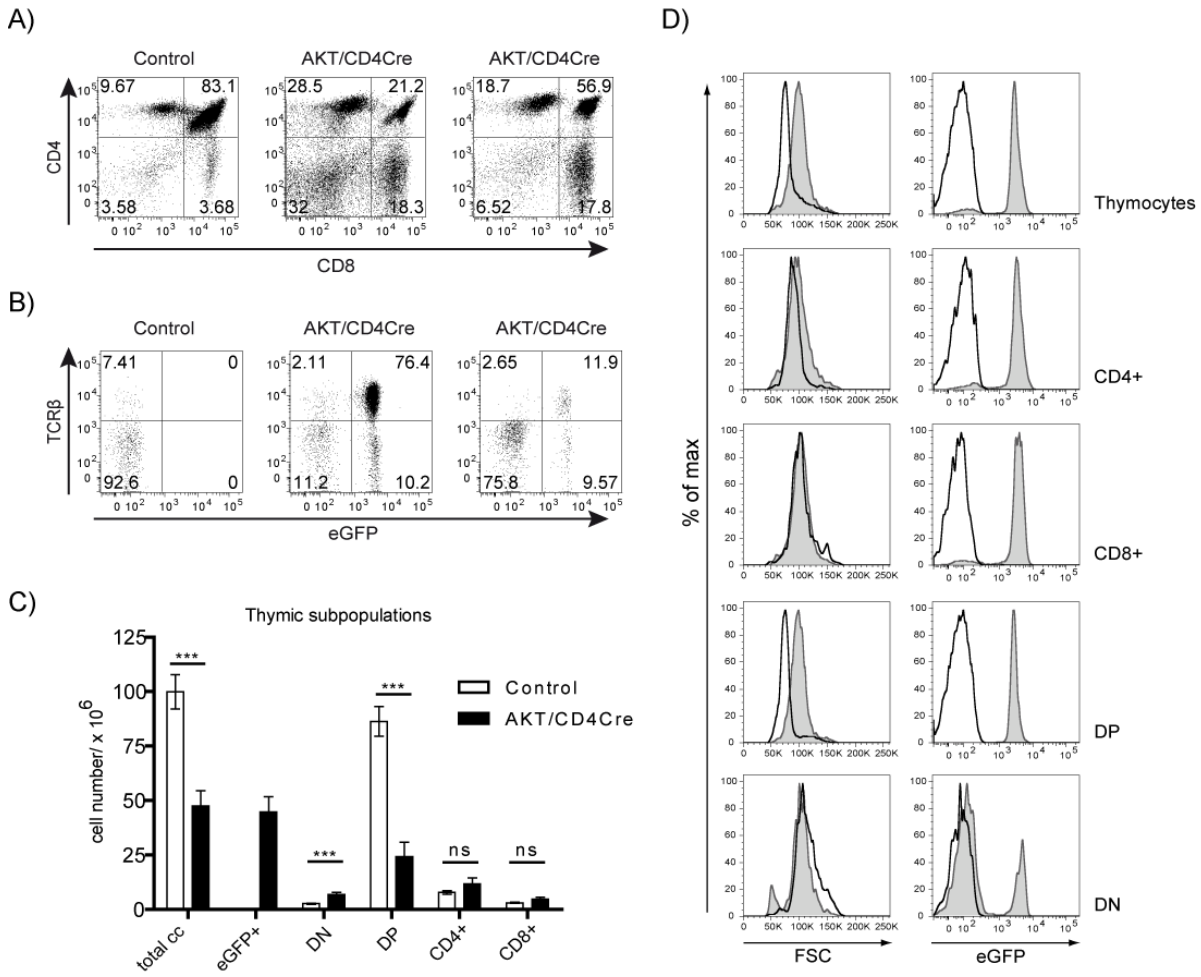

**Figure 14: Altered thymic subpopulations with reduced number of DP cells in AKT/CD4Cre mice.**
A) Single cell suspensions were prepared from thymi of 5 weeks old AKT/CD4Cre and control mice. $1x10^6$ cells were stained with antibodies against CD4, CD8 and TCRβ and analyzed by flow cytometry. AKT/CD4Cre thymi showed an altered population distribution compared to control animals that varied also between littermates (two representative AKT/CD4Cre littermates are depicted). B) Thymocytes analyzed in A) were gated on the DN population. eGFP+ cells partly also showed positive staining for TCRβ. C) Thymi of 5 weeks old AKT/CD4Cre or control mice (n = 17) were taken out and total cell number was determined by counting in a Neubauer chamber. $1x10^6$ cells were stained with antibodies against CD4 and CD8 and analyzed by flow cytometry. The mean cell number with SEM for each population is shown. Significantly decreased cellularity of AKT/CD4Cre thymi due to a significant decrease in the DP compartment was observed. CD4 and CD8 SP compartments had a slightly enhanced, but not significantly different, cell count, whereas the increase in DN cell number was significant. Populations were compared with unpaired Student's t-test. Nearly all thymocytes in AKT/CD4Cre mice expressed eGFP. D) Representative flow cytometric analysis of AKT/CD4Cre (tinted) and control thymocytes stained for CD4 and CD8. Overlays of FSC and eGFP fluorescence for total thymocytes, gated on the indicated populations are shown. DP and CD4 SP thymocytes were bigger in the FSC (representing cell size) and exept for the DN compartment nearly all cells were eGFP positive. Also some of the cells in the DN compartment showed eGFP expression.

### 3.5 Massive Increase of CD4+ T-Cells in the Spleens of Transgenic AKT Mice

To characterize the lymphocyte population in more detail, organs of AKT transgenic mice were collected and analyzed at the age of 5 to 10 weeks or at the appearance of disease signs. Depending on the severity of the disease, AKT/CD4Cre mice showed pronounced splenomegaly and lymphadenopathy (Figure 15A). The spleen weights of AKT/CD4Cre mice were significantly increased (control vs. AKT/CD4Cre, mean ± SEM, 81.66mg ± 2.63 vs. 448mg ± 47.1, n = 50, $p < 0.0001$) and subsequently also the ratio of spleen to total body weight (0.422% ± 0.013 vs. 2.753% ± 0.275, $p < 0.0001$) (Figure 15B). This already indicated an accumulation of cells in the peripheral immune organs.

Flow cytometric analysis of splenocytes further revealed an increase in the percentage of TCRβ+ T-cells (29.53% ± 1.27 vs. 43.49% ± 3.9, n = 32, $p = 0.0012$) and a decrease of B220+ B-cells in the AKT transgenic animals (59.42% ± 1.11 vs. 21.86% ± 3.19, n = 32, $p < 0.0001$) (Figure 15C). The normal ratio of T- and B-cells, as observed for the controls, was completely disturbed. Primarily responsible for this alteration was an increased percentage of CD4+ T-cells (16.43% ± 0.88 vs. 36.96% ± 3.05, n = 32, $p < 0.0001$), whereas the CD8+ subpopulation was decreased (13.14% ± 0.83 vs. 5.49% ± 0.86, n = 32, $p < 0.0001$). Comparing the total splenic cell counts of control and AKT/CD4Cre mice, confirmed a massive increase in overall splenic cellularity ($56.29 \pm 3.99 \times 10^6$ vs. $172.9 \pm 22.43 \times 10^6$, n = 32, $p < 0.0001$) (Figure 15D). Precisely, a strong and significant increase of in TCRβ+ ($16.60 \pm 1.42 \times 10^6$ vs. $70.64 \pm 14.77 \times 10^6$, n = 32, $p = 0.0006$) and also CD4+ T-cells ($9.26 \pm 0.88 \times 10^6$ vs. $58.85 \pm 11.58 \times 10^6$, n = 32, $p < 0.0001$) was detected in AKT

transgenic animals. At the same time, no significant change in the cell number of CD8+ T-cells (7.3 ± 0.70 x$10^6$ vs. 6.08 ± 0.71 x$10^6$, n = 32, p = 0.227) and B220+ B-cells (33.56 ± 2.47 x$10^6$ vs. 35.36 ± 5.85 x$10^6$, n = 32, p = 0.7781) was observed.

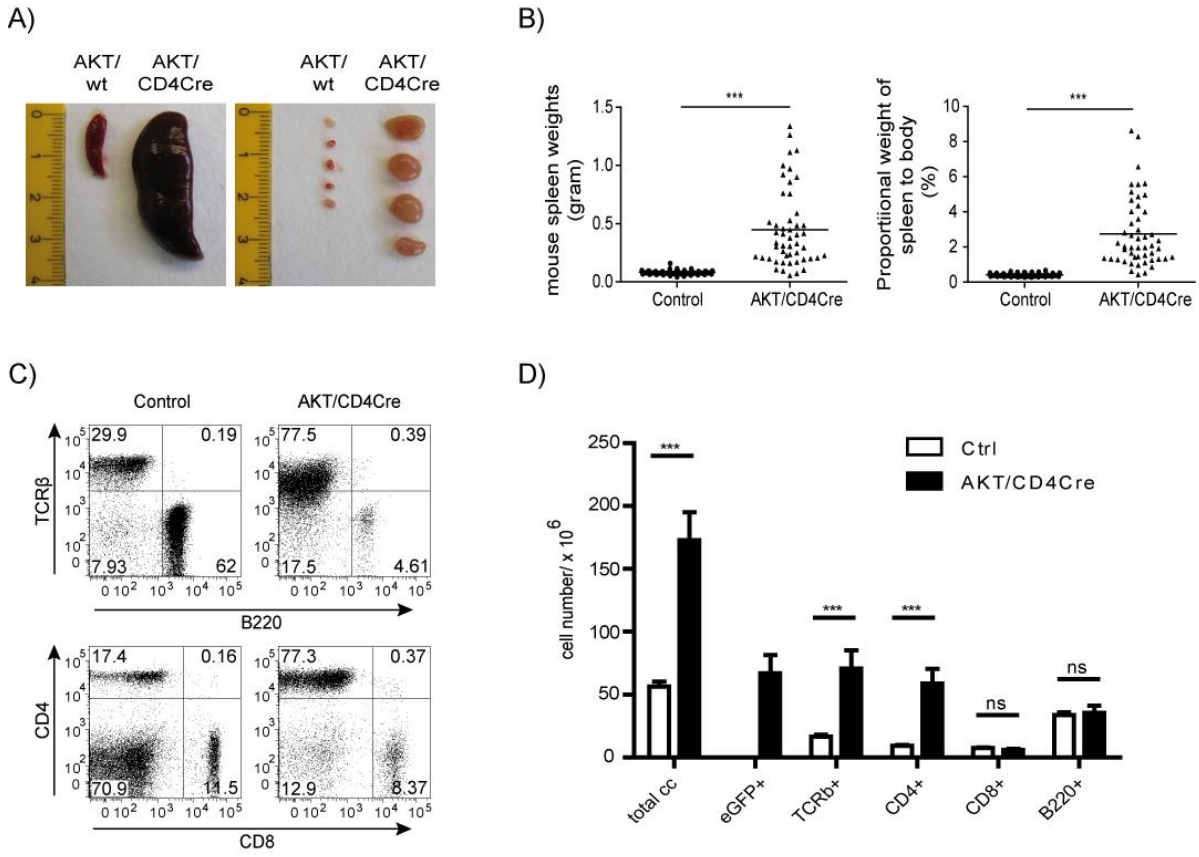


**Figure 15: Splenomegaly, lymphadenopathy and increased CD4+ T-cell numbers in AKT/CD4Cre mice.** A) Representative pictures of spleen (left) and lymph nodes (right) dissected from control (AKT/wt) and AKT/CD4Cre mice (52 days old). Massive splenomegaly and lymphadenopathy were observed. B) Sick AKT/CD4Cre and healthy control mice (n = 50 mice per genotype) were analyzed when signs of illness were detected (mainly 5 to 9 weeks old). Body and spleen weights were taken and the percentage of the spleen weight relative to the body weight was calculated. C) Splenocytes of 5 weeks old control and AKT/CD4Cre mice were stained for CD4, CD8, TCRβ and B220 and analyzed by flow cytometry. An altered population distribution due to enlargement of the CD4+ and TCRβ+ T-cell population and a reduction of the B220+ B-cell compartment was observed. D) The total cell number of spleens from 4-9 weeks old AKT/CD4Cre or control mice (n = 32) was determined by counting in a Neubauer chamber. Flow cytometric analysis with antibodies against TCRβ, CD4, CD8 or B220 was performed. The mean value of the calculated cell number with its SEM for each population is depicted. A strong increase in splenic cellularity and also in the CD4+ and TCRβ+ T-cell population was detected. Nearly no change in the CD8+ T-cell population and in the B220+ B-cell population was detected.

All populations, remarkably also the B-cells, presented a clear shift in the FSC (forward scatter), indicating an increase in size. As already mentioned before, eGFP expression was

detected in nearly all CD4+ (AKT/CD4Cre, mean ± SEM, 88.87% ± 0.78, n = 32) and CD8+ T-cells (70.46% ± 3.53) in the spleen, but not in B-cells (Figure 16A).

Next, the expression of the common T-cell activation markers CD25, CD69, CD44 and CD62L was analyzed. These cell surface molecules are known to be regulated after T-cell activation and have important functions in trafficking, homing, growth, differentiation and proliferation. Compared to control cells, all transgenic T-lymphocytes (CD4+ and CD8+) in spleen and lymph node exhibited an activated status. CD69, a marker for early T-cell activation, was found to be upregulated in transgenic animals. Also CD44 expression was markedly increased, whereas CD62L expression was strongly downregulated. Interestingly, CD25 expression, the IL-2 receptor α chain, was not affected at all (Figure 16B).

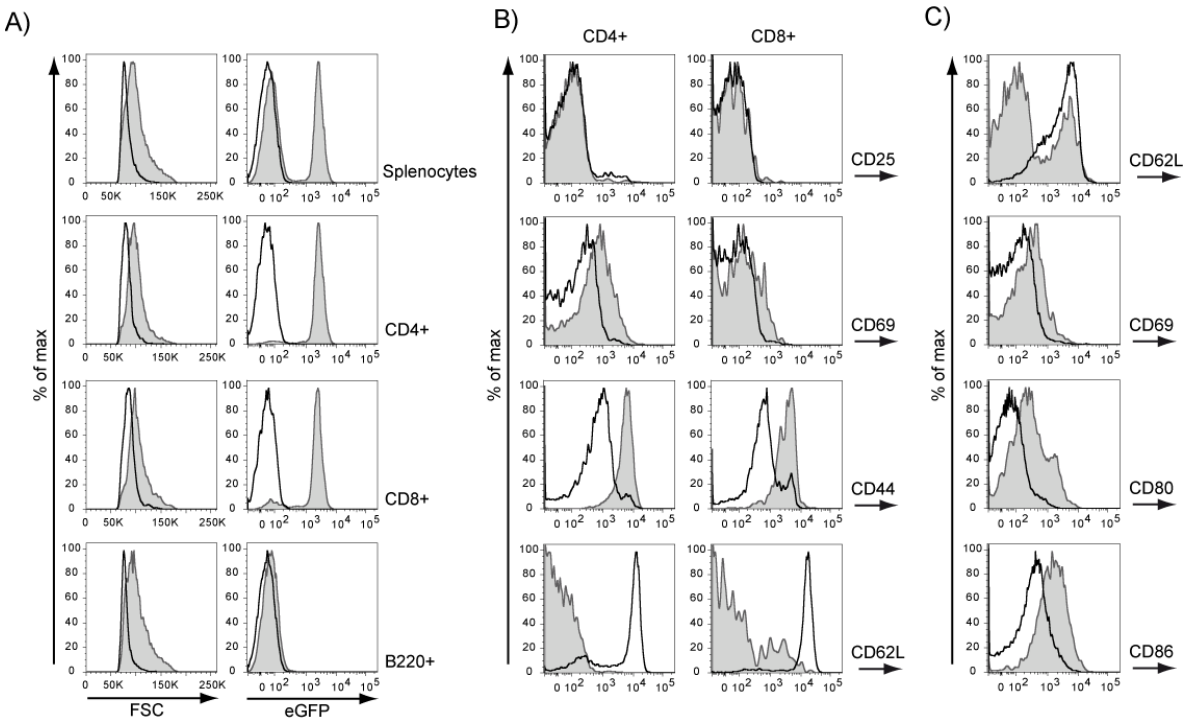


**Figure 16: T- and B-cells of AKT/CD4Cre mice show increased expression of activation markers.**
A) Representative flow cytometric analyses of AKT/CD4Cre (tinted) and control splenic cells stained for CD4, CD8 and B220. Overlays of FSC and eGFP fluorescence for total splenocytes, gated on the indicated populations are shown. CD4, CD8 and also B220 positive cells were bigger in FSC. Most of the T-cells, but not the B-cells, were eGFP+. B) Flow cytometric analysis of AKT/CD4Cre (tinted) and control splenic cells stained for CD4, CD8, CD25, CD69, CD44 and CD62L. Compared to control CD4+ or CD8+ T-cells, AKT/CD4Cre cells stained stronger for CD69 and CD44 and less strong for CD62L, indicating an activated phenotype. No change in CD25 expression was observed. Data are representative for at least 17 independent experiments. C) Flow cytometric analysis of AKT/CD4Cre (tinted) and control splenic cells stained for B220, CD62L, CD69, CD80 and CD86. All histogram overlays were made from the B220 positive population. CD69, CD80 and CD86 upregulation and CD62L downregulation was observed. This suggested that also the B-cell compartment resided in an activated state. One analysis, representative for 17 independent experiments, is depicted.

As an increase in size was also noticed for B-cells (Figure 16A), these were analyzed for common activation markers, too. CD69, CD80 and CD86 were upregulated and CD62L was downregulated on the surface of B-cells in spleen and lymph nodes of transgenic mice (Figure 16C). This indicated that also the B-cells in AKT/CD4Cre mice reside in an activated state, even though they do not express AKT1-E17K. Hence, the B-cell activation has to be a secondary effect.

### 3.6 Clonal Expansion of T-Cells in AKT/CD4Cre Mice

The assessment of clonal TCR locus rearrangements is an important part of the evaluation of potential lymphoproliferative disorders concerning the T-cell lineage. To further define the disease of AKT/CD4Cre mice, two tests for T-cell clonality were performed.

Firstly, T-cells of control and AKT/CD4Cre mice were stained with a panel of different TCRvβ chain-specific antibodies and analyzed by flow cytometry. Overrepresentation of one specific chain in the transgenic T-cell population compared to the control can be interpreted as an indication for a clonal expansion of T-cells and a clonal nature of the disease.

The overlay of all fluorescent signals obtained from the different TCRvβ chains of a control mouse resulted in a distinct pattern, representing their common distribution within the CD4+ or CD8+ T-cell population (Figure 17, control). In general, no abnormal occurrence of TCRvβ chains was detected in AKT/CD4Cre mice. But the assay is limited due to the limited availability of different TCRvβ specific antibodies. However, a reduction of the fluorescent signals was observed in six out of seven AKT transgenic mice analyzed (Figure 17, AKT/CD4Cre, upper panel). The reason for this phenomenon is maybe an increased internalization rate of the TCR due to increased activation. In contrast to that, only one out of seven AKT transgenic mice showed an augmented use of a single TCRvβ chain (TCRvβ 8.1/8.2, bright green), indicating a clear clonal expansion of T-cells (Figure 17, bottom panel).

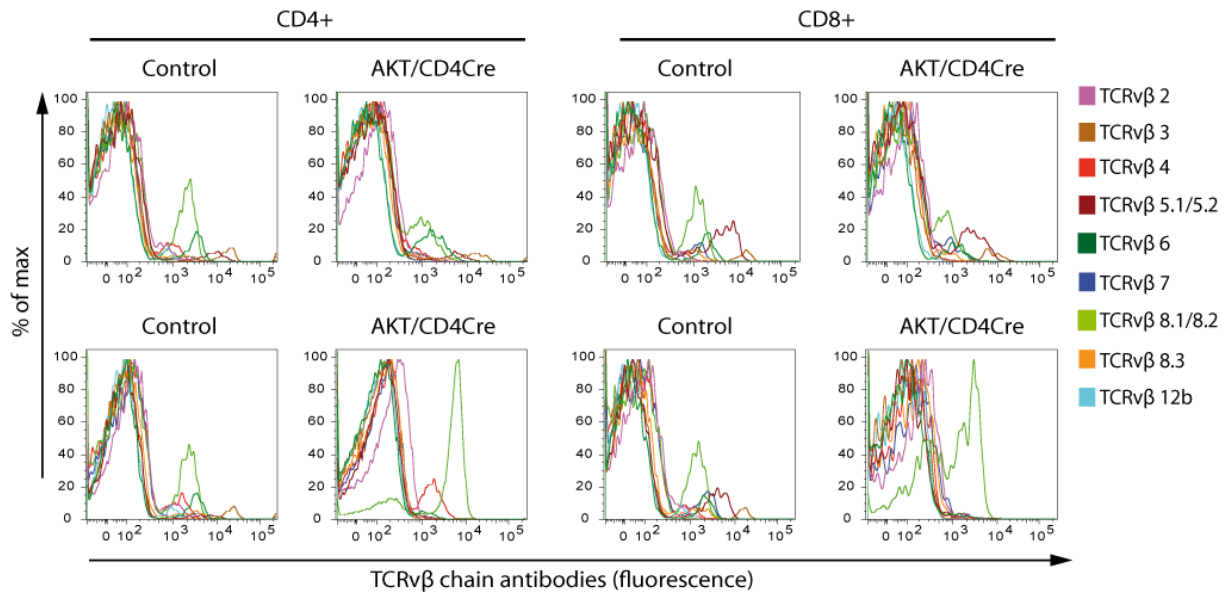


**Figure 17: Evaluation of TCRvβ chain usage by flow cytometry.**
Splenocytes of 8-9 weeks old control and AKT/CD4Cre animals were stained with specific antibodies against CD4, CD8 and a panel of different TCRvβ chains (TCRvβ 2 (violet), TCRvβ 3 (brown), TCRvβ 4 (red), TCRvβ 5.1/5.2 (dark red), TCRvβ 6 (dark green), TCRvβ 8.1/8.2 (bright green), TCRvβ 8.3 (orange), TCRvβ 12b (bright blue)) and analyzed by flow cytometry. Each histogram shows an overlay of the respective TCRvβ chains expressed on CD4+ or CD8+ T-cells of one mouse. For the experiment depicted, two control mice and two AKT/CD4Cre mice were used. Control cells showed a distinct normal usage of TCRvβ chains. Compared to the control cells, a reduced signal for the different chains was observed for the transgenic cells (top panel). Only in one out of seven experiments, an antibody recognized a specific TCRvβ chain, which was overrepresented within the T-cell population (bottom panel).

Secondly, due to the obvious limitations of the flow cytometric approach, the Genescan technique, a PCR based and computer-assisted fragment length analysis for TCRβ and TCRγ locus gene rearrangements, was used for further characterization (Kneba et al., 1995; Pechloff et al., 2010; van Dongen et al., 2003). Thereby, T-cell specific rearrangements can be amplified by a set of PCRs using a mixture of TCRβ gene segment-specific primers. The length of the resulting 5'-FAM labelled PCR products corresponds to the particular rearrangements present in the sample. Subsequently, the PCR products can be separated by capillary electrophoresis and detected by their fluorescence. Because of the random insertion or deletion of nucleotides at the joining gene segments of the TCR loci, the length of PCR products for one specific rearrangement varies within the normal polyclonal T-cell population and results in a Gaussian distribution. If a predominant rearrangement is present, this size distribution will be changed and the PCR product which is specific for the rearrangement will emerge over the polyclonal background.

To test for clonal TCRβ rearrangements, genomic DNA was extracted from splenocytes of one control and four AKT/CD4Cre mice and a set of six different PCR reactions for each sample was performed. A characteristic size distribution of PCR products was observed for the control sample, representing the usual diversity within a polyclonal T-cell population. As expected, the PCR products of reaction B, which is specific for the Dβ2/Jβ2 junction, formed a Gaussian distribution. The multiplex PCR reaction A (Vβ1-Vβ20/Jβ2) resulted in a more complex, but still distinct distribution (Figure 18, top row). Compared to the control, all tested AKT/CD4Cre mice (n = 4) displayed changes in the size distribution of the generated PCR products. Three out of four mice showed one or more predominant PCR products, which emerged as high peaks over the normal polyclonal background, indicating the presence of a clonal rearrangement (Figure 18, bottom row). Additionally, one out the four mice even showed a complete loss of the polyclonal background and only one or two prominent PCR products were detected, which is a sign for a very pronounced T-cell clonality (Figure 18, middle row).

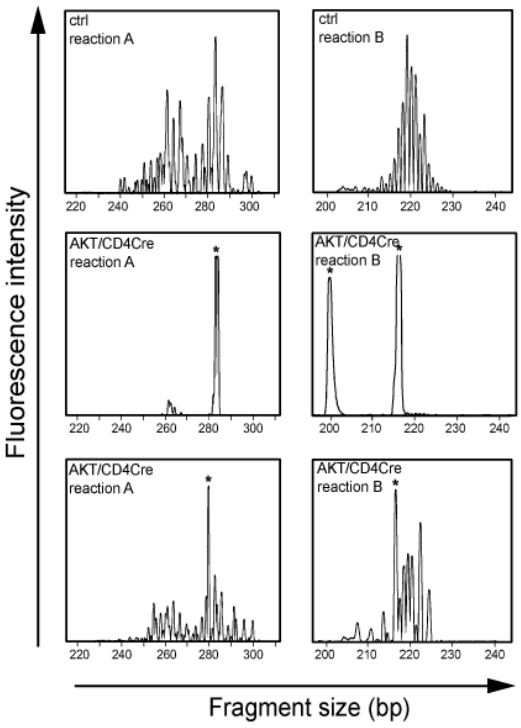


**Figure 18: Detection of clonal TCR rearrangements in AKT/CD4Cre mice by Genescan analysis.**
The genomic DNA was extracted from splenocytes of four diseased AKT/CD4Cre mice and one control mouse. The representative fragment size distribution of fluorochrome-labelled PCR products of the Vβ1-Vβ20/Jβ2 junction (Reaction A) and of the Dβ2/Jβ2 junction (Reaction B) for one control mouse (first row) and two AKT/CD4Cre mice is shown. A regular size distribution was observed for the control animal. This normal distribution was completely lost in one out of four transgenic animals. Only one or two PCR products appeared (depending on the PCR reaction used), indicating clonality (middle row). The other mice showed one or more overrepresented clones peaking over the normal polyclonal background (last row).

### 3.7 Infiltration of T-Cells into Organs and Histopathological Characterization

A hallmark of lymphoma is the infiltration of neoplastic cells into different organs of the diseased organism. Therefore, organs of control and transgenic mice were harvested, to determine the present fraction of lymphocytes by flow cytometry and to perform a histopathological characterization. A strong increase in the percentage of transgenic T-cells (TCRβ+ and GFP+) was found in liver, lung, kidney, bone marrow and peritoneal cavity of AKT/CD4Cre mice compared to T-cells (only TCRβ+) in the corresponding organs of control mice (Figure 19). These results clearly indicate an infiltration of transgenic T-cells into the organs of AKT/CD4Cre mice.

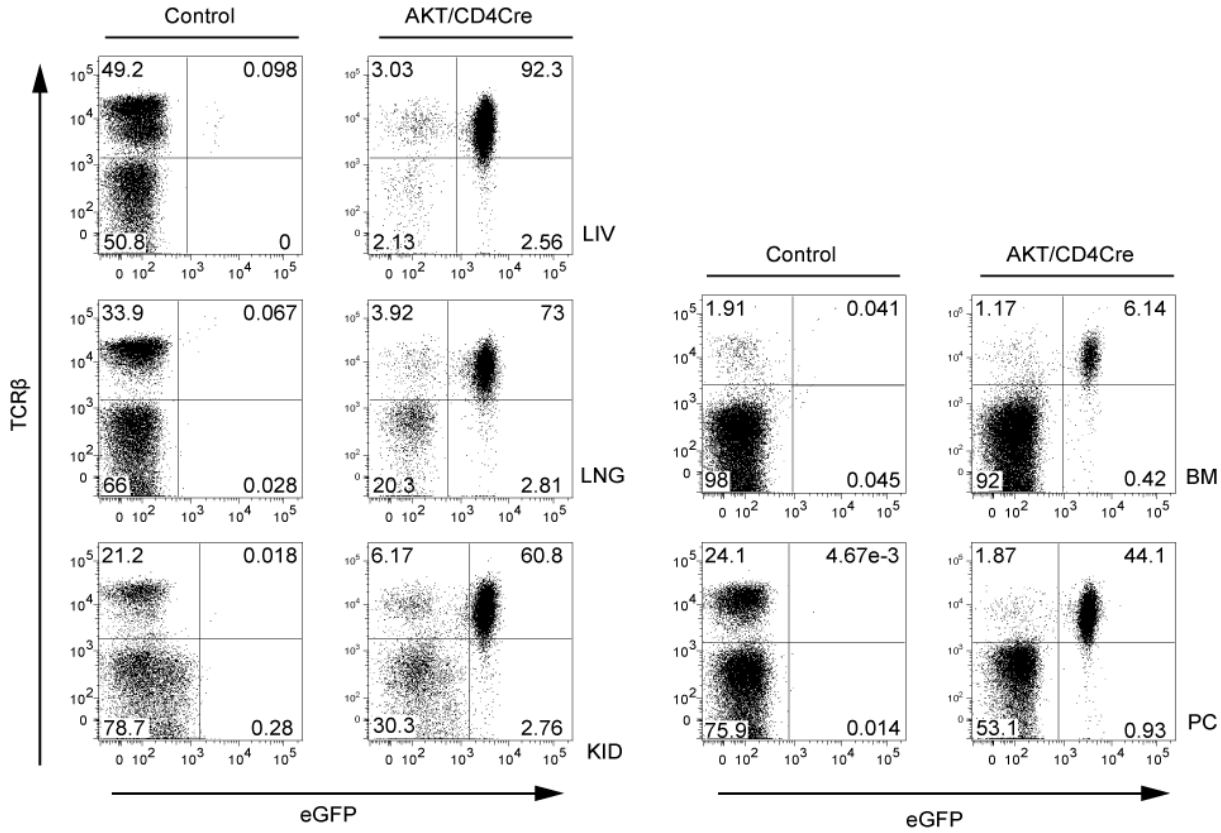


**Figure 19: Increased percentage of transgenic T-cells in the various organs.**
Single cell suspensions were made out of the liver (LIV), lung (LNG), and kidney (KID) of 6 weeks old control and AKT/CD4Cre mice. The lymphocyte fraction was further enriched by centrifugation within a Percoll gradient. Additionally, cells from the bone marrow (BM) and the peritoneal cavity (PC) were obtained. Cells were stained for TCRβ and analyzed by flow cytometry. All organs from AKT/CD4Cre mice had a higher percentage of TCRβ and GFP double positive cells compared to only TCRβ positive cells in the controls. Data are representative for at least three independent experiments.

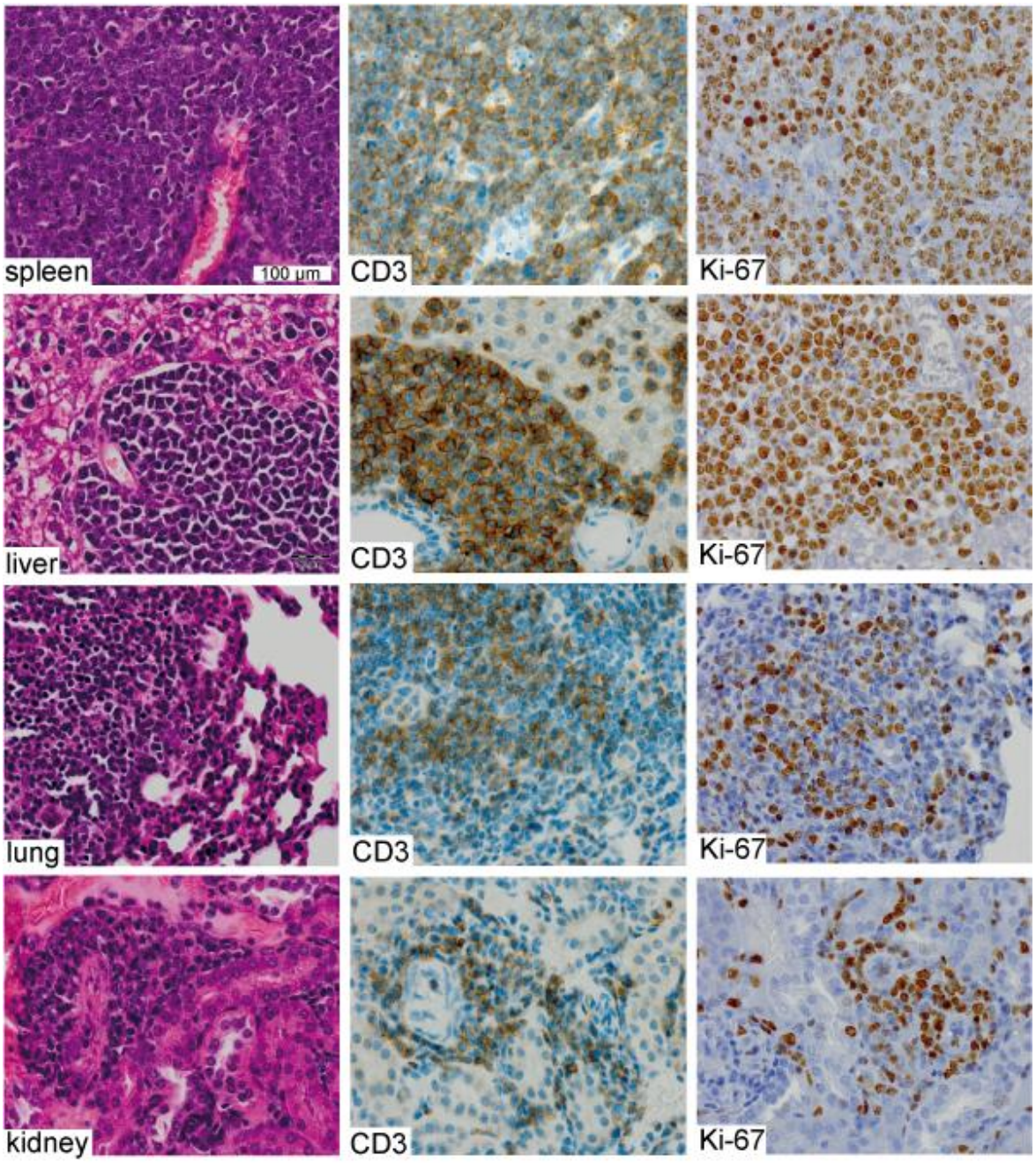


**Figure 20: Infiltration of proliferating T-cells into organs of AKT/CD4Cre mice.**
Immunohistochemistry made of organ tissue sections from 6 week old AKT/CD4Cre mice. Hematoxylin and Eosin (H+E) staining of spleen, liver, lung and kidney showed infiltrations of abnormal lymphocytes (left panel). Anti-CD3 staining (brown) confirmed T-cell identity of the infiltrating cells (middle panel), which also displayed a moderate to strong proliferation, marked by Ki-67 positive staining (brown, right panel). A representative AKT/CD4Cre mouse is depicted. Control mice were without pathological findings (data not shown).

The histopathological analysis of multiple organs (liver, lung, kidney) and the bone marrow confirmed an infiltration of abnormal lymphocytes into these organs (Figure 20, left panel). Especially in the liver, broad lymphocyte infiltrates were observed, surrounding the portal

tract and spreading into the parenchyma. Other organs showed perivascular infiltration of lymphoid cells that spread into the surrounding tissue. Additionally, the enlarged spleen and lymph nodes were packed with abnormal lymphocytes and showed architectural changes. The infiltrating cells were of a mature T-cell phenotype, characterized by the expression of CD3 (Figure 20, middle panel). No expression of TdT, a nuclear protein widely used as a marker for lymphoblastic leukemia, was detected. This result ruled out lymphoblastic leukemia as a potential classification of the disease. Moreover, the majority of infiltrating cells stained positive for the proliferation marker Ki-67, resulting in a high proliferation index with up to 70% (Figure 20, right panel). In conclusion, flow cytometry and histopathological evaluation described a lymphoid neoplasm, consistent with a Peripheral T-cell lymphoma (NOS).

### 3.8 Increased Proliferation of AKT/CD4Cre Transgenic T-Cells

Since the immunohistochemical analysis showed a high proliferative index of AKT transgenic T-cells, their proliferative potential was investigated in more detail. CD4+ lymphocytes were purified from spleens of AKT/CD4Cre and control mice, respectively, and labelled with a fluorescent dye, which allows monitoring cell division. The T-cells then were stimulated with plate-bound anti-CD3, or anti-CD3 and anti-CD28 antibodies, or left unstimulated. After 72h of incubation, the cells were harvested and subsequently analyzed by flow cytometry.

Due to the already mentioned increase in size of the transgenic T-cells and the associated heterogeneity of the population, the labelling resulted in a broader peak in the histograms. Consistent with the positive staining for Ki-67 in the histologic analysis, CD4+ T-cells derived from AKT/CD4Cre mice proliferated faster in comparison to control cells when stimulated with plate-bound anti-CD3 alone or anti-CD3/CD28 antibodies. Without stimulation, both genotypes showed no noticeable cell proliferation. In contrast to the control cells, no difference in proliferation was observed between transgenic cells stimulated with either anti-CD3- or CD3/CD28 antibodies, respectively. Control cells proliferated faster when stimulated with both anti-CD3/CD28 antibodies, compared to stimulation with anti-CD3 alone. However, the transgenic cells exhibited the same level of proliferation in both stimulation settings (Figure 21).

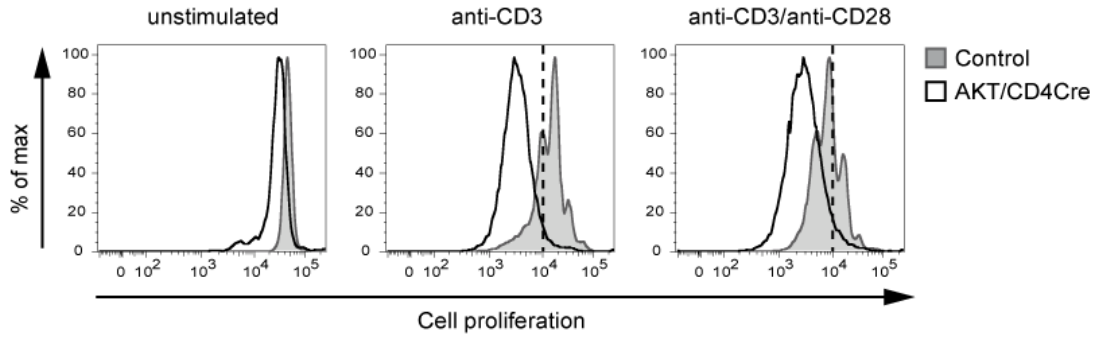


**Figure 21: Increased proliferation of AKT1-E17K transgenic T-cells compared to control T-cells.**
CD4+ T-cells were purified with MACS beads from control (grey tinted curve) and AKT/CD4Cre (open curve) mice and labelled with the dye CPD-AF670. 0.25 x$10^6$ cells per well were seeded in a 96 well plate and stimulated with plate-bound anti-CD3 (0.5µg/ml) or plate-bound anti-CD3 in combination with soluble CD28 (2µg/ml) antibody. The cells were harvested and analyzed by flow cytometry at several timepoints after stimulation. The 72h timepoint, depicted here, represented the strongest difference between the two genotypes. AKT/CD4Cre T-cells clearly proliferated faster than control cells after stimulation. But the proliferation of transgenic cells was not increased by the addition of anti-CD28 antibodies, in contrast to the control cells. The depicted histogram overlays are representative for at least 3 independent experiments.

### 3.9 Transplantation of AKT Transgenic T-Cells Can Induce the Disease

An additional criterion to evaluate the aggressive potential of cancer cells is their ability to grow in recipient mice after transplantation. This is often done by subcutaneous injection of cancer cell lines into immunocompromised mouse strains, which cannot mount an immune response. The tumor growth is subsequently analysed using diverse techniques. Leukemia and lymphoma cells are known to be the most difficult tumors to be transplanted (Watanabe et al., 1980). However, to answer the question, if AKT-transformed cells are transplantable, CD4+ T-cells were purified from diseased AKT/CD4Cre mice. Hence, 1x$10^7$ cells were injected intravenously into siblings and non-siblings of the donor. These immunocompetent recipients were then monitored for disease signs.

CD4+ T-cells from AKT transgenic mice were able to induce the same disease in the recipients as observed for the donors. However, 6 out of 13 transplanted recipients showed the described phenotype, while the remaining recipients showed no signs of disease. If successful, the recipient mice died or had to be sacrificed due to ethical reasons 25 days after transplantation on average (Figure 22). No difference in the development of the disease was observed for recipients being siblings or non-siblings of the donor. These observations confirmed an aggressive phenotype of the lymphoma, emphasizing the potential of AKT transgenic T-cells to induce the disease even in immunocompetent mice after transplantation.

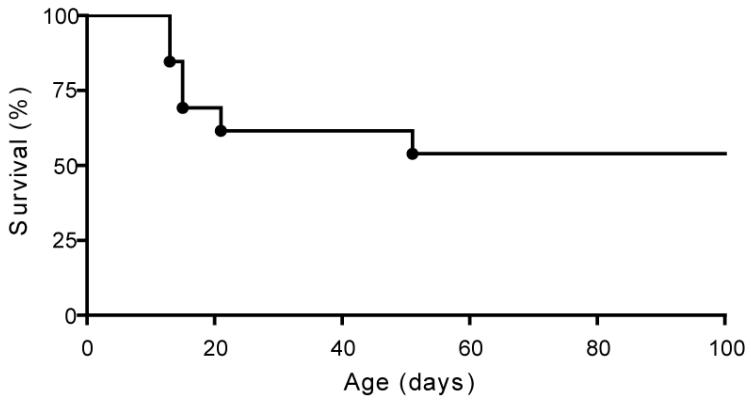


**Figure 22: Survival of mice transplanted with AKT transgenic T-cells.**
CD4+ T-cells of donor AKT/CD4Cre mice were purified with MACS beads and injected intravenously ($1x10^7$ cells) into siblings or non-siblings of the donor mouse. The transplanted mice were monitored for signs of disease. 6 out of 13 recipients developed a similar disease as observed for the donors with an average latency of 25 days. The remaining recipients stayed healthy with no signs of disease.

### 3.10 Bcl10 Deficiency Slightly Prolongs Survival of AKT/CD4Cre Mice

Several reports have implicated a role for AKT in contributing to the activation of the transcription factor NFκB (Bai et al., 2009; Jones et al., 2000; Ozes et al., 1999; Romashkova and Makarov, 1999). Even a link between AKT and the Carma-Bcl10-Malt1 (CBM) complex and subsequent IKK activation has been described lately (Cheng et al., 2011; Narayan et al., 2006). The CBM complex is formed upon antigen receptor stimulation and is crucial for signal transduction to the IKK complex, which then is able to phosphorylate IκBα. Phosphorylated IκBα gets ubiquitinated and is subsequently degraded by the proteasome. Thereby, NFκB is released and translocates to the nucleus to mediate its various functions. In cells lacking Bcl10 the pathway is interrupted and NFκB activation upon several stimuli is abolished (Ruland et al., 2001).

To investigate if a loss of Bcl10 could influence AKT driven lymphomagenesis, AKT/CD4Cre mice were crossed onto a Bcl10-deficient background (AKT/CD4Cre/$Bcl10^{-/-}$). The breeding resulted in a normal Mendelian distribution of sex and genotypes within the offspring (Figure 23A). AKT/CD4Cre/$Bcl10^{-/-}$ mice also developed typical disease signs and died or had to be sacrificed due to ethical reasons. But a significant survival advantage was observed compared to the breeding on the wildtype genetic background. The median survival of transgenic AKT/CD4Cre/$Bcl10^{-/-}$ mice was 74 days, compared to 52 days of

survival of the normal AKT transgenic mice (Figure 23B). The body weights were not significantly changed (Bcl10$^{-/-}$ vs. AKT/CD4Cre/Bcl10$^{-/-}$, mean ± SEM, 18.14g ± 0.76 vs. 17.74g ± 0.63, n = 21, p = 0.6901) when mice were analyzed upon disease signs (Figure 23E). One very striking difference of the AKT/CD4Cre/Bcl10$^{-/-}$ breeding was that more than half of the mice (15 out of 29 mice examined) developed a tumor in the pleural cavity, where normally the thymus is located (Figure 23C). Flow cytometric analysis of the abnormal tissue showed thymocytes, which all were eGFP positive, revealing the thymic origin of the tumor (Figure 23D).

A)

| total mice | male | female | AKT/wt Bcl10 -/- | AKT/ CD4Cre Bcl10 -/- | wt/ CD4Cre Bcl10 -/- | wt/wt Bcl10 -/- |
|---|---|---|---|---|---|---|
| 155 | 78 | 77 | 42 | 37 | 41 | 35 |
| 100% | 50.3% | 49.7% | 27.1% | 23.9% | 26.5% | 22.5% |

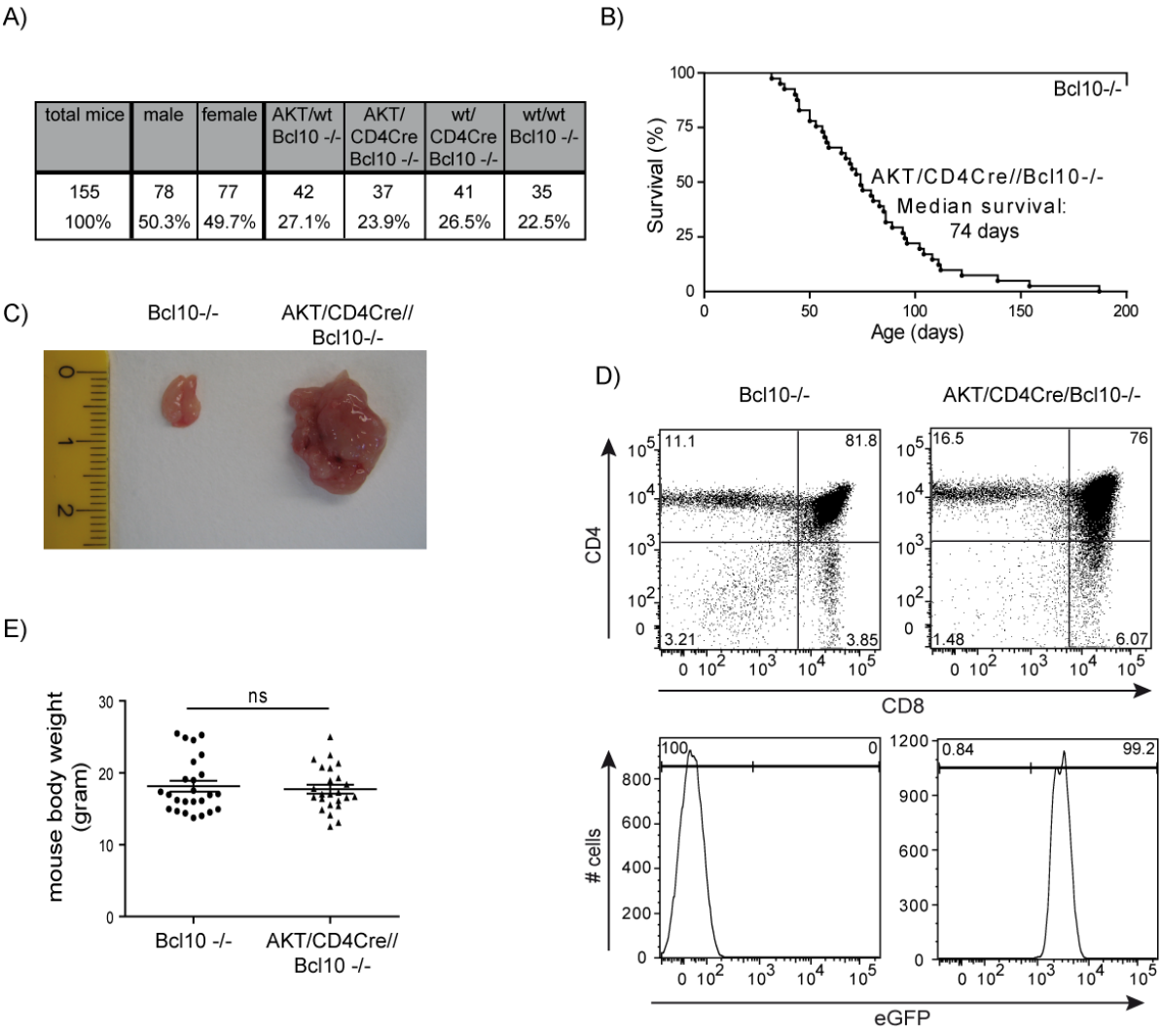


**Figure 23: Survival and thymic tumor development of AKT/CD4Cre/Bcl10$^{-/-}$ mice.**
A) Offspring analysis of the heterozygous breeding of AKT transgenic and CD4Cre mice (both on Bcl10-deficient background). Normal Mendelian ratios of sex and genotypes were observed. B) Kaplan-Meier survival curve of AKT/CD4Cre//Bcl10$^{-/-}$ (n = 41) and control Bcl10$^{-/-}$ (n = 18) animals. The curve includes animals that died or that had to be sacrificed due to a fatal illness or ethical reasons. The median survival of

AKT/CD4Cre/Bcl10$^{-/-}$ mice was 74 days. C) Representative picture of thymi derived from 8 weeks old control Bcl10$^{-/-}$ and AKT/CD4Cre/Bcl10$^{-/-}$ animals. Approximately 50-60% of the animals expressing AKT on a Bcl10$^{-/-}$ background developed abnormal thymic tissue growth. D) Flow cytometric analysis of a Bcl10$^{-/-}$ control thymus and an abnormal tissue of one AKT/CD4Cre/Bcl10$^{-/-}$ mouse, found in the pleural cavity. The tissue consisted of thymocytes, which nearly all were eGFP positive. The data shown is representative of at least 3 independent experiments. E) Statistical analysis of body weights of Bcl10$^{-/-}$ (n = 21) and AKT/CD4Cre//Bcl10$^{-/-}$ (n = 21) mice. Unpaired Student's t test was used to compare both genotypes.

Similar to the analysis of AKT transgenic mice on a genetic wildtype background, changes of thymic subpopulations in AKT/CD4Cre/Bcl10$^{-/-}$ mice compared to Bcl10$^{-/-}$ mice were investigated. A dramatic increase in thymic cell counts of AKT/CD4Cre/Bcl10$^{-/-}$ mice which harbor a thymic tumor compared to those who did not (37.6 ± 7.1 x10$^6$ vs. 243.8 ± 42.1 x10$^6$, n = 14 vs.15, p < 0.0001) was observed. To prevent a falsification of thymic cell counts, the animals were divided into two groups and analyzed separately, depending on tumor development.

The thymic cell counts of mice not having a thymic tumor compared to controls mostly resembled the phenotype seen in AKT/CD4Cre thymi. A decrease in total thymic cell number (Bcl10$^{-/-}$ vs. AKT/CD4Cre/Bcl10$^{-/-}$, mean ± SEM, 62.73 ± 8.64 x10$^6$ vs. 37.61 ± 7.05 x10$^6$, n = 14, p = 0.033), due to a decrease of DP cell numbers (50.67 ± 7.29 x10$^6$ vs. 10.45 ± 3.42 x10$^6$, n = 14, p < 0.0001), as well as the increase of cells in the DN compartment (2.08 ± 0.31 x10$^6$ vs. 11.19 ± 1.96 x10$^6$, n = 14, p < 0.0001) were observed. CD4+ T-cell numbers were slightly lower than in control mice (7.17 ± 0.9 x10$^6$ vs. 3.3 ± 0.7 x10$^6$, n = 14, p < 0.0022) and CD8+ T-cell numbers were slightly increased (2.82 ± 0.34 x10$^6$ vs. 12.67 ± 2.38 x10$^6$, n = 14, p = 0.0004) (Figure 24A, upper panel).

Mice which had developed a thymic tumor showed a different phenotype: the total thymic cell counts were strongly increased (67.89 ± 9.37 x10$^6$ vs. 243.8 ± 42.07 x10$^6$, n = 15, p = 0.0003), mainly due to elevated DP cell counts (54.6 ± 7.86 x10$^6$ vs. 188.6 ± 46.16 x10$^6$, n = 15, p = 0.0079). Also the other subsets showed an increase in cell numbers (DN: 2.41 ± 0.3 x10$^6$ vs. 13.61 ± 2.48 x10$^6$, n = 15, p = 0.0001; CD4 SP: 7.98 ± 1.055 x10$^6$ vs. 26.7 ± 6.24 x10$^6$, n = 15, p = 0.0062; CD8 SP: 2.91 ± 0.35 x10$^6$ vs. 15.05 ± 4.6 x10$^6$, n = 15, p = 0.0137) (Figure 24A, lower panel). In both groups all changes in cell numbers were significant. Similar to thymi of AKT/CD4Cre mice, in each thymic subset of both groups (also in the DN subset) strong eGFP expression, indicating AKT1-E17K expression, and an increase in cell size was detected (Figure 24B).

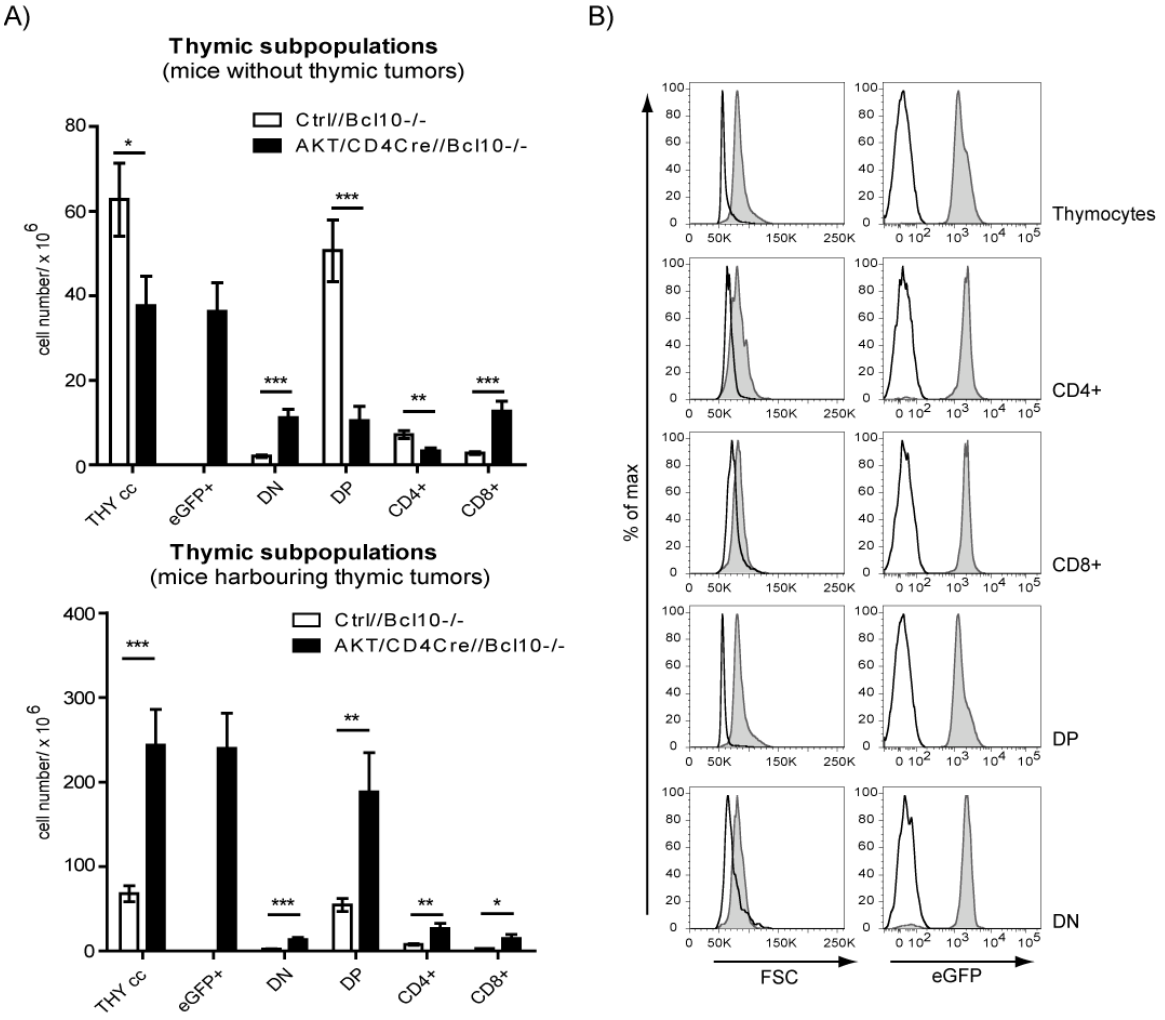


**Figure 24: Thymic subpopulations of AKT/CD4Cre/Bcl10$^{-/-}$ mice divided into two groups.**
A) Thymi of AKT/CD4Cre//Bcl10$^{-/-}$ or control mice were taken and the total cell number was determined by counting in a Neubauer chamber. Afterwards 1x10$^6$ cells were stained with antibodies against CD4 and CD8 and analyzed by flow cytometry. Depending on the incidence of a thymic tumor, AKT/CD4Cre/Bcl10$^{-/-}$ mice were grouped into two categories. Mice having no signs of thymic tumor resemble the phenotype seen in AKT/CD4Cre mice in most aspects. Mice with thymic tumor showed increased cellularity that is mostly due to an increase in the DP subpopulation. Mean cell numbers with SEM for each population of control mice, mice with a thymic tumor (bottom panel, n = 15) and mice with none (top panel, n = 14) are depicted. Populations were compared by unpaired Student's t-test analysis. B) Flow cytometric analysis of AKT/CD4Cre//Bcl10$^{-/-}$ (tinted) and Bcl10$^{-/-}$ thymocytes stained for CD4 and CD8. Overlays of FSC or eGFP fluorescence gated on the indicated population are shown. All AKT transgenic subpopulations showed an increase in FSC, indicating a bigger cell size, and nearly all expressed eGFP. Results are representative for at least six independent experiments.

### 3.11 Diminished Secondary B-Cell Activation in AKT/CD4Cre//Bcl10$^{-/-}$ Mice

In analogy to the situation on a wildtype background, the spleens of the Bcl10-deficient animals (control and AKT-E17K transgenic) were analyzed in more detail. Splenomegaly was observed in AKT/CD4Cre/Bcl10$^{-/-}$ mice, but in a lower frequency and to a lesser extent than in AKT/CD4Cre mice. Consistent with this observation, the spleens showed a small but significant increase in weight, compared to Bcl10$^{-/-}$ controls (Bcl10$^{-/-}$ vs. AKT/CD4Cre/Bcl10$^{-/-}$, mean ± SEM , 0.056g ± 0.002 vs. 0.189g ± 0.057, n = 21, p = 0.024) (Figure 25A).

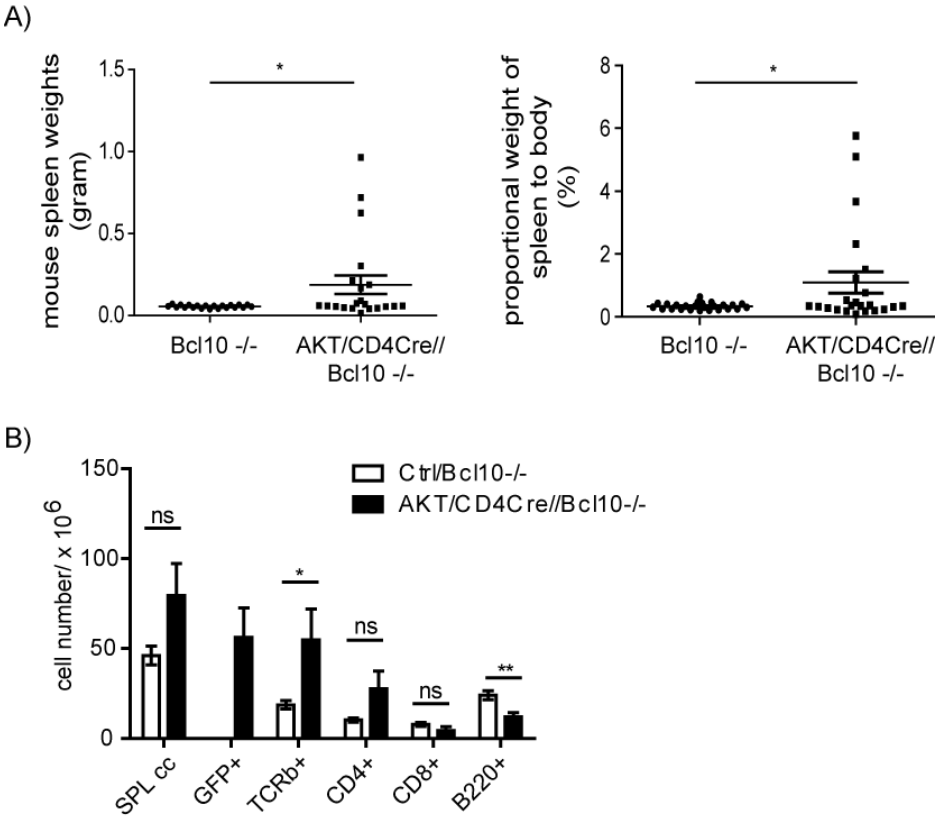


**Figure 25: Splenic weights and splenic subpopulations of AKT/CD4Cre/Bcl10$^{-/-}$ mice.**
A) Statistical analysis of spleen weights of Bcl10$^{-/-}$ (n = 21) and AKT/CD4Cre//Bcl10$^{-/-}$ (n = 21) mice. B) Spleens of 4 - 9 weeks old AKT/CD4Cre//Bcl10$^{-/-}$ (n = 22) or Bcl10$^{-/-}$ mice (n = 22) were isolated and total cell numbers were determined by counting in a Neubauer chamber. Flow cytometric analysis with antibodies against TCRβ, CD4, CD8 or B220 was performed. Mean value of the calculated cell number with SEM for each population is depicted. An increase in splenic cellularity and also in the CD4+ and TCRβ+ T-cell population, but no change in the CD8+ T-cell population was observed. The B220+ B-cell population was reduced. Unpaired Student's t test was used to compare both genotypes.

In general, the spleens of AKT/CD4Cre/Bcl10$^{-/-}$ mice seemed to harbour more cells than those of control mice, but the difference was not significant because of a much higher

variation of splenic cell counts (46.02 ± 5.25 x$10^6$ vs. 79.46 ± 17.69 , n = 22, p = 0.0772) (Figure 25B). A significantly increased TCRβ+ splenic T-cell number (18.58 ± 2.31 x$10^6$ vs. 54.86 ± 17.26 x$10^6$, n = 22, p = 0.0436) was observed in comparison to control mice, whereas the B-cells were significantly reduced (23.97 ± 2.56 x$10^6$vs. 11.98 ± 2.35 x$10^6$, n = 22, p = 0.0013), indicating a negative effect of Bcl10 deficiency on this population. The CD4+ cell number tended to an increase (10.14 ± 1.11 x$10^6$ vs. 27.53 ± 9.92 x$10^6$, n = 22, p = 0.089), but this increase was not significant. Also, the CD8+ T-cells showed no significant change in cell number (7.70 ± 1.09 x$10^6$ vs. 4.32 ± 2.17 x$10^6$, n = 22, p = 0.1706).

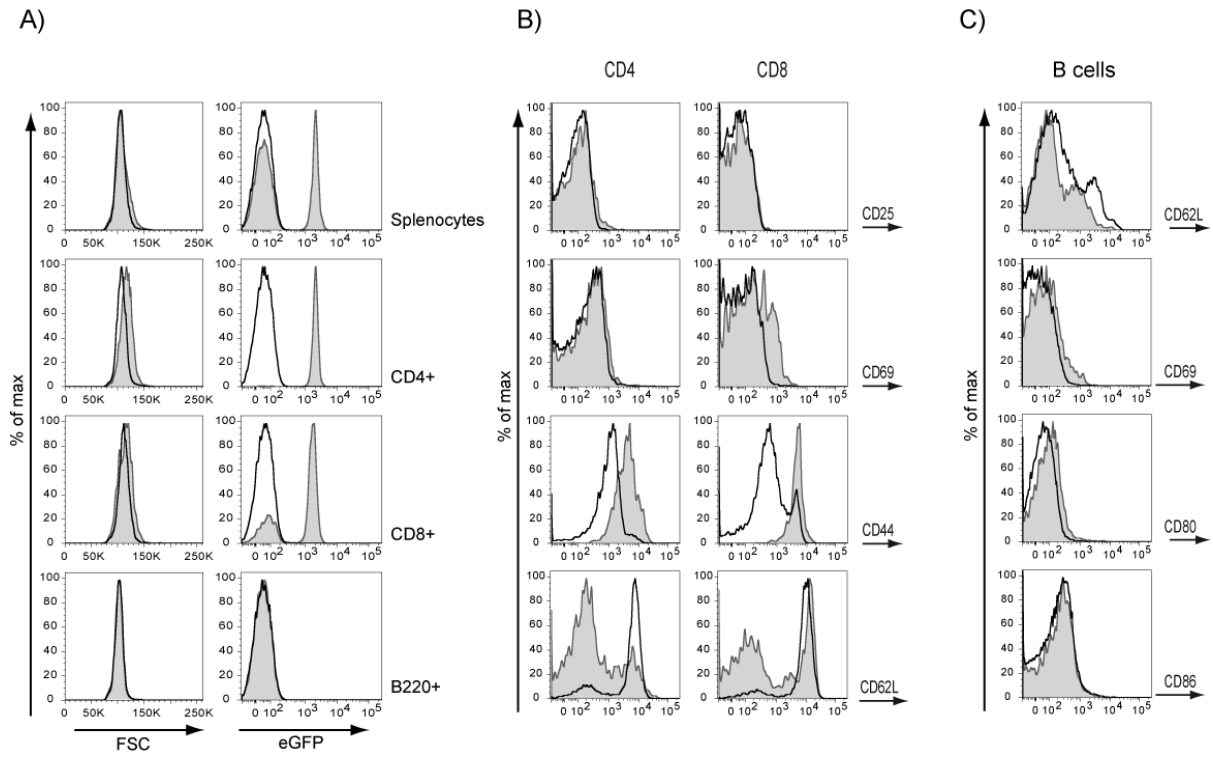


**Figure 26: T- but not B-cells of AKT/CD4Cre/Bcl10$^{-/-}$ show an activated phenotype.**
A) Representative flow cytometric analysis of 5 week old AKT/CD4Cre/Bcl10$^{-/-}$ (tinted) and control Bcl10$^{-/-}$ splenic cells stained for CD4, CD8 and B220. Overlays of FSC or GFP fluorescence, respectively, for total splenocytes, gated on the indicated populations are shown. CD4 and CD8 but not B220 positive cells showed an increase in size in the FSC. Except for the B-cells, most of the T-cells were GFP+. B) Flow cytometric analysis of AKT/CD4Cre/Bcl10$^{-/-}$ (tinted) and control Bcl10$^{-/-}$ splenic cells stained for CD4, CD8, CD25, CD69, CD44 and CD62L. Compared to controls, CD4+ and CD8+ AKT transgenic T-cells showed downregulation of CD62L and upregulation of CD44. Upregulation of CD69 is stronger in the CD8+ compartment. No change in CD25 expression was observed. Data is representative for at least 10 independent experiments. C) Flow cytometric analysis of AKT/CD4Cre/Bcl10$^{-/-}$ (tinted) and control Bcl10$^{-/-}$ splenic cells stained for B220, CD62L, CD69, CD80 and CD86. All histogram overlays were created from the B220+ population. Only a moderate downregulation of CD62L and upregulation of CD69, CD80 and CD86 was observed. The data is representative for 8 independent experiments.

The transgenic T-cells of AKT/CD4Cre/Bcl10$^{-/-}$ mice were marked by an increase in size, similar to the observations in AKT/CD4Cre mice. But in contrast to the original model, where Bcl10 is present, the B-cells of AKT/CD4Cre/Bcl10$^{-/-}$ mice consistently showed no increase in size (Figure 26A).

Flow cytometric analysis of activation markers on splenic T-cells again revealed an activated phenotype, similar to AKT/CD4Cre mice (Figure 26B). While CD25 expression levels were unaffected, CD44 expression was always upregulated in the AKT transgenic T-cells. Increased expression of CD69 was also detected, but appeared to be more pronounced for CD8+ T-cells. In line with the activated phenotype, CD62L was constantly downregulated in all transgenic T-cells.

Additionally, activation markers on the B-cell compartment were analyzed (Figure 26C). Compared to the controls, CD62L expression was found to be very slightly downregulated and expression of CD69, CD80 and CD86 was not altered or only marginally upregulated. Together with the lacking increase in size, this indicates that B-cells of AKT/CD4Cre/Bcl10$^{-/-}$ mice did not show signs for activation by a secondary effect, in contrast to AKT/CD4Cre mice.

### 3.12 Similar Phenotype of AKT1-E17K Transgenic Mice with or without Bcl10

To evaluate if other characteristics of the disease point to the same phenotype as on a wildtype background, the AKT/CD4Cre/Bcl10$^{-/-}$ mice were also tested for clonality. T-cells of control and AKT transgenic mice on a Bcl10-deficient background were stained with TCRvβ chain-specific antibodies and analyzed by flow cytometry. Control cells showed a distinct expression pattern of TCRvβ chains, representing their usage within the normal T-cell population. In four out of five experiments, the fluorescence intensity for the TCRvβ chains was diminished in AKT transgenic T-cells compared to the controls, indicating a reduced usage of these chains (Figure 27A, top panel). Only in one out of five experiments, an overrepresented TCRvβ chain was identified, pointing to a clonal expansion of T-cells (Figure 27A, bottom panel).

Again, Genescan analysis was performed and confirmed T-cell clonality. As expected, a characteristic length distribution of PCR products was observed for the Bcl10$^{-/-}$ control cells, representing the usual diversity within a polyclonal T-cell population (Figure 27B, top panel). However, all tested AKT/CD4Cre/Bcl10$^{-/-}$ mice showed changes in this

characteristic distribution of PCR products. In three out of four mice specific rearrangements were identified to be overrepresented in the T-cell population, visible as high peaks emerging over the still present polyclonal background (Figure 27B, bottom panel). One mouse even showed a complete loss of the polyclonal background combined with the appearance of one or two defined peaks, indicating a pronounced clonal rearrangement (Figure 27B, mid panel).

In addition to clonality, the organs of AKT/CD4Cre/Bcl10$^{-/-}$ mice were tested for infiltrations of transgenic cells by flow cytometry. Similar to AKT/CD4Cre mice on a wildtype background, an increased eGFP+ T-cell fraction was found in liver, lung, kidney and bone marrow. This indicated a massive infiltration of transgenic T-cells into these organs (Figure 27C).

Furthermore, the proliferative potential of CD4+ T-cells derived from AKT/CD4Cre/ Bcl10$^{-/-}$ mice and Bcl10$^{-/-}$ controls was evaluated using flow cytometry. In general, AKT transgenic cells proliferated faster than control cells, even when they were not stimulated (Figure 27D). Combined stimulation with anti-CD3 and anti-CD28 antibodies did not induce stronger proliferation of transgenic cells than anti-CD3 stimulation alone did. Surprisingly, the culture and stimulation conditions used here, also induced proliferation of the Bcl10$^{-/-}$ control cells, which is not described in the original publication (Ruland et al., 2001). But a more recent work has shown that also Bcl10-deficient cells can proliferate depending on the cell type and the strength of the stimulation (Kingeter and Schaefer, 2008).

In summary, the disease characteristics of AKT/CD4Cre/Bcl10$^{-/-}$ mice were similar to those of AKT/CD4Cre mice. Signs of disease, expansion and infiltration of T-cells into various organs, T-cell clonality and an increased T-cell proliferation again pointed to a PTCL (NOS) similar to the wildtype situation. However, the loss of Bcl10 also altered some characteristics of the disease. It resulted in a prolonged survival, an increased incidence of thymic tumors, and a loss of the secondary B-cells activation in comparison to AKT/CD4Cre mice.

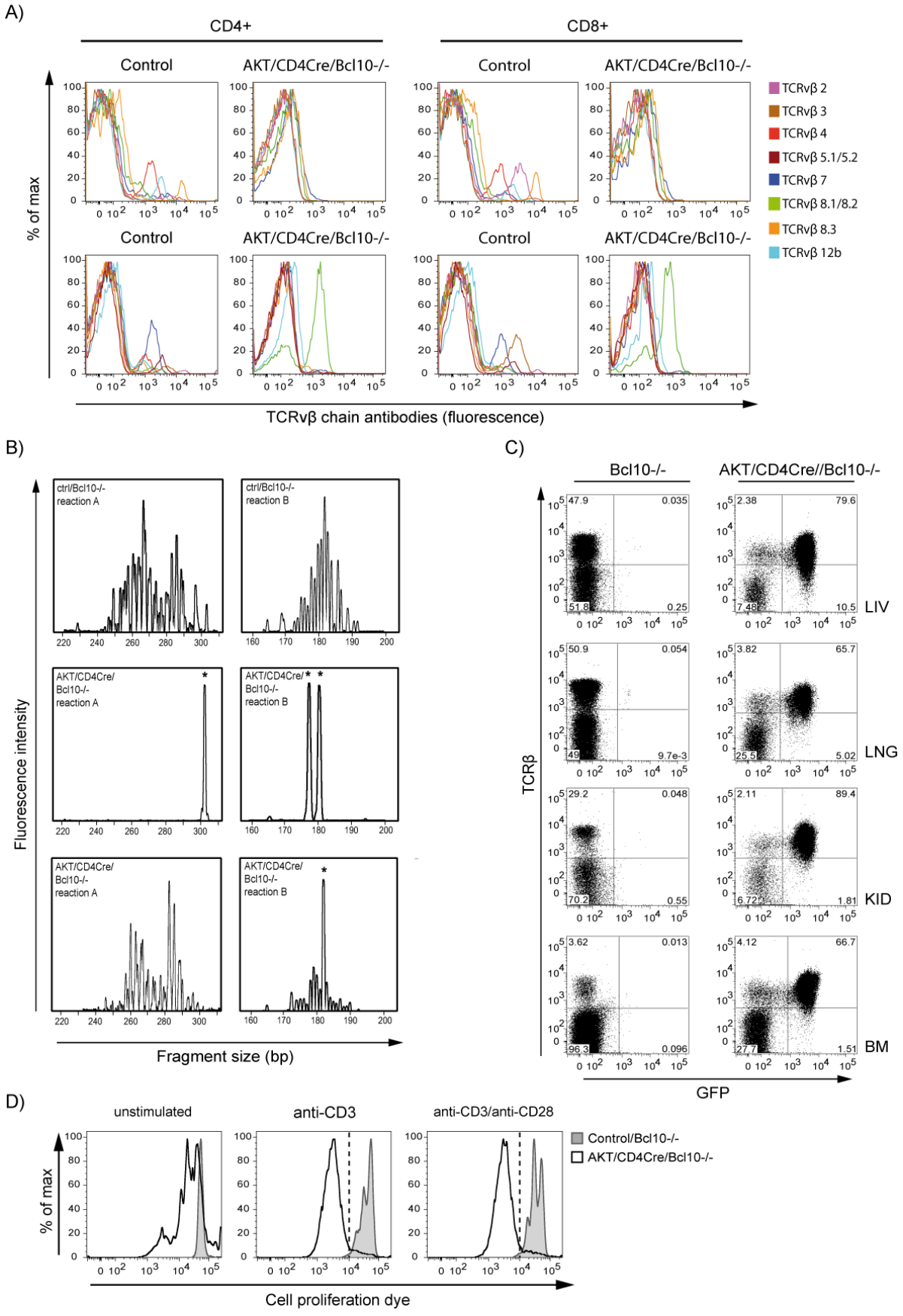


**Figure 27: Clonality, infiltrations and increased T-cell proliferation in AKT/CD4Cre/Bcl10$^{-/-}$ mice.**

A) Splenocytes of 8-9 week old Bcl10$^{-/-}$ and AKT/CD4Cre//Bcl10$^{-/-}$ animals were stained with specific antibodies against CD4, CD8 and a panel of different TCRvβ chains (TCRvβ 4 (red), TCRvβ 7 (dark blue), TCRvβ 8.1/8.2 (bright green), TCRvβ 8.3 (orange), TCRvβ 12b (bright blue), TCRvβ 2 (violet), TCRvβ 3 (bright brown), TCRvβ 5.1/5.2 (dark brown)) and analyzed by flow cytometry. Two Bcl10$^{-/-}$ mice (control) and two AKT/CD4Cre/Bcl10$^{-/-}$ mice are shown. Each histogram depicts an overlay of the TCRvβ chains expressed on the CD4+ or CD8+ T-cells of one mouse. Control cells showed a characteristic pattern of TCRvβ chain expression. The transgenic cells of one mouse displayed a reduced fluorescence for the different chains (top panel). In the other mouse, one antibody recognized an increased usage of a specific chain, indicating clonal growth of the T-cells (bottom panel). B) Genescan analysis of splenic DNA from four diseased AKT/CD4Cre/Bcl10$^{-/-}$ mice and one Bcl10$^{-/-}$ mouse. Representative fragment size distribution of fluorochrome-labelled PCR products of the Vβ1-Vβ20/Jβ2 junction (Reaction A) and of the Dβ1/Jβ1 junction (Reaction B) for one Bcl10$^{-/-}$ mouse (first row) and two AKT/CD4Cre/Bcl10$^{-/-}$ mice is shown. For the control animal, normal fragment size distributions of the PCR products was observed (first row). In three out of four mice, one or more peaks emerged out of the normal polyclonal background (third row), indicating clonal rearrangements. One out of four AKT/CD4Cre/Bcl10$^{-/-}$ mice analyzed showed a complete loss of the polyclonal background combined with the appearance of one or two very clear peaks, indicating a pronounced clonality.C) Infiltration of eGFP expressing T-cells into different organs. Single cell suspensions from the peritoneal cavity, liver, lung, kidney and bone marrow of 6 week old control Bcl10$^{-/-}$ and AKT/CD4Cre/ Bcl10$^{-/-}$ animals were stained for TCRβ and analyzed by flow cytometry. All organs from AKT/CD4Cre/Bcl10$^{-/-}$ mice contained a higher percentage of TCRβ and GFP double positive cells compared to only TCRβ positive cells in the controls. Data shown is representative for at least three independent experiments. D) Proliferation of CD4+ T-cells from control/Bcl10$^{-/-}$ and AKT/CD4Cre/Bcl10$^{-/-}$ mice. Cells were purified with MACS beads, labelled with CPD-AF670, seeded out in a 96 well plate and stimulated with the indicated agents. After different timepoints cells were harvested and analyzed by flow cytometry. The T-cells from AKT/CD4Cre/Bcl10$^{-/-}$ mice proliferated faster than control T-cells.

### 3.13 Constitutive Activation of AKT1-E17K in T-Cells

The activation of AKT was shown to be dependent on two important phosphorylation sites, Thr308 and Ser473. Full kinase activity is only achieved if both residues are phosphorylated. In order to evaluate the phosphorylation status of AKT1-E17K and to investigate the effects of the kinase on cellular signalling, CD4+ T-cells of AKT/CD4Cre, AKT/CD4Cre/Bcl10$^{-/-}$ and appropriate control mice were purified and analyzed by Western Blot (Figure 28). As already observed before, AKT1-E17K was strongly expressed in both transgenic lines and, due to its additional flag-tag, displayed a slight shift in its molecular weight, in contrast to the endogenous AKT.

In both mouse strains expressing AKT1-E17K (wildtype and Bcl10$^{-/-}$ background), the basal levels of AKT phosphorylation at Thr308 and Ser473 were strongly increased compared to the corresponding controls, indicating the constitutive activity of the kinase. The phosphorylation of the Thr308 site generally was weaker than Ser473 phosphorylation. Interestingly, stimulation of control T-cells with PMA/Ionomycin resulted in phosphorylation of AKT Ser473 but not of Thr308. The stimulus PMA/Ionomycin therefore

did not result in the activation of PDK1, which is responsible for Thr308 phosphorylation, but in the activation of mTORc2 or an alternative AKT Ser473-specific kinase.

To gain more information on the activation state of AKT1-E17K, phosphorylation of direct AKT downstream targets was analyzed. CD4+ T-cells of both AKT transgenic genotypes displayed an increased phosphorylation of the FoxO family proteins FoxO1 and FoxO4, implying that the AKT-FoxO axis is constitutively switched on. Also GSK3α and GSK3β showed increased phosphorylation, but to a lesser extent compared to FoxO1. In the controls, a phosphorylation of these two targets was only observed after stimulation with PMA/Ionomycin. These results confirmed the constitutive activation of AKT1-E17K and revealed permanent downstream signalling, even without stimulation.

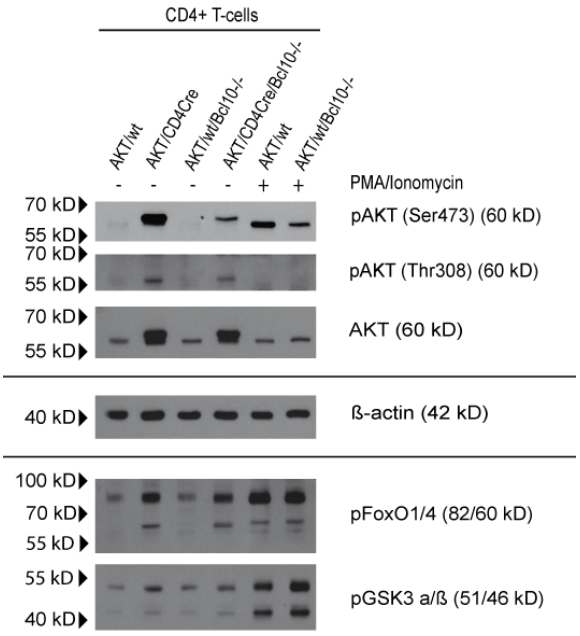


**Figure 28: Constitutive activation and downstream signalling of AKT1-E17K.**
Western Blot analysis of purified CD4+ T-cells of AKT/CD4Cre, AKT/CD4Cre/Bcl10$^{-/-}$, wildtype and Bcl10$^{-/-}$ mice was performed. Control cells were stimulated with PMA/Ionomycin (100 nM each) where indicated. The analysis included examination of AKT expression, its phosphorylation at two critical sites (Thr308, Ser473) and phosphorylation of FoxO1/4 and GSK3α/β as direct targets of the AKT kinase. β-actin served as a loading control. The results shown are representative for at least 3 independent experiments.

### 3.14 Transgenic T-Cells Show No Consistent Activation of the NFκB pathway

In normal T-cells, the transcription factor NFκB can be activated in many ways, e.g. by engagement of the TCR. A complex signalling cascade transduces the signal, resulting in the phosphorylation and activation of the IKK complex. This complex consists of the proteins IKKα, IKKβ and IKKγ in the canonical and of IKKα dimers in the non-canonical pathway. The IKK complex subsequently phosphorylates IκBα and targets it for ubiquitination and degradation. Thereby, NFκB is set free and can translocate to the nucleus to regulate gene transcription. Several studies implicated a role for AKT in NFκB activation or in modulation of the NFκB response. Therefore, the influence of AKT1-E17K on NFκB signalling was investigated in more detail.

To determine NFκB activity in purified CD4+ T-cells of control or AKT/CD4Cre mice, nuclear extracts were prepared and Electrophoretic Mobility Shift Assays (EMSAs) were performed. Binding of the active transcription factor in the nuclear lysates to a fluorescently labelled DNA consensus site was detected for some transgenic samples, but was not consistently present in all of them (Figure 29A). Three out of eight mice analyzed, showed stronger NFκB binding than the control mice, indicating that in AKT-driven lymphomas constitutive NFκB activation exists but is not obligatory.

Further, the potential effects of constitutively active AKT on pathway components directly upstream of NFκB were examined. In CD4+ T-cell lysates, obtained from control and AKT/CD4Cre mice (also from $Bcl10^{-/-}$ background), no activation of the pathway was detected (Figure 29B). IKKα and IKKβ phosphorylation levels turned out to be equally low in unstimulated T-cells of all four genotypes analyzed. Additionally, neither a stronger phosphorylation nor an increased degradation of IκBα was observed in AKT transgenic T-cells, compared to the controls. Only wildtype cells stimulated with PMA/Ionomycin showed phosphorylation of the IKK complex and IκBα degradation as hallmarks of NFκb pathway activation. In contrast to that, Bcl10-deficient cells were unresponsive to stimulation, due to a previously described defect in signal transduction (Ruland et al., 2001). Hence, AKT1-E17K expression did not lead to a basal engagement of the pathway, concerning the components directly upstream of NFκB.

The activation of NFκB in T-cells via the canonical pathway involves the Carma1-Bcl10-Malt1 (CBM) complex. After TCR stimulation, the three proteins form a complex that mediates further downstream signalling including activation of the IKK complex. Therefore, the formation of the CBM complex was analyzed by coimmunoprecipitation of the Bcl10

protein. In unstimulated control T-cells, coimmunoprecipitation resulted only in low amounts of Carma1 associated with Bcl10. Following stimulation with PMA/Ionomycin, substantially more Carma1 protein was coprecipitated with Bcl10. Constitutively active AKT did not influence the CBM complex formation, since no enhanced formation of the CBM complex was found in AKT-E17K T-cells (Figure 29C). Additionally, neither in unstimulated or stimulated control cells nor in AKT transgenic cells, AKT was coimmunoprecipitated with Bcl10. This indicates that the kinase does not bind to any of the components of the CBM complex. In summary, AKT1-E17K seemed to have no effect on NFκB pathway components and activation mediated through these.

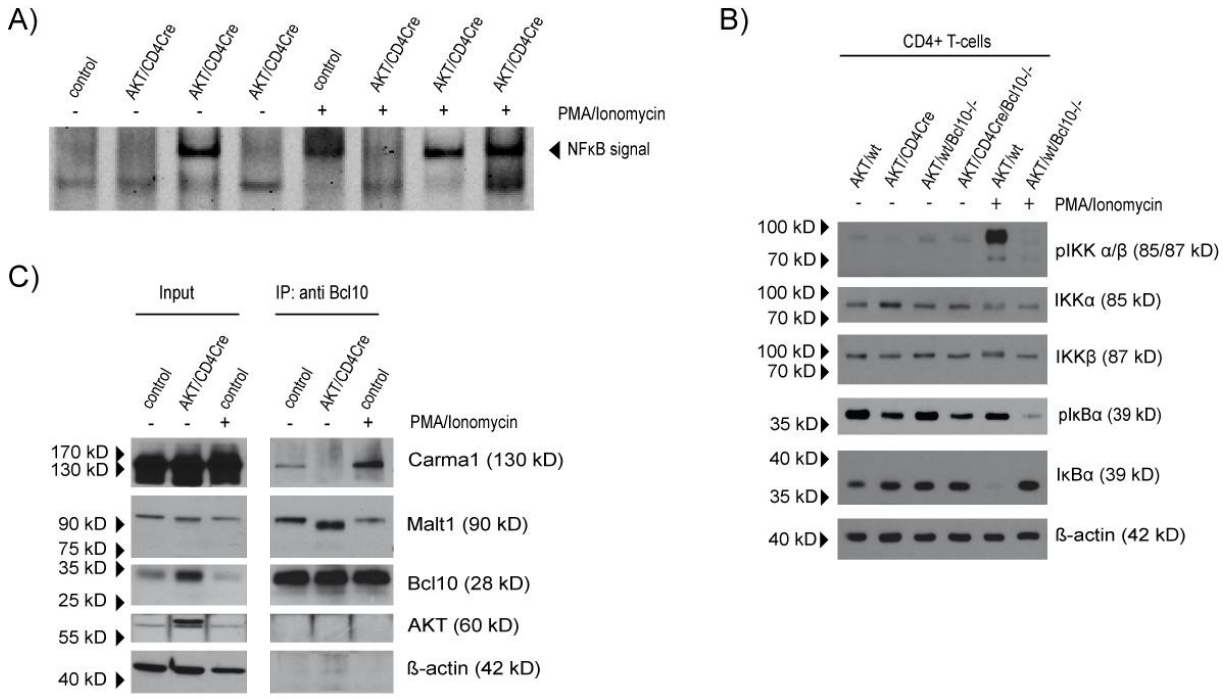


**Figure 29: Constitutive active AKT1-E17K signalling has no effect on NFκB pathway components.**
A) EMSA of purified CD4+ T-cells of AKT/CD4Cre and control mice. Where indicated, cells were stimulated with PMA/Ionomycin (100 nM each) for 20 min. Nuclear extracts were prepared and used for the NFκB binding assay. One control and three AKT/CD4Cre mice are depicted. Unstimulated control cells only showed a very faint signal but NFκB binding to the consensus sequence could be induced by stimulation with PMA/Ionomycin. NFκB activity was observed in unstimulated AKT/CD4Cre T-cells, but not in all samples. Stimulation further induced NFκB activity only in one transgenic sample. B) Western Blot analysis of purified CD4+ T-cells of AKT/CD4Cre, AKT/CD4Cre/Bcl10$^{-/-}$, wildtype and Bcl10$^{-/-}$ mice. Control cells were stimulated with PMA/Ionomycin (100 nM each) as indicated. Analyses included several components of the NFκB signalling pathway (IKKα/β, IκBα). β-actin served as a loading control. Results shown are representative for at least 3 independent experiments. C) Coimmunoprecipitation of Bcl10. Where indicated, control cells were stimulated with PMA/Ionomycin. CD4+ T-cells were purified, lysed and used in a co-IP with antibodies against Bcl10. Upon stimulation, a stronger CBM complex formation was observed in the control cells. In AKT/CD4Cre transgenic T-cells no Carma1 band was detected, indicating that the complex is not forming in unstimulated cells that express constitutive active AKT. Additionally, no direct or indirect binding of AKT to Bcl10 or the complex was observed.

### 3.15 AKT1-E17K Influences p65 Phosphorylation and Other Crucial Signalling Pathways

It has been demonstrated that AKT is able to influence the transactivation function of NFκB through stimulating the transactivation domain of p65 (Madrid et al., 2000). The transactivation domains of a transcription factor can modulate or stimulate transcription by interaction with other transcription factors. Therefore, the phosphorylation status of two sites of the NFκB subunit p65 was evaluated by Phosflow analysis. This technique uses phosphosite-specific antibodies directed against signalling proteins for intracellular staining of fixed and permeabilized cells. The mean fluorescence intensities (MFIs) generated by flow cytometric analysis of T-cells from both control and AKT/CD4Cre mice were used to calculate the relative changes in the phosphorylation levels. The results of several experiments were pooled and two p65 phosphorylation sites, which are supposed to play a role in modulating the transactivation potential of the protein, were analyzed. The residue Ser529 showed enhanced phosphorylation (1.45 ± 0.18, n = 9) compared to control cells, indicating an increased transactivation potential. On the contrary, the Ser536 site seemed to be unaffected in AKT transgenic T-cells (0.89 ± 0.12, n = 7) (Figure 30).

To further evaluate the impact of constitutively activated AKT on other important signalling pathways, crucial phosphorylation sites of several proteins also were investigated by Phosflow analysis. As already shown by Western Blot, an increased phosphorylation of AKT at the critical sites Thr308 (mean relative change ± SEM, 1.33 ± 0.10, n = 8) and Ser473 (1.66 ± 0.30, n = 12) (Figure 30) was detected, indicating a constitutive active AKT kinase.

Additionally, enforced signals for Erk1/2 (1.81 ± 0.2, n = 10) and Stat3 (1.88 ± 0.16, n = 11) phosphorylation, two important proteins often implicated in oncogenesis, were found. The analyzed Stat3 Ser727 phosphorylation site is thought to influence the transcriptional activity and the oncogenic potential of the protein, whereas Tyr705 phosphorylation was shown to be a prerequisite for Stat3 dimerization, nuclear translocation and activation of gene transcription (Bowman et al., 2000; Reich, 2009). Only minor effects of constitutive active AKT signalling on the phosphorylation levels of p38 (1.19 ± 0.16, n = 11) and Stat5 (1.13 ± 0.11, n = 12), again players in the MAPK and Stat pathways, were detected.

The CD3ζ chain is a part of the TCR/CD3 complex, important for coupling antigen recognition to intracellular signalling. This protein appeared to be less phosphorylated (0.75 ± 0.15, n = 10) in transgenic cells. The receptor-proximal proteins PLCγ1 (0.93 ± 0.09, n =

9), PLCγ2 (1.16 ± 0.11, n = 8), and SLP76 (1.13 ± 0.12, n = 5) were only slightly altered, except for Lck which showed a markedly increased phosphorylation (1.32 ± 0.17, n = 10). Besides, staining against KI-67 and Bcl-2 with common antibodies (not phosphosite-specific) showed increased KI-67 protein levels (1.28 ± 0.31, n = 11) in AKT transgenic cells, indicating again enhanced proliferation. Interestingly, the antiapoptotic Bcl2 protein was always downregulated in AKT/CD4Cre T-cells (0.13 ± 0.02, n = 10). Thus, constitutive AKT signalling also affected other important pathways involved in cell survival and cell transformation, in a direct or indirect way.

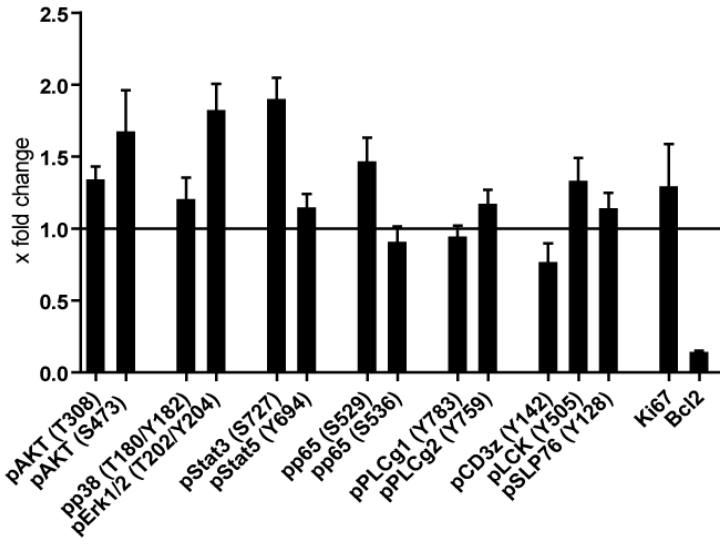


**Figure 30: Phosflow analysis of several important signalling proteins.**
Splenocytes of control and AKT/CD4Cre mice were stained with a live/dead cell marker and an antibody against CD4. The cells were fixed, permeabilized and incubated with antibodies specific for the phoshorylation sites of the indicated proteins. Cells were analyzed subsequently by flow cytometry. The mean fluorescence intensity (MFI) of the phospho-site-specific antibodies was set into relation to the MFI obtained from the living fraction of cells stained with the live/dead cell marker. The fold change of the phosphorylation status was calculated for each experiment and the mean values (with SEM) of pooled results (at least 5 independent experiments) are shown.

# 4 Discussion

The crucial role of the PI3K/AKT pathway and its importance in diverse cellular functions has been worked out over the last couple of years. With increasing knowledge about the functional details and components also the frequent disturbance of the pathway, especially in neoplastic diseases, was noticed. Numerous alterations of pathway components leading to aberrant signalling were identified in human tumors.

The novel mutation found by Carpten et al. within the AKT1 gene describes a single point mutation in the pleckstrin homology domain of the protein. Mechanistically, it leads to an increased affinity to the membrane lipids PIP2 and PIP3, which induces constitutive activation of the kinase (Carpten et al., 2007; Landgraf et al., 2008). The mutation was detected in several tumor types but only in a low frequency, indicating that the serine/threonine kinase AKT is rarely affected by mutations in a direct way. However, diverse mutations, such as PTEN deletions or PIK3CA mutation, were identified all over the pathway resulting in a frequent activation of AKT in human cancers. Because this kinase and its numerous downstream targets have crucial functions in cellular processes, AKT is a very interesting research topic and a potential therapeutic target. Thus, it is necessary to characterize the consequences of constitutive AKT activation *in vivo* for a better understanding of its contribution to oncogenesis and to provide a tool for the evaluation of therapeutic approaches.

To be able to evaluate the oncogenic potential of the AKT1-E17K mutation and to investigate the impact of constitutively activated AKT signalling, a new mouse line was successfully generated in this study. The Rosa26-AKT1-E17K mouse can conditionally express the transgene under the control of the Rosa26 promotor by breeding with celltype-specific Cre mice. This opens up the possibility to study the effects of AKT1-E17K in diverse cell types *in vivo*. Thereby, the mouse line can serve as an important model for the investigation of various AKT driven diseases and aberrant PI3K/AKT signalling.

## 4.1 Expression of AKT1-E17K in T-Cells Results in a Peripheral T-Cell Lymphoma

Since activation of the PI3K/AKT pathway is a recurring phenomenon in T-cell lymphomas, the impact of AKT1-E17K expression in T-cells has been of special intrest to this work. The pathogenesis of these lymphomas is poorly understood because experimental models are lacking and large series of similar cases are few (Cotta and Hsi, 2008). Yet, gene expression profiling using DNA microarrays and immunohistological analyses have improved the knowledge about T-cell lymphoma pathobiology (Rodriguez et al., 2009). For example, PDGFRα, NPM-ALK and Syk expression, as well as activation of Syk or AKT were shown to be altered in Peripheral T-Cell Lymphoma (PTCL), indicating an involvement of the PI3K/AKT pathway (Feldman et al., 2008).

The category PTCL comprises of a biologically heterogeneous group of lymphomas, derived from mature (post-thymic) T-cells (de Leval and Gaulard, 2011). Even though it is a rare disease, accounting for less than 15% of all cases of Non-Hodgkin lymphoma worldwide, most entities are clinically aggressive with overall poor responses to classical treatments and unfavourable prognosis (Vose, 2008). PTCL is subdivided by the Revised European-American Lymphoma/WHO classification into specified (Angioimmoblastic T-cell lymphoma, ALK positive/negative anaplastic large-cell lymphoma) and not otherwise specified (NOS) forms (Harris et al., 1994; Jaffe, 2001; Piccaluga et al., 2007). In reality diagnosis and classification is hampered by several difficulties, including morphological and immunophenotypic overlap across different entities and the lack of characteristic genetic alterations for most of them (de Leval and Gaulard, 2008).

In order to evaluate the role of constitutive active AKT signalling in T-cell lymphomagenesis, AKT1-E17K mice were bred with CD4Cre mice, to specifically induce expression in T-cells. The resulting mice were viable, but soon displayed typical signs of disease such as loss of weight and died around 52 days after birth. Compared to the controls, some pubs were smaller right after birth, what could be an indicator for a very early onset and a wasting character of the disease. Spleen and lymph nodes of transgenic mice were enlarged and contained massively altered lymphocyte populations, caused by an accumulation of CD4+ T-cells with an activated phenotype. Spectratyping (GeneScan analysis) of TCR rearrangements revealed clonal expansion of the T-cells and the histopathological analysis further showed infiltration of the T-cells into various organs, both typical features of a T-cell lymphoma. Additionally, the transgenic T-cells had a high proliferative potential and were transplantable to other mice. Biochemically, increased basal

phosphorylation of the AKT1-E17K protein showed constitutive activation of the kinase, which was confirmed by enhanced phosphorylation of the direct downstream targets FoxO and GSK3. Also, influences of AKT1-E17K expression on the phosphorylation of other signalling proteins such as Erk and Stat3, which are implicated in oncogenesis, were observed. In summary, the data presented here clearly demonstrates the potential of AKT1-E17K to induce a T-cell lymphoma with a 100% penetrance and a very short latency *in vivo*. In combination with the histopathological evaluation, the disease resembles a Peripheral T-cell lymphoma (NOS), similar to the human situation. So the AKT1-E17K/CD4Cre mouse represents an *in vivo* model for PTCL and for AKT-driven lymphomagenesis in general.

## 4.2 AKT1-E17K/CD4Cre Mice - A Valuable Model for Constitutive Activated AKT in T-Cells

Prior to this work, other researchers have generated transgenic mice expressing a constitutively active AKT in T-cells by adding a myristoylation signal to the AKT sequence (myrAKT). Myristoylation targets a protein towards the membrane compartment of the cell and thereby seems to recruit the AKT kinase into an environment which facilitates activation. Two independent groups expressed myrAKT under the control of the human CD2 promotor, but were not able to observe lymphomagenesis in these mice, at least for several months (Jones et al., 2000; Na et al., 2003). Two other groups decided to use the Lck promotor for myrAKT expression and could induce the development of lymphomas, which have a slower onset than AKT1-E17K mice (Malstrom et al., 2001; Rathmell et al., 2003). This difference could lie in a stronger oncogenic potential of AKT1-E17K *in vivo* compared to myrAKT. Further, the use of different promotors may play a role, resulting in a differing start and strength of expression.

The novel AKT1-E17K model presented here has the advantage of using a clinically relevant and mechanistically characterized point mutation instead of the artificial myristoylation. The clean conditional targeting into the well-characterized Rosa26 locus allows defined and cell type specific expression of the transgene, circumventing genetically different founder animals and a possible selection on a milder phenotype. An additional feature is the bicistronic expression of eGFP, which can be used to identify and trace recombined cells. So the model generated here is superior to the other attempts to constitutively activate AKT *in vivo*.

Nevertheless, since AKT1-E17K is expressed additionally to the endogenous AKT, the question arises, if the oncogenic effect of the kinase in this model is due to the activating mutation, or if it could also be due to a simple overexpression of AKT from the Rosa26 locus. Previous studies indicate that AKT1 expression alone does not efficiently transform cells in culture (Carpten et al., 2007; Sakoda et al., 2003). Carpten and colleagues, as well as this study, were not able to observe transforming activity of overexpressed wildtype AKT1 when performing soft agar assays with retroviral transduced Rat1 cells. In contrast to that, AKT1-E17K gave rise to multiple colonies and therefore definitely had transforming abilities. This rules out the possibility of simple AKT1 overexpression leading to the described phenotype in mice and confirms the oncogenic role of the E17K mutation. Additionally, it was shown in this work that the transforming capacity of AKT1-E17K depends on its kinase activity, because a kinase dead version of AKT1-E17K (K179M) was no longer able to transform Rat1 cells and grow colonies in softagar assays.

## 4.3 Impact of AKT1-E17K on Antigen- and Coreceptor Mediated Signalling in Mature T-Cells

T-cells mediate many important functions in the immune system, but have to be controlled tightly to prevent them from harming the body they are supposed to defend. This is necessary because loss of control can cause grave diseases, such as leukemia, lymphoma or autoimmune disorders. Therefore, the activation, proliferation, function and even the development of T-cells is strictly dependent on antigen receptor signalling, triggered by an appropriate antigen or stimulus.

After antigen recognition, the expression of certain surface molecules is up- or downregulated and can be used to determine the activation status of T-cells. In AKT1-E17K/CD4Cre mice, the T-cells showed an expression pattern of surface markers which is typical for an activated status. Marked downregulation of CD62L and upregulation of CD69 as well as CD44 were found compared to control cells. Only CD25 expression was not affected. These results implicate that AKT1-E17K expression is capable of activating T-cells without TCR stimulation.

Also, the size of T-cells increases in response to activation, most likely due to the altered metabolic demands of the cells, which have to initiate proliferation and cytokine secretion. It was reported that CD28 costimulation of T-cells enhances cell size, expression of glucose

transporters, glucose uptake, and glycolysis. This enhancement was dependent on PI3K/AKT activation downstream of the CD28 coreceptor (Frauwirth et al., 2002). Additionally, in a myrAKT transgenic mouse model, naïve T-cells were found to be significantly enlarged and less dependent on CD28-mediated costimulation for cell growth, proliferation and cytokine production compared to control cells (Rathmell et al., 2003). Also the AKT1-E17K transgenic mouse model presented here, showed an increase in T-cell size, when the transgene was expressed. This indicates a positive effect of AKT1-E17K on T-cell growth and activation and a role downstream of the TCR/CD28 signalling pathway.

Further, an accumulation of transgenic T-cell in lymphoid as well as other organs could be observed in AKT1-E17K/CD4Cre mice. These T-cells were found to have a high proliferative index (up to 70%), determined by immunohistochemical staining for the marker Ki-67. In *ex vivo* experiments no marked proliferation of unstimulated, purified CD4+ T-cells of control and transgenic mice was detected. But stimulation with plate-bound anti-CD3 antibodies resulted in a higher proliferation rate compared to the controls. It is known that antigen receptor stimulation alone is not sufficient for an optimal T-cell response. Both, antigen- and coreceptor stimulation are needed to fully induce activation, proliferation and interleukin secretion (Shahinian et al., 1993). Indeed, additional costimulation with soluble anti-CD28 antibody increased the proliferation rate of control T-cells compared to stimulation with anti-CD3 alone. Interestingly, this was not the case for the transgenic T-cells. Additional anti-CD28 stimulation did not lead to further acceleration. AKT transgenic T-cells seem not to be dependent on an exogenous costimulatory signal to reach the full proliferative potential, indicating that AKT-E17K can provide the missing input.

Similar observations were also obtained for the myrAKT mouse model mentioned above (Rathmell et al., 2003). Transgenic T-cells from myrAKT mice were found to be independent of CD28 costimulation not only for achieving maximal increase in cell size, but also for cell division. Also the induction of the $T_H1$ cytokines IL-2 and IFNγ was shown to be elevated in myrAKT transgenic T-cells upon CD3 stimulation alone, again indicating partial independence from CD28 costimulation. There are several reports that suggest a participation of the PI3K/AKT pathway downstream of the CD28 coreceptor (Kane et al., 2001; Lafont et al., 2000; Parry et al., 2007). For instance, Kane et al. demonstrated that the AKT kinase can mediate some of the functional consequences of CD28 costimulation. Expression of myrAKT was able to rescue production of the cytokines IL-2 and IFNγ in experiments with CD28-deficient T-cells. But not all responses were reconstituted by AKT,

as proliferation and IL-4 and IL-5 ($T_H2$) were not affected in this study (Kane et al., 2001; Kane and Weiss, 2003).

In summary, AKT1-E17K expression in T-cells leads to enhanced cell growth, upregulation of activation markers, increased proliferation *in vivo* and *ex vivo*, and abolishes the need of CD28 costimulation to reach full proliferative capacity. Taken together with the results obtained from previous studies, the data suggests an important role for AKT in antigen- and coreceptor regulated processes, such as activation and proliferation, in T-cells.

### 4.4 Impact of AKT1-E17K on T-Cell Development

Antigen receptor signalling is also involved in the development of T-cells. During maturation, thymocytes have to undergo several selection processes before they are finally released into the periphery. The β-selection takes place at the DN3 stage of thymocyte development. There, functional rearrangement of the TCRβ locus has to be confirmed by successful signalling through the pre-TCR, which subsequently leads to proliferation and progression to later stages. If pre-TCR signals are absent, the thymocytes will undergo apoptosis. At the DP stage, the cells have to survive positive and negative selection to proceed to the CD4 or CD8 SP stage. Again, a positive signal from the now mature TCR is necessary to verify functionality and to induce another round of proliferation, whereas a missing signal leads to apoptosis via death-by-neglect. On the other hand, if the signal from the TCR is too strong, indicating reactivity of the TCR towards self-peptides, the T-cell is eliminated to prevent autoimmunity (Carpenter and Bosselut, 2010). Also the PI3K/AKT pathway was shown to play a role in T-cell development. Mice deficient for the PI3K subunits p110δ and p110γ had small thymi and reduced numbers of DP thymocytes (Webb et al., 2005; Swat et al., 2006). Treatment with PI3K inhibitors blocked the transition from DN to DP stage in FOTC (Fetal Thymic Organ Culture) (Mao et al., 2007) and a T-cell specific deletion of PTEN rescued thymocyte development in mice with defective IL-7 or pre-TCR signalling (Shiroki et al., 2007; Hagenbeek et al., 2004). Moreover, the deletion of AKT1 or AKT1/AKT2 resulted in defects at the DN3 to DN4 transition (Fayard et al., 2007; Juntilla et al., 2007) and AKT activation was shown to depend on pre-TCR and p110δ (Janas et al., 2010). So, the PI3K/AKT pathway is involved in the process of β-selection in the thymus. Unfortunately, there is not much known about its function beyond that point.

In the mouse model analyzed in this study, the expression of constitutively active AKT1-E17K starts with CD4 and subsequent Cre expression at the DP stage of T-cell development. The mice showed a strong decrease of total thymic cellularity, but the DN and CD4 SP cell number were found to be elevated and the CD8 SP cell number stayed unaffected. A direct effect of AKT1-E17K on the DN population is unlikely, since it is not even expressed at this timepoint. However, a significant portion of the DN cells from AKT/CD4Cre thymi showed expression of the transgenic Rosa26 locus, detected by eGFP fluorescence. Thus the DN cells must have expressed CD4 and Cre at a former timepoint. Additionally, stronger TCRß expression was observed, indicating a more mature T-cell population as normally present in this compartment, which somehow lost expression of the CD4 or CD8 surface markers. The reduction of total thymic cellularity in AKT/CD4Cre thymi was traced back to a massively decreased DP cell number, exactly at the stage where transgene expression starts. Of course, in diseased mice diverse processes could influence and alter thymic cellularity. But the reduction of DP cells was also observed in apparently not diseased, younger mice. An effect of a deregulated PI3K/AKT signalling on DN thymocytes can be excluded since AKT expression starts not until the DP stage. So AKT1-E17K possibly influences positive or negative selection at the DP stage. A reduced cell number could mean either the DP thymocytes become unreactive and die through death-by-neglect, or they react to strong and go into apoptosis trying to prevent autoimmunity. Considering, the positive role of AKT in T-cell activation, AKT1-E17K expression at the DP stage of thymocyte development could provide a strong stimulatory signal, mimicking high affinity binding of the TCR to self-peptides. This would lead to enforced negative selection, which is responsible for the decrease of DP cells. Indeed, TCR signal strength was shown to be one factor which determines the outcome of positive and negative selection of thymocytes, whereas the role of CD28 mediated costimulation in these processes is still controversial (Palmer, 2003).

Also a mouse model expressing myrAKT under the control of the human CD2 promotor showed reduced thymic cellularity and even a decrease in the DP cell number. Combined with the observation that a higher percentage of transgenic DP thymocytes had upregulated expression of TCR, CD5 and CD69, the study concluded that more transgenic DP cells receive signals that qualify them for further differentiation or induction of apoptosis (Na et al., 2003). Further, also Malstrom et al. observed a low cellularity of thymi derived from myrAKT transgenic mice, in combination with a larger size of the thymocytes. This group hypothesized that the size of the thymus is regulated by intrinsic signals and that the increase in cell size leads to a decrease in the total cell number in a way that the total thymic

cell mass remains constant (Malstrom et al., 2001). A similar increase in size was also observed for the thymocytes of AKT/CD4Cre mice.

In the end, it can be concluded that AKT1-E17K has an effect on T-cell development. But in this model one more question arises: Is there an additional selection pressure on transgenic T-cells depending on AKT signal strength? In theory, all transgenic T-cells which express AKT1-E17K should receive a strong stimulatory signal and subsequently die due to an increased negative selection process. But in AKT/CD4Cre mice mature T-cells can be found in the periphery in large numbers. These could be cells which were able to compensate or dampen the AKT mediated signal and thereby escaped the selection process, survived and migrated to the periphery. Further evaluation of this theory will have to take place in the future.

### 4.5 Influence of AKT1-E17K Expression on NFκB Activation

Activation of the transcription factor NFκB is one of the most important consequences of antigen receptor signalling. The AKT1-E17K mouse model was further used to evaluate the role of AKT signalling in NFκB activation, since a potential but controversial connection was proposed. Due to its implications in many critical cellular functions, such as proliferation or apoptosis, the NFκB pathway is also involved in oncogenesis. Aberrations and constitutive activity of the pathway are often associated with human malignancies (Dolcet et al., 2005). One interesting study even was able to differentiate two groups within the PTCL category, characterized by the expression levels of NFκB pathway genes (Martinez-Delgado et al., 2005). Therefore, the activation status of the NFκB pathway was analyzed on different levels in the AKT1-E17K transgenic mouse model.

In conclusion, no consistent activation of several NFκB pathway components could be observed in this study. Constitutive active AKT1 in T-cells did not lead to an enhanced formation of the CBM complex, which is necessary for classical NFκB activation. Neither stronger phosphorylation of IKKα, IKKβ or IκBα, nor stronger degradation of IκBα was detected in the majority of CD4+ T-cells compared to the controls. Nevertheless, enhanced NFκB nuclear translocation and binding activity were detected in T-cells of several, but not all, AKT1-E17K transgenic mice.

However, other studies demonstrated that an AKT inhibitor (AKTi 1/2) reduces CBM complex formation after stimulation of a T-cell line (Cheng et al., 2011). The inhibitor was

shown to have no effect on IKK phosphorylation, but slightly affecting IKK complex kinase activity and IKKγ ubiquitination. Moreover, inhibition of AKT led to a slight delay of IκBα degradation. A reason for the differing outcome could be an unknown off-target effect of AKTi 1/2, a problem which can never be excluded when working with inhibitors. Also the usage of a cell line in contrast to *ex vivo* CD4+ purified T-cells could have influenced the results. In fact, most of the studies, which report an impact of AKT on NFκB, used cell lines and therefore do not necessarily resemble the situation *in vivo*. For example, experiments from the group around Lawrence Kane showed that NFκB activation by AKT is dependent on CARMA1 (Narayan et al., 2006). But these and other findings were obtained from the Jurkat T-cell line, which is known to have a PTEN deletion and therefore already has an altered PI3K/AKT signalling. Another, often cited publication by Ozes et al. describes the dependency of TNF mediated induction of NFκB on AKT activation in HeLa and other cell lines (Ozes et al., 1999). But these observations could not be confirmed in the laboratory of Michael Karin (Delhase et al., 2000). In their hands TNFα did not induce AKT activation. The treatment of TNF-stimulated HeLa cells with a PI3K inhibitor had no influence on NFκB induction. The discrepancy here could be due to the varying properties of HeLa cells among different laboratories and underlines the disadvantages of cell line usage.

Earlier findings, using a myrAKT transgenic mouse model, implicated that activated AKT is not sufficient to induce NFκB on its own, but to enhance NFκB activity after stimulation of the TCR and coreceptor (Jones 2000; Jones 2005). These studies also showed that AKT-mediated survival of T-cells after cytokine withdrawal is independent of NFκB. Nevertheless, NFκB1 (p105/p50) was reported to be a critical molecule downstream of the PI3K/PTEN/AKT axis to block apoptosis triggered by the Fas death receptor.

Consistently, AKT1-E17K expression alone did not result in an effect on NFκB pathway components such as the CBM complex, IKKα/β and IκBα. Moreover, the fact that stimulation of transgenic T-cells with anti-CD3 antibody alone led to a strong proliferative response in contrast to control cells and that the proliferation could not further be increased by additional costimulation with CD28 coreceptor, also suggests a role for AKT in enhancing T-cell reactivity after stimulation.

In the literature also the assumption is present that AKT is not able to induce NFκB on its own, but plays a role in modulating NFκB driven expression by influencing the transactivation potential of p65 (Dan et al., 2008; Madrid et al., 2001).

It is known that several stimuli do not only induce phosphorylation of IκBα but also of NFκB proteins. Additionally several studies demonstrated that signal induced phosphorylation of p65 is critical for NFκB dependent transcription (Bonnard et al., 2000). For example, inducible phosphorylation of p65 increases the transcriptional activity of NFκB, but does not appear to affect its nuclear translocation or DNA binding activity (Wang and Baldwin, 1998).

Madrid et al. postulate an indirect role for AKT in increasing the transactivation potential of the NFκB subunit p65 mediated by phosphorylation of Ser529 and Ser536 dependent on IKKβ and p38. In line with this theory, increased phosphorylation of p65 at Ser529, a site suggested to be involved in this process, was found in this work in AKT transgenic T-cells. But phosphorylation levels at Ser536 appeared to be unchanged in comparison to control cells. Nevertheless, this could be an important effect of AKT on the transactivation potential of NFκB, modulating the transcription of target genes after stimulation.

Although constitutive active AKT1-E17K did not affect several NFκB pathway components examined in the AKT1-E17K mouse model an increased binding activity of the transcription factor itself was detected in part of the analyzed mice. This could be due to unidentified secondary mutational events occurring during lymphomagenesis. If such events affect a pathway involved in NFκB signalling, this could lead to constant NFκB activation, generating a survival advantage for the affected cell and promote further lymphomagenesis. An interesting aspect of the AKT transgenic mouse model favours this theory. Clonal expansion of transgenic T-cell was observed, although AKT1-E17K expression had been switched on in nearly all developing T-cells. This suggests a role for secondary events in this system. One could think of a scenario where constitutively active AKT initially promotes proliferation of the T-cells, which then acquire “second hits” over time. Thereby some of them gain an advantage over other T-cell clones and finally grow out. Clear monoclonality was not always reached in the presented mouse model, since perhaps more than one clone acquires additional mutations or, due to the rapid progression of the disease within the mice, there is not enough time left for the outgrowth of one single clone. It would be very interesting to investigate if some pathways are favoured in harbouring secondary mutational events in the AKT driven model. This could be useful for identification of additional therapeutic targets in cancer patients and for subsequent administration of a combination of different inhibitors to target the disease more efficiently.

### 4.6 Bcl10-Deficiency Is Not Able to Rescue the Disease but Prolongs Survival of Mice

The Bcl10 protein is a crucial component of the antigen receptor induced signal transduction pathway and acts as a positive regulator connecting antigen receptor signalling in lymphocytes to NFκB activation. Bcl10-deficient mice are severely immunodeficient and the lymphocytes are defective in antigen receptor induced NFκB activation, which results in a general defect in lymphocyte activation (Ruland et al., 2001).

Crossing the AKT-E17K/CD4Cre mice on a Bcl10-deficient background should therefore answer the question, if a functional NFκB cascade is necessary for the development of an AKT driven lymphoma. An earlier study observed that aging myrAKT transgenic mice are prone to develop lymphoid hyperplasia, but this process was blocked by genetic inhibition of NFκB nuclear translocation (Jones et al., 2005). However, AKT1-E17K transgenic mice deficient for Bcl10 developed a disease very similar to those on a wildtype background. Clonal expansion of T-cells with an activated phenotype, infiltration into various organs, enhanced proliferation and stronger phosphorylation of direct downstream targets of AKT were observed. The AKT/CD4Cre/Bcl10-/- mice also seem to suffer from a PTCL. Therefore, the development of an AKT driven lymphoma is not dependent on the presence of the Bcl10 protein and its function in NFκB activation. A reason for this discrepancy could lie in the use of different mouse strains, which abrogate NFκB activation at different levels. Expression of a constitutive repressor of NFκB (IκBΔN) could have other effects than the removal of Bcl10. Furthermore, the distinct ways of AKT activation could contribute to the difference between both studies.

Additionally, some differences to the wildtype background were noticed when analyzing AKT-E17K transgenic mice deficient for Bcl10. For instance, an increased incidence of mice developing a thymic lymphoma was observed. Maybe, the lack of Bcl10 protein protects the thymocytes from cell death due to overstimulation, as discussed for the normal AKT transgenic cells. Less thymocytes dying increase the possibility one cell harbouring a crucial second hit already during the proliferative stage in the thymus. Therefore at this timepoint transformation takes place and cells grow and stay predominantly in the thymus.

Comparing the median survival of AKT/CD4Cre mice with and without Bcl10, a survival benefit of 22 days was noticed, despite the kinase was not found to directly influence several investigated NFκB pathway components. A possible explanation for the prolonged survival could be the missing secondary B-cell activation observed in these mice. In normal AKT transgenic mice the B-cell number is comparable to controls but the B-cell population

exhibited an increase in size and an expression pattern of surface markers, indicating an activated state. Since only T-cells express AKT1-E17K in this system, it has to be a secondary effect influencing the B-cell population. This could influence progression of the lymphoma. On a Bcl10-deficient background the secondary effect is missing, suggesting that B-cells or other cell types are not able to react to or do not receive signals and therefore lymphoma progression is slowed down.

Another explanation for the prolonged survival could be the Bcl10-deficient background itself. A reduction of DP cells in thymi of Bcl10 k.o. mice was described earlier (Ruland et al., 2001). Fewer cells enter the DP stage of thymocyte development, where in our model AKT1-E17K expression is turned on and the cell number is reduced again. So, in AKT transgenic Bcl10 k.o. mice, two effects are leading to a reduction of thymocytes, whereas in normal transgenic mice only one takes place. As a consequence, less malignant cells reach the periphery which could slow down lymphoma progression.

## 4.7 Conclusion and Outlook

In this study a new transgenic mouse model conditionally expressing constitutively active AKT1-E17K was successfully generated. T-cell specific expression of the mutant kinase led to a severe lymphoproliferative disorder that was characterized as a PTCL. AKT1-E17K influenced not only its direct downstream targets, but also other crucial signalling proteins such as Erk and Stat3, components of pathways known to be implicated in oncogenesis. Even though a direct effect of the AKT1-E17K kinase on several NFκB pathway components was not detected some of the mice analyzed showed enhanced NFκB activation levels and an increase in p65 transactivation potential. Further analysis showed that loss of Bcl10, a crucial adaptor protein within the classical NFκB pathway, did not prevent lymphoma development, but altered some characteristics of the disease.

These results highlight the oncogenic potential of the AKT1-E17K mutation in T-cells and help to understand the role of constitutive AKT activation in T-cell lymphomagenesis. The mouse model presented here has profound clinical relevance, since useful *in vivo* models for PTCL are rare. It provides a valuable tool to identify new targets and and to test and evaluate new therapeutic strategies. For example, the PI3Kδ inhibitor CAL-101, which shows promising results in clinical trials, could be less efficient in patients harbouring mutations uncoupling AKT from upstream events, such as the E17K mutation. Identification

of particular mutations in the PI3K/AKT pathway combined with the development of inhibitors, which are tailored to the specific situation, will increase therapeutic success and minimize possible side effects.

Additionally, in this model AKT1-E17K expression is not only restricted to one specific cell type and therefore can be also used to investigate other AKT driven diseases and constitutive AKT signalling in general. Considering the high frequency of mutations in the PI3K/AKT pathway in human malignancies, many patients could benefit of ongoing progress made in this field.

# 5 Citation index

**Abubaker, J., Bavi, P. P., Al-Harbi, S., Siraj, A. K., Al-Dayel, F., Uddin, S. & Al-Kuraya, K.** (2007). "PIK3CA mutations are mutually exclusive with PTEN loss in diffuse large B-cell lymphoma". *Leukemia,* 21**,** 2368-70.

**Altomare, D. A. & Testa, J. R.** (2005). "Perturbations of the AKT signaling pathway in human cancer". *Oncogene,* 24**,** 7455-64.

**Arranz, E., Robledo, M., Martinez, B., Gallego, J., Roman, A., Rivas, C. & Benitez, J.** (1996). "Incidence of homogeneously staining regions in non-Hodgkin lymphomas". *Cancer Genet Cytogenet,* 87**,** 1-3.

**Askham, J. M., Platt, F., Chambers, P. A., Snowden, H., Taylor, C. F. & Knowles, M. A.** (2010). "AKT1 mutations in bladder cancer: identification of a novel oncogenic mutation that can co-operate with E17K". *Oncogene,* 29**,** 150-5.

**Backer, J. M.** (2008). "The regulation and function of Class III PI3Ks: novel roles for Vps34". *Biochem J,* 410**,** 1-17.

**Bai, D., Ueno, L. & Vogt, P. K.** (2009). "Akt-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of PI3K and Akt". *Int J Cancer,* 125**,** 2863-70.

**Baohua, Y., Xiaoyan, Z., Tiecheng, Z., Tao, Q. & Daren, S.** (2008). "Mutations of the PIK3CA gene in diffuse large B cell lymphoma". *Diagn Mol Pathol,* 17**,** 159-65.

**Bellacosa, A., Testa, J. R., Staal, S. P. & Tsichlis, P. N.** (1991). "A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region". *Science,* 254**,** 274-7.

**Bleeker, F. E., Felicioni, L., Buttitta, F., Lamba, S., Cardone, L., Rodolfo, M., Scarpa, A., Leenstra, S., Frattini, M., Barbareschi, M., Grammastro, M. D., Sciarrotta, M. G., Zanon, C., Marchetti, A. & Bardelli, A.** (2008). "AKT1(E17K) in human solid tumours". *Oncogene,* 27**,** 5648-50.

**Bleeker, F. E., Lamba, S., Zanon, C., Van Tilborg, A. A., Leenstra, S., Troost, D., Hulsebos, T., Vandertop, W. P. & Bardelli, A.** (2009). "Absence of AKT1 mutations in glioblastoma". *PLoS One,* 4**,** e5638.

**Bonnard, M., Mirtsos, C., Suzuki, S., Graham, K., Huang, J., Ng, M., Itie, A., Wakeham, A., Shahinian, A., Henzel, W. J., Elia, A. J., Shillinglaw, W., Mak, T. W., Cao, Z. & Yeh, W. C.** (2000). "Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription". *EMBO J,* 19**,** 4976-85.

**Borlado, L. R., Redondo, C., Alvarez, B., Jimenez, C., Criado, L. M., Flores, J., Marcos, M. A., Martinez, A. C., Balomenos, D. & Carrera, A. C.** (2000). "Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo". *FASEB J,* 14**,** 895-903.

**Bowman, T., Garcia, R., Turkson, J. & Jove, R.** (2000). "STATs in oncogenesis". *Oncogene,* 19**,** 2474-88.

**Butler, M. P., Wang, S. I., Chaganti, R. S., Parsons, R. & Dalla-Favera, R.** (1999). "Analysis of PTEN mutations and deletions in B-cell non-Hodgkin's lymphomas". *Genes Chromosomes Cancer,* 24**,** 322-7.

**Cai, Q., Deng, H., Xie, D., Lin, T. & Lin, T.** (2012). "Phosphorylated AKT protein is overexpressed in human peripheral T-cell lymphomas and predicts decreased patient survival". *Clin Lymphoma Myeloma Leuk,* 12**,** 106-12.

**Cao, Z., Song, J. H., Kim, C. J., Cho, Y. G., Kim, S. Y., Nam, S. W., Lee, J. Y. & Park, W. S.** (2008). "Absence of E17K mutation in the pleckstrin homology domain of AKT1 in gastrointestinal and liver cancers in the Korean population". *Apmis,* 116**,** 530-3.

**Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S. & Reed, J. C.** (1998). "Regulation of cell death protease caspase-9 by phosphorylation". *Science,* 282**,** 1318-21.

**Carnero, A.** (2010). "The PKB/AKT pathway in cancer". *Curr Pharm Des,* 16**,** 34-44.

**Carpenter, A. C. & Bosselut, R.** (2010). "Decision checkpoints in the thymus". *Nat Immunol,* 11**,** 666-73.

**Carpten, J. D., Faber, A. L., Horn, C., Donoho, G. P., Briggs, S. L., Robbins, C. M., Hostetter, G., Boguslawski, S., Moses, T. Y., Savage, S., Uhlik, M., Lin, A., Du, J., Qian, Y. W., Zeckner, D. J., Tucker-Kellogg, G., Touchman, J., Patel, K., Mousses, S., Bittner, M., Schevitz, R., Lai, M. H., Blanchard, K. L. & Thomas, J. E.** (2007). "A transforming mutation in the pleckstrin homology domain of AKT1 in cancer". *Nature,* 448**,** 439-44.

**Chalhoub, N. & Baker, S. J.** (2009). "PTEN and the PI3-kinase pathway in cancer". *Annu Rev Pathol,* 4**,** 127-50.

**Chan, T. O., Rittenhouse, S. E. & Tsichlis, P. N.** (1999). "AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation". *Annu Rev Biochem,* 68**,** 965-1014.

**Chang, F., Lee, J. T., Navolanic, P. M., Steelman, L. S., Shelton, J. G., Blalock, W. L., Franklin, R. A. & Mccubrey, J. A.** (2003). "Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy". *Leukemia,* 17**,** 590-603.

**Chantry, D., Vojtek, A., Kashishian, A., Holtzman, D. A., Wood, C., Gray, P. W., Cooper, J. A. & Hoekstra, M. F.** (1997). "p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes". *J Biol Chem,* 272**,** 19236-41.

**Cheng, J., Phong, B., Wilson, D. C., Hirsch, R. & Kane, L. P.** (2011). "Akt fine-tunes NF-kappaB-dependent gene expression during T cell activation". *J Biol Chem,* 286**,** 36076-85.

**Coffer, P. J. & Woodgett, J. R.** (1991). "Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families". *Eur J Biochem,* 201**,** 475-81.

**Cohen, Y., Shalmon, B., Korach, J., Barshack, I., Fridman, E. & Rechavi, G.** (2009). "AKT1 pleckstrin homology domain E17K activating mutation in endometrial carcinoma". *Gynecol Oncol,* 116**,** 88-91.

**Cotta, C. V. & Hsi, E. D.** (2008). "Pathobiology of mature T-cell lymphomas". *Clin Lymphoma Myeloma,* 8 Suppl 5**,** S168-79.

**Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. & Hemmings, B. A.** (1995). "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B". *Nature,* 378**,** 785-9.

**Dan, H. C., Cooper, M. J., Cogswell, P. C., Duncan, J. A., Ting, J. P. & Baldwin, A. S.** (2008). "Akt-dependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK". *Genes Dev,* 22**,** 1490-500.

**Dan, H. C., Sun, M., Kaneko, S., Feldman, R. I., Nicosia, S. V., Wang, H. G., Tsang, B. K. & Cheng, J. Q.** (2004). "Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP)". *J Biol Chem,* 279**,** 5405-12.

**Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M. E.** (1997). "Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery". *Cell,* 91**,** 231-41.

**Davies, M. A., Stemke-Hale, K., Tellez, C., Calderone, T. L., Deng, W., Prieto, V. G., Lazar, A. J., Gershenwald, J. E. & Mills, G. B.** (2008). "A novel AKT3 mutation in melanoma tumours and cell lines". *Br J Cancer,* 99**,** 1265-8.

**Dbouk, H. A., Pang, H., Fiser, A. & Backer, J. M.** (2010). "A biochemical mechanism for the oncogenic potential of the p110beta catalytic subunit of phosphoinositide 3-kinase". *Proc Natl Acad Sci U S A,* 107**,** 19897-902.

**De Keersmaecker, K., Marynen, P. & Cools, J.** (2005). "Genetic insights in the pathogenesis of T-cell acute lymphoblastic leukemia". *Haematologica,* 90**,** 1116-27.

**De Leval, L. & Gaulard, P.** (2008). "Pathobiology and molecular profiling of peripheral T-cell lymphomas". *Hematology Am Soc Hematol Educ Program***,** 272-9.

**De Leval, L. & Gaulard, P.** (2011). "Pathology and biology of peripheral T-cell lymphomas". *Histopathology,* 58**,** 49-68.

**Delhase, M., Li, N. & Karin, M.** (2000). "Kinase regulation in inflammatory response". *Nature,* 406**,** 367-8.

**Dolcet, X., Llobet, D., Pallares, J. & Matias-Guiu, X.** (2005). "NF-kB in development and progression of human cancer". *Virchows Arch,* 446**,** 475-82.

**Downward, J.** (2004). "PI 3-kinase, Akt and cell survival". *Semin Cell Dev Biol,* 15**,** 177-82.

**Drexler, H. G., Gignac, S. M., Von Wasielewski, R., Werner, M. & Dirks, W. G.** (2000). "Pathobiology of NPM-ALK and variant fusion genes in anaplastic large cell lymphoma and other lymphomas". *Leukemia,* 14**,** 1533-59.

**Du, K. & Tsichlis, P. N.** (2005). "Regulation of the Akt kinase by interacting proteins". *Oncogene,* 24**,** 7401-9.

**Durand, C. A., Hartvigsen, K., Fogelstrand, L., Kim, S., Iritani, S., Vanhaesebroeck, B., Witztum, J. L., Puri, K. D. & Gold, M. R.** (2009). "Phosphoinositide 3-kinase p110 delta regulates natural antibody production, marginal zone and B-1 B cell function, and autoantibody responses". *J Immunol,* 183**,** 5673-84.

**Engelman, J. A., Luo, J. & Cantley, L. C.** (2006). "The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism". *Nat Rev Genet,* 7**,** 606-19.

**Eom, H. S., Kim, M. S., Hur, S. Y., Yoo, N. J. & Lee, S. H.** (2009). "Absence of oncogenic AKT1 E17K mutation in prostate, esophageal, laryngeal and urothelial carcinomas, hepatoblastomas, gastrointestinal stromal tumors and malignant meningiomas". *Acta Oncol,* 48**,** 1084-5.

**Fabre, S., Lang, V., Harriague, J., Jobart, A., Unterman, T. G., Trautmann, A. & Bismuth, G.** (2005). "Stable activation of phosphatidylinositol 3-kinase in the T cell immunological synapse stimulates Akt signaling to FoxO1 nuclear exclusion and cell growth control". *J Immunol,* 174**,** 4161-71.

**Falasca, M. & Maffucci, T.** (2012). "Regulation and cellular functions of class II phosphoinositide 3-kinases". *Biochem J,* 443**,** 587-601.

**Fayard, E., Gill, J., Paolino, M., Hynx, D., Hollander, G. A. & Hemmings, B. A.** (2007). "Deletion of PKBalpha/Akt1 affects thymic development". *PLoS One,* 2**,** e992.

**Feldman, A. L., Sun, D. X., Law, M. E., Novak, A. J., Attygalle, A. D., Thorland, E. C., Fink, S. R., Vrana, J. A., Caron, B. L., Morice, W. G., Remstein, E. D., Grogg, K. L., Kurtin, P. J., Macon, W. R. & Dogan, A.** (2008). "Overexpression of Syk tyrosine kinase in peripheral T-cell lymphomas". *Leukemia,* 22**,** 1139-43.

**Fillmore, G. C., Wang, Q., Carey, M. J., Kim, C. H., Elenitoba-Johnson, K. S. & Lim, M. S.** (2005). "Expression of Akt (protein kinase B) and its isoforms in malignant lymphomas". *Leuk Lymphoma,* 46**,** 1765-73.

**Finlay, D. & Cantrell, D. A.** (2011). "Metabolism, migration and memory in cytotoxic T cells". *Nat Rev Immunol,* 11**,** 109-17.

**Frauwirth, K. A., Riley, J. L., Harris, M. H., Parry, R. V., Rathmell, J. C., Plas, D. R., Elstrom, R. L., June, C. H. & Thompson, C. B.** (2002). "The CD28 signaling pathway regulates glucose metabolism". *Immunity,* 16**,** 769-77.

**Friedrich, G. & Soriano, P.** (1991). "Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice". *Genes Dev,* 5**,** 1513-23.

**Fruman, D. A. & Bismuth, G.** (2009). "Fine tuning the immune response with PI3K". *Immunol Rev,* 228**,** 253-72.

**Fukuda, R., Hayashi, A., Utsunomiya, A., Nukada, Y., Fukui, R., Itoh, K., Tezuka, K., Ohashi, K., Mizuno, K., Sakamoto, M., Hamanoue, M. & Tsuji, T.** (2005). "Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL)". *Proc Natl Acad Sci U S A,* 102**,** 15213-8.

**Gardai, S. J., Hildeman, D. A., Frankel, S. K., Whitlock, B. B., Frasch, S. C., Borregaard, N., Marrack, P., Bratton, D. L. & Henson, P. M.** (2004). "Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils". *J Biol Chem,* 279**,** 21085-95.

**Greer, E. L. & Brunet, A.** (2005). "FOXO transcription factors at the interface between longevity and tumor suppression". *Oncogene,* 24**,** 7410-25.

**Gronbaek, K., Zeuthen, J., Guldberg, P., Ralfkiaer, E. & Hou-Jensen, K.** (1998). "Alterations of the MMAC1/PTEN gene in lymphoid malignancies". *Blood,* 91**,** 4388-90.

**Gustin, J. A., Korgaonkar, C. K., Pincheira, R., Li, Q. & Donner, D. B.** (2006). "Akt regulates basal and induced processing of NF-kappaB2 (p100) to p52". *J Biol Chem,* 281**,** 16473-81.

**Gustin, J. A., Ozes, O. N., Akca, H., Pincheira, R., Mayo, L. D., Li, Q., Guzman, J. R., Korgaonkar, C. K. & Donner, D. B.** (2004). "Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation". *J Biol Chem,* 279**,** 1615-20.

**Gutierrez, A., Sanda, T., Grebliunaite, R., Carracedo, A., Salmena, L., Ahn, Y., Dahlberg, S., Neuberg, D., Moreau, L. A., Winter, S. S., Larson, R., Zhang, J., Protopopov, A., Chin, L., Pandolfi, P. P., Silverman, L. B., Hunger, S. P., Sallan, S. E. & Look, A. T.** (2009). "High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia". *Blood,* 114**,** 647-50.

**Hagenbeek, T. J., Naspetti, M., Malergue, F., Garcon, F., Nunes, J. A., Cleutjens, K. B., Trapman, J., Krimpenfort, P. & Spits, H.** (2004). "The loss of PTEN allows TCR alphabeta lineage thymocytes to bypass IL-7 and Pre-TCR-mediated signaling". *J Exp Med,* 200**,** 883-94.

**Hanahan, D. & Weinberg, R. A.** (2011). "Hallmarks of cancer: the next generation". *Cell,* 144**,** 646-74.

**Harriague, J. & Bismuth, G.** (2002). "Imaging antigen-induced PI3K activation in T cells". *Nat Immunol,* 3**,** 1090-6.

**Harris, N. L., Jaffe, E. S., Stein, H., Banks, P. M., Chan, J. K., Cleary, M. L., Delsol, G., De Wolf-Peeters, C., Falini, B., Gatter, K. C. & Et Al.** (1994). "A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group". *Blood,* 84**,** 1361-92.

**Hayden, M. S. & Ghosh, S.** (2008). "Shared principles in NF-kappaB signaling". *Cell,* 132**,** 344-62.

**Ismail, S. I., Mahmoud, I. S., Msallam, M. M. & Sughayer, M. A.** (2010). "Hotspot mutations of PIK3CA and AKT1 genes are absent in multiple myeloma". *Leuk Res,* 34**,** 824-6.

**Jaffe, E. S.** (2001). "Anaplastic large cell lymphoma: the shifting sands of diagnostic hematopathology". *Mod Pathol,* 14**,** 219-28.

**Janas, M. L., Varano, G., Gudmundsson, K., Noda, M., Nagasawa, T. & Turner, M.** (2010). "Thymic development beyond beta-selection requires phosphatidylinositol 3-kinase activation by CXCR4". *J Exp Med,* 207**,** 247-61.

**Jiang, B. H. & Liu, L. Z.** (2008). "AKT signaling in regulating angiogenesis". *Curr Cancer Drug Targets,* 8**,** 19-26.

**Jones, P. F., Jakubowicz, T., Pitossi, F. J., Maurer, F. & Hemmings, B. A.** (1991). "Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily". *Proc Natl Acad Sci U S A,* 88**,** 4171-5.

**Jones, R. G., Elford, A. R., Parsons, M. J., Wu, L., Krawczyk, C. M., Yeh, W. C., Hakem, R., Rottapel, R., Woodgett, J. R. & Ohashi, P. S.** (2002). "CD28-dependent activation of protein kinase B/Akt blocks Fas-mediated apoptosis by preventing death-inducing signaling complex assembly". *J Exp Med,* 196**,** 335-48.

**Jones, R. G., Parsons, M., Bonnard, M., Chan, V. S., Yeh, W. C., Woodgett, J. R. & Ohashi, P. S.** (2000). "Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo". *J Exp Med,* 191**,** 1721-34.

**Jones, R. G., Saibil, S. D., Pun, J. M., Elford, A. R., Bonnard, M., Pellegrini, M., Arya, S., Parsons, M. E., Krawczyk, C. M., Gerondakis, S., Yeh, W. C., Woodgett, J. R., Boothby, M. R. & Ohashi, P. S.** (2005). "NF-kappaB couples protein kinase B/Akt signaling to distinct survival pathways and the regulation of lymphocyte homeostasis in vivo". *J Immunol,* 175**,** 3790-9.

**Jucker, M., Sudel, K., Horn, S., Sickel, M., Wegner, W., Fiedler, W. & Feldman, R. A.** (2002). "Expression of a mutated form of the p85alpha regulatory subunit of phosphatidylinositol 3-kinase in a Hodgkin's lymphoma-derived cell line (CO)". *Leukemia,* 16**,** 894-901.

**Juntilla, M. M., Wofford, J. A., Birnbaum, M. J., Rathmell, J. C. & Koretzky, G. A.** (2007). "Akt1 and Akt2 are required for alphabeta thymocyte survival and differentiation". *Proc Natl Acad Sci U S A,* 104**,** 12105-10.

**Kane, L. P., Andres, P. G., Howland, K. C., Abbas, A. K. & Weiss, A.** (2001). "Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-gamma but not TH2 cytokines". *Nat Immunol,* 2**,** 37-44.

**Kane, L. P., Shapiro, V. S., Stokoe, D. & Weiss, A.** (1999). "Induction of NF-kappaB by the Akt/PKB kinase". *Curr Biol,* 9**,** 601-4.

**Kane, L. P. & Weiss, A.** (2003). "The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3". *Immunol Rev,* 192**,** 7-20.

**Kim, M. S., Jeong, E. G., Yoo, N. J. & Lee, S. H.** (2008). "Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias". *Br J Cancer,* 98**,** 1533-5.

**Kingeter, L. M. & Schaefer, B. C.** (2008). "Loss of protein kinase C theta, Bcl10, or Malt1 selectively impairs proliferation and NF-kappa B activation in the CD4+ T cell subset". *J Immunol,* 181**,** 6244-54.

**Kneba, M., Bolz, I., Linke, B. & Hiddemann, W.** (1995). "Analysis of rearranged T-cell receptor beta-chain genes by polymerase chain reaction (PCR) DNA sequencing and automated high resolution PCR fragment analysis". *Blood,* 86**,** 3930-7.

**Koyasu, S.** (2003). "The role of PI3K in immune cells". *Nat Immunol,* 4**,** 313-9.

**Lafont, V., Astoul, E., Laurence, A., Liautard, J. & Cantrell, D.** (2000). "The T cell antigen receptor activates phosphatidylinositol 3-kinase-regulated serine kinases protein kinase B and ribosomal S6 kinase 1". *FEBS Lett,* 486**,** 38-42.

**Landgraf, K. E., Pilling, C. & Falke, J. J.** (2008). "Molecular mechanism of an oncogenic mutation that alters membrane targeting: Glu17Lys modifies the PIP lipid specificity of the AKT1 PH domain". *Biochemistry,* 47**,** 12260-9.

**Laplante, M. & Sabatini, D. M.** (2012). "mTOR signaling in growth control and disease". *Cell,* 149**,** 274-93.

**Lee, P. P., Fitzpatrick, D. R., Beard, C., Jessup, H. K., Lehar, S., Makar, K. W., Perez-Melgosa, M., Sweetser, M. T., Schlissel, M. S., Nguyen, S., Cherry, S. R., Tsai, J. H., Tucker, S. M., Weaver, W. M., Kelso, A., Jaenisch, R. & Wilson, C. B.** (2001). "A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival". *Immunity,* 15**,** 763-74.

**Lemmon, M. A. & Ferguson, K. M.** (2000). "Signal-dependent membrane targeting by pleckstrin homology (PH) domains". *Biochem J,* 350 Pt 1**,** 1-18.

**Liang, J. & Slingerland, J. M.** (2003). "Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression". *Cell Cycle,* 2**,** 339-45.

**Liao, Y. & Hung, M. C.** (2010). "Physiological regulation of Akt activity and stability". *Am J Transl Res,* 2**,** 19-42.

**Lindhurst, M. J., Sapp, J. C., Teer, J. K., Johnston, J. J., Finn, E. M., Peters, K., Turner, J., Cannons, J. L., Bick, D., Blakemore, L., Blumhorst, C., Brockmann, K., Calder, P., Cherman, N., Deardorff, M. A., Everman, D. B., Golas, G., Greenstein, R. M., Kato, B. M., Keppler-Noreuil, K. M., Kuznetsov, S. A., Miyamoto, R. T., Newman, K., Ng, D., O'brien, K., Rothenberg, S., Schwartzentruber, D. J., Singhal, V., Tirabosco, R., Upton, J., Wientroub, S., Zackai, E. H., Hoag, K., Whitewood-Neal, T., Robey, P. G., Schwartzberg, P. L., Darling, T. N., Tosi, L. L., Mullikin, J. C. & Biesecker, L. G.** (2011). "A mosaic activating mutation in AKT1 associated with the Proteus syndrome". *N Engl J Med,* 365**,** 611-9.

**Macintyre, A. N., Finlay, D., Preston, G., Sinclair, L. V., Waugh, C. M., Tamas, P., Feijoo, C., Okkenhaug, K. & Cantrell, D. A.** (2011). "Protein kinase B controls transcriptional programs that direct cytotoxic T cell fate but is dispensable for T cell metabolism". *Immunity,* 34**,** 224-36.

**Madge, L. A. & Pober, J. S.** (2000). "A phosphatidylinositol 3-kinase/Akt pathway, activated by tumor necrosis factor or interleukin-1, inhibits apoptosis but does not activate NFkappaB in human endothelial cells". *J Biol Chem,* 275**,** 15458-65.

**Madrid, L. V., Mayo, M. W., Reuther, J. Y. & Baldwin, A. S., Jr.** (2001). "Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38". *J Biol Chem,* 276**,** 18934-40.

**Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S., Jr. & Mayo, M. W.** (2000). "Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB". *Mol Cell Biol,* 20**,** 1626-38.

**Mahmoud, I. S., Sughayer, M. A., Mohammad, H. A., Awidi, A. S., Ms, E. L.-K. & Ismail, S. I.** (2008). "The transforming mutation E17K/AKT1 is not a major event in B-cell-derived lymphoid leukaemias". *Br J Cancer,* 99**,** 488-90.

**Malanga, D., Scrima, M., De Marco, C., Fabiani, F., De Rosa, N., De Gisi, S., Malara, N., Savino, R., Rocco, G., Chiappetta, G., Franco, R., Tirino, V., Pirozzi, G. & Viglietto, G.** (2008). "Activating E17K mutation in the gene encoding the protein

kinase AKT1 in a subset of squamous cell carcinoma of the lung". *Cell Cycle,* 7**,** 665-9.

**Malstrom, S., Tili, E., Kappes, D., Ceci, J. D. & Tsichlis, P. N.** (2001). "Tumor induction by an Lck-MyrAkt transgene is delayed by mechanisms controlling the size of the thymus". *Proc Natl Acad Sci U S A,* 98**,** 14967-72.

**Manning, B. D. & Cantley, L. C.** (2007). "AKT/PKB signaling: navigating downstream". *Cell,* 129**,** 1261-74.

**Mao, C., Tili, E. G., Dose, M., Haks, M. C., Bear, S. E., Maroulakou, I., Horie, K., Gaitanaris, G. A., Fidanza, V., Ludwig, T., Wiest, D. L., Gounari, F. & Tsichlis, P. N.** (2007). "Unequal contribution of Akt isoforms in the double-negative to double-positive thymocyte transition". *J Immunol,* 178**,** 5443-53.

**Martinez-Delgado, B., Cuadros, M., Honrado, E., Ruiz De La Parte, A., Roncador, G., Alves, J., Castrillo, J. M., Rivas, C. & Benitez, J.** (2005). "Differential expression of NF-kappaB pathway genes among peripheral T-cell lymphomas". *Leukemia,* 19**,** 2254-63.

**Maurer, U., Charvet, C., Wagman, A. S., Dejardin, E. & Green, D. R.** (2006). "Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1". *Mol Cell,* 21**,** 749-60.

**Mohamedali, A., Lea, N. C., Feakins, R. M., Raj, K., Mufti, G. J. & Kocher, H. M.** (2008). "AKT1 (E17K) mutation in pancreatic cancer". *Technol Cancer Res Treat,* 7**,** 407-8.

**Na, S. Y., Patra, A., Scheuring, Y., Marx, A., Tolaini, M., Kioussis, D., Hemmings, B. A., Hunig, T. & Bommhardt, U.** (2003). "Constitutively active protein kinase B enhances Lck and Erk activities and influences thymocyte selection and activation". *J Immunol,* 171**,** 1285-96.

**Narayan, P., Holt, B., Tosti, R. & Kane, L. P.** (2006). "CARMA1 is required for Akt-mediated NF-kappaB activation in T cells". *Mol Cell Biol,* 26**,** 2327-36.

**Okkenhaug, K., Patton, D. T., Bilancio, A., Garcon, F., Rowan, W. C. & Vanhaesebroeck, B.** (2006). "The p110delta isoform of phosphoinositide 3-kinase controls clonal expansion and differentiation of Th cells". *J Immunol,* 177**,** 5122-8.

**Osaki, M., Oshimura, M. & Ito, H.** (2004). "PI3K-Akt pathway: its functions and alterations in human cancer". *Apoptosis,* 9**,** 667-76.

**Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M. & Donner, D. B.** (1999). "NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase". *Nature,* 401**,** 82-5.

**Palmer, E.** (2003). "Negative selection--clearing out the bad apples from the T-cell repertoire". *Nat Rev Immunol,* 3**,** 383-91.

**Parry, R. V., Riley, J. L. & Ward, S. G.** (2007). "Signalling to suit function: tailoring phosphoinositide 3-kinase during T-cell activation". *Trends Immunol,* 28**,** 161-8.

**Patra, A. K., Na, S. Y. & Bommhardt, U.** (2004). "Active protein kinase B regulates TCR responsiveness by modulating cytoplasmic-nuclear localization of NFAT and NF-kappa B proteins". *J Immunol,* 172**,** 4812-20.

**Pechloff, K., Holch, J., Ferch, U., Schweneker, M., Brunner, K., Kremer, M., Sparwasser, T., Quintanilla-Martinez, L., Zimber-Strobl, U., Streubel, B., Gewies, A., Peschel, C. & Ruland, J.** (2010). "The fusion kinase ITK-SYK mimics a T cell receptor signal and drives oncogenesis in conditional mouse models of peripheral T cell lymphoma". *J Exp Med,* 207**,** 1031-44.

**Piccaluga, P. P., Agostinelli, C., Califano, A., Rossi, M., Basso, K., Zupo, S., Went, P., Klein, U., Zinzani, P. L., Baccarani, M., Dalla Favera, R. & Pileri, S. A.** (2007).

"Gene expression analysis of peripheral T cell lymphoma, unspecified, reveals distinct profiles and new potential therapeutic targets". *J Clin Invest,* 117**,** 823-34.

**Pierau, M., Engelmann, S., Reinhold, D., Lapp, T., Schraven, B. & Bommhardt, U. H.** (2009). "Protein kinase B/Akt signals impair Th17 differentiation and support natural regulatory T cell function and induced regulatory T cell formation". *J Immunol,* 183**,** 6124-34.

**Ramadani, F., Bolland, D. J., Garcon, F., Emery, J. L., Vanhaesebroeck, B., Corcoran, A. E. & Okkenhaug, K.** (2010). "The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development". *Sci Signal,* 3**,** ra60.

**Rathmell, J. C., Elstrom, R. L., Cinalli, R. M. & Thompson, C. B.** (2003). "Activated Akt promotes increased resting T cell size, CD28-independent T cell growth, and development of autoimmunity and lymphoma". *Eur J Immunol,* 33**,** 2223-32.

**Rauch, B. H., Weber, A., Braun, M., Zimmermann, N. & Schror, K.** (2000). "PDGF-induced Akt phosphorylation does not activate NF-kappa B in human vascular smooth muscle cells and fibroblasts". *FEBS Lett,* 481**,** 3-7.

**Reich, N. C.** (2009). "STAT3 revs up the powerhouse". *Sci Signal,* 2**,** pe61.

**Riener, M. O., Bawohl, M., Clavien, P. A. & Jochum, W.** (2008). "Analysis of oncogenic AKT1 p.E17K mutation in carcinomas of the biliary tract and liver". *Br J Cancer,* 99**,** 836.

**Rodriguez, J., Gutierrez, A., Martinez-Delgado, B. & Perez-Manga, G.** (2009). "Current and future aggressive peripheral T-cell lymphoma treatment paradigms, biological features and therapeutic molecular targets". *Crit Rev Oncol Hematol,* 71**,** 181-98.

**Romashkova, J. A. & Makarov, S. S.** (1999). "NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling". *Nature,* 401**,** 86-90.

**Rossig, L., Badorff, C., Holzmann, Y., Zeiher, A. M. & Dimmeler, S.** (2002). "Glycogen synthase kinase-3 couples AKT-dependent signaling to the regulation of p21Cip1 degradation". *J Biol Chem,* 277**,** 9684-9.

**Rudd, C. E., Taylor, A. & Schneider, H.** (2009). "CD28 and CTLA-4 coreceptor expression and signal transduction". *Immunol Rev,* 229**,** 12-26.

**Rudelius, M., Pittaluga, S., Nishizuka, S., Pham, T. H., Fend, F., Jaffe, E. S., Quintanilla-Martinez, L. & Raffeld, M.** (2006). "Constitutive activation of Akt contributes to the pathogenesis and survival of mantle cell lymphoma". *Blood,* 108**,** 1668-76.

**Ruland, J., Duncan, G. S., Elia, A., Del Barco Barrantes, I., Nguyen, L., Plyte, S., Millar, D. G., Bouchard, D., Wakeham, A., Ohashi, P. S. & Mak, T. W.** (2001). "Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure". *Cell,* 104**,** 33-42.

**Ruland, J. & Mak, T. W.** (2003). "Transducing signals from antigen receptors to nuclear factor kappaB". *Immunol Rev,* 193**,** 93-100.

**Sakai, A., Thieblemont, C., Wellmann, A., Jaffe, E. S. & Raffeld, M.** (1998). "PTEN gene alterations in lymphoid neoplasms". *Blood,* 92**,** 3410-5.

**Sakoda, H., Gotoh, Y., Katagiri, H., Kurokawa, M., Ono, H., Onishi, Y., Anai, M., Ogihara, T., Fujishiro, M., Fukushima, Y., Abe, M., Shojima, N., Kikuchi, M., Oka, Y., Hirai, H. & Asano, T.** (2003). "Differing roles of Akt and serum- and glucocorticoid-regulated kinase in glucose metabolism, DNA synthesis, and oncogenic activity". *J Biol Chem,* 278**,** 25802-7.

**Sale, E. M. & Sale, G. J.** (2008). "Protein kinase B: signalling roles and therapeutic targeting". *Cell Mol Life Sci,* 65**,** 113-27.

**Samuels, Y. & Ericson, K.** (2006). "Oncogenic PI3K and its role in cancer". *Curr Opin Oncol,* 18**,** 77-82.

**Sarbassov, D. D., Guertin, D. A., Ali, S. M. & Sabatini, D. M.** (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex". *Science,* 307**,** 1098-101.

**Sasaki, Y., Derudder, E., Hobeika, E., Pelanda, R., Reth, M., Rajewsky, K. & Schmidt-Supprian, M.** (2006). "Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation". *Immunity,* 24**,** 729-39.

**Shahinian, A., Pfeffer, K., Lee, K. P., Kundig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B. & Mak, T. W.** (1993). "Differential T cell costimulatory requirements in CD28-deficient mice". *Science,* 261**,** 609-12.

**Shiroki, F., Matsuda, S., Doi, T., Fujiwara, M., Mochizuki, Y., Kadowaki, T., Suzuki, H. & Koyasu, S.** (2007). "The p85alpha regulatory subunit of class IA phosphoinositide 3-kinase regulates beta-selection in thymocyte development". *J Immunol,* 178**,** 1349-56.

**Shoji, K., Oda, K., Nakagawa, S., Hosokawa, S., Nagae, G., Uehara, Y., Sone, K., Miyamoto, Y., Hiraike, H., Hiraike-Wada, O., Nei, T., Kawana, K., Kuramoto, H., Aburatani, H., Yano, T. & Taketani, Y.** (2009). "The oncogenic mutation in the pleckstrin homology domain of AKT1 in endometrial carcinomas". *Br J Cancer,* 101**,** 145-8.

**Sinclair, L. V., Finlay, D., Feijoo, C., Cornish, G. H., Gray, A., Ager, A., Okkenhaug, K., Hagenbeek, T. J., Spits, H. & Cantrell, D. A.** (2008). "Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking". *Nat Immunol,* 9**,** 513-21.

**Sizemore, N., Leung, S. & Stark, G. R.** (1999). "Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit". *Mol Cell Biol,* 19**,** 4798-805.

**So, L. & Fruman, D. A.** (2012). "PI3K signalling in B- and T-lymphocytes: new developments and therapeutic advances". *Biochem J,* 442**,** 465-81.

**Song, G., Ouyang, G. & Bao, S.** (2005). "The activation of Akt/PKB signaling pathway and cell survival". *J Cell Mol Med,* 9**,** 59-71.

**Staal, S. P.** (1987). "Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma". *Proc Natl Acad Sci U S A,* 84**,** 5034-7.

**Staal, S. P., Hartley, J. W. & Rowe, W. P.** (1977). "Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma". *Proc Natl Acad Sci U S A,* 74**,** 3065-7.

**Sun, Z., Arendt, C. W., Ellmeier, W., Schaeffer, E. M., Sunshine, M. J., Gandhi, L., Annes, J., Petrzilka, D., Kupfer, A., Schwartzberg, P. L. & Littman, D. R.** (2000). "PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes". *Nature,* 404**,** 402-7.

**Suzuki, A., De La Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., Del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., Fukumoto, M. & Mak, T. W.** (1998). "High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice". *Curr Biol,* 8**,** 1169-78.

**Suzuki, A., Yamaguchi, M. T., Ohteki, T., Sasaki, T., Kaisho, T., Kimura, Y., Yoshida, R., Wakeham, A., Higuchi, T., Fukumoto, M., Tsubata, T., Ohashi, P. S., Koyasu, S., Penninger, J. M., Nakano, T. & Mak, T. W.** (2001). "T cell-specific loss of Pten leads to defects in central and peripheral tolerance". *Immunity,* 14**,** 523-34.

**Swat, W., Montgrain, V., Doggett, T. A., Douangpanya, J., Puri, K., Vermi, W. & Diacovo, T. G.** (2006). "Essential role of PI3Kdelta and PI3Kgamma in thymocyte survival". *Blood,* 107**,** 2415-22.

**Tao, W. J., Lin, H., Sun, T., Samanta, A. K. & Arlinghaus, R.** (2008). "BCR-ABL oncogenic transformation of NIH 3T3 fibroblasts requires the IL-3 receptor". *Oncogene,* 27**,** 3194-200.

**Teitell, M. A.** (2005). "The TCL1 family of oncoproteins: co-activators of transformation". *Nat Rev Cancer,* 5**,** 640-8.

**Tsuruta, F., Masuyama, N. & Gotoh, Y.** (2002). "The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria". *J Biol Chem,* 277**,** 14040-7.

**Uddin, S., Hussain, A. R., Siraj, A. K., Manogaran, P. S., Al-Jomah, N. A., Moorji, A., Atizado, V., Al-Dayel, F., Belgaumi, A., El-Solh, H., Ezzat, A., Bavi, P. & Al-Kuraya, K. S.** (2006). "Role of phosphatidylinositol 3'-kinase/AKT pathway in diffuse large B-cell lymphoma survival". *Blood,* 108**,** 4178-86.

**Uner, A. H., Saglam, A., Han, U., Hayran, M., Sungur, A. & Ruacan, S.** (2005). "PTEN and p27 expression in mature T-cell and NK-cell neoplasms". *Leuk Lymphoma,* 46**,** 1463-70.

**Van Dongen, J. J., Langerak, A. W., Bruggemann, M., Evans, P. A., Hummel, M., Lavender, F. L., Delabesse, E., Davi, F., Schuuring, E., Garcia-Sanz, R., Van Krieken, J. H., Droese, J., Gonzalez, D., Bastard, C., White, H. E., Spaargaren, M., Gonzalez, M., Parreira, A., Smith, J. L., Morgan, G. J., Kneba, M. & Macintyre, E. A.** (2003). "Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936". *Leukemia,* 17**,** 2257-317.

**Vanhaesebroeck, B. & Alessi, D. R.** (2000). "The PI3K-PDK1 connection: more than just a road to PKB". *Biochem J,* 346 Pt 3**,** 561-76.

**Vanhaesebroeck, B., Welham, M. J., Kotani, K., Stein, R., Warne, P. H., Zvelebil, M. J., Higashi, K., Volinia, S., Downward, J. & Waterfield, M. D.** (1997). "P110delta, a novel phosphoinositide 3-kinase in leukocytes". *Proc Natl Acad Sci U S A,* 94**,** 4330-5.

**Vivanco, I. & Sawyers, C. L.** (2002). "The phosphatidylinositol 3-Kinase AKT pathway in human cancer". *Nat Rev Cancer,* 2**,** 489-501.

**Vose, J. M.** (2008). "Update on T-cell lymphoma". *Ann Oncol,* 19 Suppl 4**,** iv74-6.

**Wang, D. & Baldwin, A. S., Jr.** (1998). "Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529". *J Biol Chem,* 273**,** 29411-6.

**Watanabe, S., Shimosato, Y., Kuroki, M., Sato, Y. & Nakajima, T.** (1980). "Transplantability of human lymphoid cell line, lymphoma, and leukemia in splenectomized and/or irradiated nude mice". *Cancer Res,* 40**,** 2588-95.

**Webb, L. M., Vigorito, E., Wymann, M. P., Hirsch, E. & Turner, M.** (2005). "Cutting edge: T cell development requires the combined activities of the p110gamma and p110delta catalytic isoforms of phosphatidylinositol 3-kinase". *J Immunol,* 175**,** 2783-7.

**Wullschleger, S., Loewith, R. & Hall, M. N.** (2006). "TOR signaling in growth and metabolism". *Cell,* 124**,** 471-84.

**Xu, N., Lao, Y., Zhang, Y. & Gillespie, D. A.** (2012). "Akt: a double-edged sword in cell proliferation and genome stability". *J Oncol,* 2012**,** 951724.

**Yamaguchi, H. & Wang, H. G.** (2001). "The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change". *Oncogene,* 20**,** 7779-86.

**Yang, L., Sun, M., Sun, X. M., Cheng, G. Z., Nicosia, S. V. & Cheng, J. Q.** (2007). "Akt attenuation of the serine protease activity of HtrA2/Omi through phosphorylation of serine 212". *J Biol Chem,* 282**,** 10981-7.

**Yang, Z. Z., Tschopp, O., Baudry, A., Dummler, B., Hynx, D. & Hemmings, B. A.** (2004). "Physiological functions of protein kinase B/Akt". *Biochem Soc Trans,* 32**,** 350-4.

**Yuan, T. L. & Cantley, L. C.** (2008). "PI3K pathway alterations in cancer: variations on a theme". *Oncogene,* 27**,** 5497-510.

**Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G. & Soriano, P.** (1997). "Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells". *Proc Natl Acad Sci U S A,* 94**,** 3789-94.

**Zenz, T., Dohner, K., Denzel, T., Dohner, H., Stilgenbauer, S. & Bullinger, L.** (2008). "Chronic lymphocytic leukaemia and acute myeloid leukaemia are not associated with AKT1 pleckstrin homology domain (E17K) mutations". *Br J Haematol,* 141**,** 742-3.

**Zha, J., Harada, H., Yang, E., Jockel, J. & Korsmeyer, S. J.** (1996). "Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L)". *Cell,* 87**,** 619-28.

**Zilberman, D. E., Cohen, Y., Amariglio, N., Fridman, E., Ramon, J. & Rechavi, G.** (2009). "AKT1 E17 K pleckstrin homology domain mutation in urothelial carcinoma". *Cancer Genet Cytogenet,* 191**,** 34-7.

**Zuurbier, L., Petricoin, E. F., 3rd, Vuerhard, M. J., Calvert, V., Kooi, C., Buijs-Gladdines, J. G., Smits, W. K., Sonneveld, E., Veerman, A. J., Kamps, W. A., Horstmann, M., Pieters, R. & Meijerink, J. P.** (2012). "The significance of PTEN and AKT aberrations in pediatric T-cell acute lymphoblastic leukemia". *Haematologica,* 97**,** 1405-13.

# 6 Acknowledgements

I would like to express my gratitude to **Prof. Dr. Jürgen Ruland** for giving me the opportunity to work in his research group, to **Prof. Dr. Bernhard Küster** for agreeing to be the supervisor of my doctoral thesis and to **Prof. Dr. Schemann** for agreeing to lead the chair of my thesis committee.

I also want to thank **Dr. Martina Rudelius** for performing the histological analysis, **Mario Teichmann** for his assisstance with the soft agar assays and **Julian Holch** for his help with the GeneScan analysis.

Further, I want mention and thank all the people in and around the lab for their help, for creating a scientific but also familiar atmosphere and for becoming more than just colleagues at work:
**Michael Bscheider, Christoph Drees, Andreas Gewies, Oliver Gorka, Nicole Hannesschläger, Ulrike Höckendorf, Stefanie Jilg, Nathalie Knies, Verena Laux, Uta Meyer zu Büschenfelde, Konstantin Neumann, Thomas Patzelt, Konstanze Pechloff, Susanne Roth, Martina Schmickl, Katrin Schweneker, Susanne Spinner, Dominik Strasser, Vera Pfänder, Monica Yabal** and many more.

In addition to that, I thank **Olaf Groß** for providing me a desk to write this thesis and **Marc Schweneker** for agreeing to be my mentor.

Especially, I would like to thank **Kristina Salzeder** for her excellent technical assistance and her extraordinary help with the daily lab work.

I am very gateful to **Wiebke Stahlschmidt** for her patience and understanding, for sharing the ups and downs during the last years, for listening and for her good advices.

Very special thanks go to my parents **Anna** and **Josef Wanninger** as well as to my brother **Martin Wanninger** for their constant support over the years.