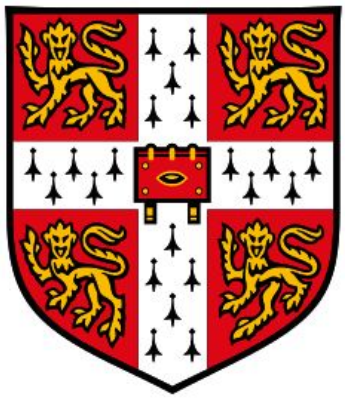
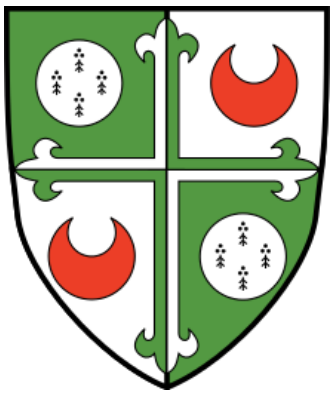
Searching for the missing T Cell Receptor (TCR) in Anaplastic Large Cell Lymphoma (ALCL): Surplus to requirements or a protagonist in lymphomagenesis?





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Searching for the missing T Cell Receptor (TCR) in Anaplastic Large Cell Lymphoma (ALCL): Surplus to requirements or a protagonist in lymphomagenesis?

Camilla Jayne Fairbairn

Summary

Anaplastic Large Cell Lymphoma (ALCL) is a peripheral T cell lymphoma divided into three distinct entities: ALCL, Anaplastic Lymphoma Kinase (ALK)+, ALCL ALK- and cutaneous ALCL. In the majority of ALCL, ALK+, ALK is expressed as the result of a chromosomal translocation generating Nucleophosmin 1(NPM)-ALK, which is considered the main driver. ALCL have an unusual immunophenotype; they rarely express a T cell receptor (TCR), but are often positive for CD4 and produce cytotoxic proteins such as perforin and Granzyme B, but in the absence of CD8, questioning the origin and pathogenesis of this malignancy. Expression of NPM-ALK in mice from the T-cell specific CD4 promoter gives rise to thymic lymphomas not modelling human ALCL suggesting that other events and/or expression of NPM-ALK at a defined stage of T cell ontogeny is required for peripheral T cell lymphoma development. Indeed, back-crossing the CD4/NPM-ALK line onto a RAG competent, MHC class I restricted ovalbumin-specific TCR, OTI transgenic line (CD4/NPM-ALK/OTI) permits peripheral lymphoma development mimicking human ALCL (but CD4/NPM-ALK/OTII mice still develop thymic lymphoma); tumours contain cells histopathologically identical to ALCL hallmark cells. Interestingly, peripheral tumours developing in this model also lack cell surface expression of the OTI TCR in fitting with observations of a lack of TCRβ expression on human ALCL. It follows that stimulation of T cells *in vivo* by infection with MHV-ova prevents lymphomagenesis suggesting that the TCR is detrimental to tumour growth. Indeed, strong stimulation via the TCR of NPM-ALK-expressing primary T cells *in vitro,*

impedes cell proliferation but cell growth is favoured when a weaker stimulus is employed. Overall, data presented in this thesis identifies a potential mechanism of lymphomagenesis accounting for the unusual immunophenotype of ALCL and an explanation as to why cells lack a TCR and associated proximal signaling.

Declaration

This dissertation is a result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. The work in this thesis has not been previously submitted for a degree, diploma or other qualification at any university. The research in this thesis was carried out under the supervision of Dr Suzanne Turner at the Division of Cellular and molecular Pathology, Department of Pathology, University of Cambridge, UK, between October 2013 and October 2017.

Camilla Jayne Fairbairn

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Summary

Anaplastic Large Cell Lymphoma (ALCL) is a peripheral T cell lymphoma divided into three distinct entities: ALCL, Anaplastic Lymphoma Kinase (ALK)+, ALCL ALK- and cutaneous ALCL. In the majority of ALCL, ALK+, ALK is expressed as the result of a chromosomal translocation generating Nucleophosmin 1(NPM)-ALK, which is considered the main driver. ALCL have an unusual immunophenotype; they rarely express a T cell receptor (TCR), but are often positive for CD4 and produce cytotoxic proteins such as perforin and Granzyme B, but in the absence of CD8, questioning the origin and pathogenesis of this malignancy. Expression of NPM-ALK in mice from the T-cell specific CD4 promoter gives rise to thymic lymphomas not modelling human ALCL suggesting that other events and/or expression of NPM-ALK at a defined stage of T cell ontogeny is required for peripheral T cell lymphoma development. Indeed, back-crossing the CD4/NPM-ALK line onto a RAG competent, MHC class I restricted ovalbumin-specific TCR, OTI transgenic line (CD4/NPM-ALK/OTI) permits peripheral lymphoma development mimicking human ALCL (but CD4/NPM-ALK/OTII mice still develop thymic lymphoma); tumours contain cells histopathologically identical to ALCL hallmark cells. Interestingly, peripheral tumours developing in this model also lack cell surface expression of the OTI TCR in fitting with observations of a lack of TCRβ expression on human ALCL. It follows that stimulation of T cells *in vivo* by infection with MHV-ova prevents lymphomagenesis suggesting that the TCR is detrimental to tumour growth. Indeed, strong stimulation via the TCR of NPM-ALK-expressing primary T cells *in vitro,* impedes cell proliferation but cell growth is favoured when a weaker stimulus is employed. Overall, data presented in this thesis identifies a potential mechanism of

lymphomagenesis accounting for the unusual immunophenotype of ALCL and an explanation as to why cells lack a TCR and associated proximal signaling.

Abbreviations:

2-ME: 2-Mercaptoethanol

ALCL: Anaplastic Large Cell Lymphoma

ALK: Anaplastic Lymphoma Kinase

AKT: Protein Kinase B

AP-1: Activator protein 1

APC: Allophycocyanin

BCR: B cell receptor

BSA: Bovine serum albumin

CCR7: C-C chemokine receptor type 7

CFSE: Carboxyfluorescein succinimidyl ester

CCL21: Chemokine (C-C motif) ligand 21

CCL25: Chemokine (C-C motif) ligand 25

CD4 SP: CD4+ single positive

CD4/NPM-ALK/OTI (occasionally denoted as OTINA)

CD4/NPM-ALK/OTII (occasionally denoted as OTIINA)

CD8 SP: CD8+ single positive

CDC42: Cell division control protein 42 homolog

CDR1: Complimentary determining region 1

CDR2: Complimentary determining region 2

CDR3: Complimentary determining region 3

CSC: Cancer stem cell

cTEC: Corticol thymic epithelial cell

CXCL12: C-X-C motif chemokine ligand 12

DAG: Diacylglycerol

DLBCL: Diffuse large b cell lymphoma

DN1: Double negative stage 1 CD44+CD25-

DN2: Double negative stage 2 CD44+CD25+

DN3: Double negative stage 3 CD44- CD25+

DN4: Double negative stage 4 CD44- CD25-

DOK1: Docking protein 1

DOK2: Docking protein 2

DP: Double positive CD8+CD4+

DSB: Double strand Breaks

DUSP22: Dual Specificity Phosphatase 22

E1: EINFEKL

EBV: Epstein Barr Virus

ERK: Extracellular signal-regulated kinases

ETP: Early Thymic progenitor

FACS: Flourescence-activated cell sorting

FITC: Fluorescein isothiocyanate

FOXP3: Forkhead Box P3

G4: SIIGFEKL

GIST: Gastrointestinal stromal tumours

GRB2: Growth factor receptor-bound protein 2

HCC: hepatocellular carcinoma

HHV-6: human herpes virus 6

HPK1: Mitogen-activated protein kinase 1

HPV: Human papilloma Virus

HRP: Horseradish peroxidase

IFN-γ: Interferon γ

IL2: Interleukin 2

IL7: Interleukin 7

ILC: Innate lymphoid cell

IP3: Inositol triphosphate

IRS1: Insulin Receptor Substrate 1

ITAMs: Immunoreceptor tyrosine-based activation motif

ITK: Interleukin-2-inducible T cell kinase

IVF: *In vitro* fertilisation

JAK3: Janus Kinase 3

KLF2: Kruppel-like factor 2

LAT: Linker of Activated T cells

LCK: Lymphocyte-specific protein tyrosine kinase

MAPK: Mitogen-activated protein kinase

MALT: MALToma

MCTP1: Multiple C2 and Transmembrane domain containing 1

MEK: Mitogen-activated protein kinase

MHC: Major histocompatibility complex

MHV-OVA: Murine Herpes Virus gamma expressing ovalbumin

mTEC: medullary thymic epithelial cell

MTT: MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

NFAT: Nuclear factor of Activation

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHL: Non-Hodgkin's Lymphoma

NPM: Nucleophosmin 1

NPM-ALK: Nucleophosmin-Anaplastic Lymphoma Kinase

OVA: SIINFEKL, OVA

PCR: polymerase chain reaction

PE: Phycoerythrin

PI3K: Phosphatidylinositol-4,5-bisphosphate-3-kinase

PIP3: Phosphatidylinositol (3,4,5)-triphosphate

PKC: Protein Kinase C

PLCg: Phospholipase C gamma

Pre TCR: pre-T cell receptor

PTCL: Peripheral T-cell Lymphoma

PTPN22: Protein tyrosine phosphatase, non-receptor type 22

Q4: SIIQFEKL

RAG: Recombination-activating gene

RSS: Recombination Signal Sequence

RT-PCR: Reverse transcriptase polymerase chain reaction

S1P1: Spingosine-1-phosphate receptor 1

SDS: Sodium dodecyl substrate

SH2: Src Homology 2

SHP-1: Src homology region 2 domain containing phosphatase-1

STAT3: Signal Transducer and Activator of transcription 3

STAT5: Signal Transducer and Activator of transcription 5

STS1: Suppressor of T cell receptor signalling 1

STS2: Suppressor of T cell receptor signalling 2

T4: SIITFEKL

T-ALL: T acute lymphoblastic leukaemia

TBS: Tris-buffered saline

TCR: T cell receptor

Tfh: T follicular helper cell

tgTCR/ TCRtg: transgenic T cell receptor

Th2: T helper cell 2

TNF-a: Tumour necrosis factor alpha

Treg: T regulatory cell

V(D)J: Variable, diversity, Joining

ZAP-70: ζ-chain associated protein kinase 70

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Introduction

1.1 Tracing the origins of cancer

Cancer is a genetic disease shaped by both the micro- and wider environment, considered to evolve from a cell of origin with survival promoting genetic defects acquiring further mutations along the way, permissive of autonomous growth, survival and proliferation. The acquisitions of these characteristics give rise to the hallmarks of cancer as defined by Weinberg and Hanahan (Hananhan & Weinberg , 2000). Weinberg and Hanahan originally identified six characteristics of cancer: the ability to sustain proliferative signalling, evasion of growth suppressors, invasion and an ability to metastasise, enabling of replicative immortality, induction of angiogenesis, and the ability to resist cell death (Hananhan & Weinberg , 2000). The Hallmarks were reviewed in 2011 adding additional characteristic traits considered as emerging hallmarks due to a greater understanding of the disease. These included: evasion of immune destruction, deregulation of cellular energetics, tumour promoting inflammation and genomic instability (Hanahan & Weinberg , 2011). These hallmarks exacerbate further the complexity of cancer and illustrates the challenges faced in the treatment and the development of treatments for this disease. This highlights the need and tendency to approach cancer treatment and prevention through identification of its specific origins.

The process of tumorigenesis is considered to be evolutionary, Darwinian selection on a cellular scale. This multifactorial process is full of randomly occurring mutations and epigenetic alterations, which can be acquired inherently or via lifestyle and environmental exposures, considered as multiple “hits” in disease generation (Vogelstein & Kinzler, 1993), (Weinberg, 2013), (Knudson , 1971). These instigators

of disease, once identified, can give an indication as to how to treat the disease and the particular “events” to target for prevention. Identification of specific contributors towards cancer development for example genetic: BRCA1, infections: HPV, lifestyle: smoking, or the cell of origin, has established successful preventative treatments and methods (Blanpain, 2013) (Lorincz, et al., 1992) (Hall , et al., 1990) (Doll & Hill , 1950).

The acquisition and accumulation of mutational events from environmental factors takes time and thus cancer is generally considered to be an age associated disease for which it can take years to decades to occur. The median age of diagnosis according to the SEER study (this is inclusive of all sex and races) is 39. An exception to this is leukaemia where the median age of diagnosis is 15 (Howlader , et al., 2014). Therefore, the majority of cancers can be assumed to be due to age, hinting more towards lifestyle and environmental contributions, evidence that the multistep process for tumorigenesis takes time. These contributions, can be in the form of mutagens, non-mutagenic events which promote proliferation, chronic inflammation and exposure to mitogens and toxic substances (Lightfoot & Roman, 2004).

Paediatric cancers on the other hand do not have this prolonged period of time in order to acquire mutations and therefore events contributing towards tumourigenesis must occur or be acquired at a much earlier stage, as early as parental germ cells. It is therefore important to identify the processes required for paediatric lymphomas and leukaemias, to allow for early diagnosis and generation of preventative treatments. Typical treatments consist mainly of chemotherapy which, although responses to treatment may be good, the long-term side effects are not as such, causing damage to organs and increasing the risk of secondary cancers (Link, et al., 1997) (Hodgson, 2015).

1.2 Risks leading to paediatric cancer development

Paediatric cancers represent around 1% of cancers diagnosed each year, (Society, 2016) thus supporting the notion that cancer is an age associated disease. This earlier onset associated with childhood cancers does suggest that events contributing towards tumorigenesis must occur at a much faster rate or that "seeds are sown" during earlier stages of development perhaps *in utero* or pre-conceptionally. Events that have been implicated in contributing towards paediatric cancer development are briefly summarised in (Figure 1.1).

Genetic acquisitions have been noted to account for 10% of paediatric cancer cases therefore inheritance of oncogenes via the gene pool could be considered as one of the main risks associated with paediatric cancers. Paediatric retinoblastoma is one of the most widely studied cancers associated with this, characterised by inheritance of the *RB1* gene (Knudson , et al., 1975). Transgenerational, pre-conceptional and periconceptional events have been implicated in the development of paediatric cancers. Mutations in parental germ cells, for example can arise from environmental factors contributing to paediatric cancer development. In the form of specific paternal lifestyle factors such as smoking, alcohol, diet and coffee consumption which have been implicated with an increased risk of paediatric leukaemia, lymphoma and brain cancers (Ji, et al., 1997) (Clavel , et al., 2005) (Bunin , 1998) (Infante-Rivard , et al., 2002) (Zhang J, 2015). These factors also contribute towards the risk of leukaemia and lymphomas *in utero* along with ionising radiation, diethylstilboestrol and chemotherapy (Lightfoot & Roman, 2004). IVF has also been linked to an increased risk of paediatric lymphoma and leukaemia (Reigstad, et al., 2016). Additional perinatal and postnatal factors can contribute such as vitamin K administration during

labour (Golding , et al., 1992), and infections associated with cancer generation. For instance, maternal transmission of EBV and *H.pylori* increases the risk of childhood liver and gastric cancers (Chang , et al., 1989) (Kitagawa, et al., 2001). These contributing factors mentioned are mainly associated with paediatric lymphomas and leukemias.

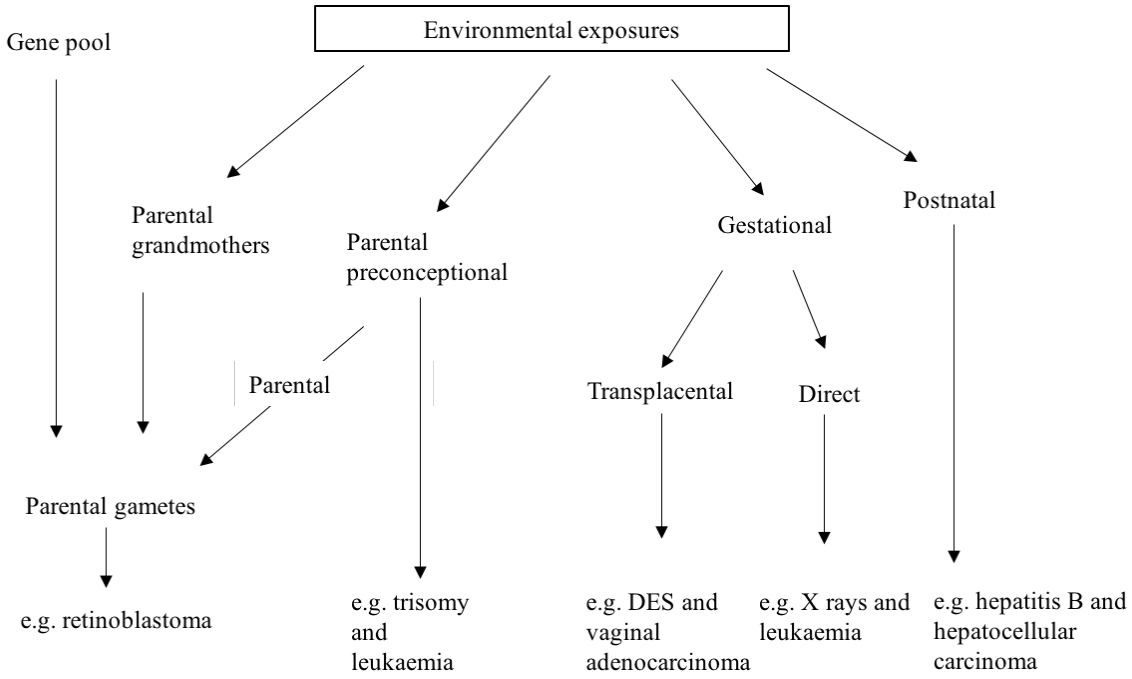


Figure 1.1 Paediatric lymphoma risks adapted from (Anderson , et al., 2000)

1.3 Lymphoma

Lymphomas are typically solid tumours, usually developing within lymph nodes but they can occur in any tissue (Shankland, et al., 2012). The classification of lymphoma umbrellas a vast range of subtypes belonging to either the Hodgkin or the Non-Hodgkin (NHL) category, identified by their morphological and cellular

appearances. Lymphomas are further classified into T, B, null or even natural killer cell types although the majority of NHL are B cell-derived accounting for 85-90% cases, the remainder for the most part are of a T cell origin (Shankland, et al., 2012). This latter category encompasses at least 35 different types of lymphoma (table 1.1), which can pose difficulty in the identification of a particular subtype. The peripheral T cell lymphomas (PTCL), the most heterogeneous, are considered to have a post-thymic origin and have a poor prognosis with a 5-year overall survival of 15-30%. Anaplastic Large Cell Lymphoma (ALCL), the focus of this thesis is considered to be a member of this particular sub-group (Savage , 2007).

Lymphoma			
Hodgkin lymphoma	**Non Hodgkin lymphoma**		
Classical (characterised by REED Sternberg cell presence)	**B cell**	**T cell**	
Nodular sclerosis	Diffuse large B-cell (DLBCL): intravascular large B-cell lymphoma, Primary mediastinal large B-cell lymphoma	Immature	Mature
Mixed cellularity	Burkitt Lymphoma	T-Cell lymphoblastic lymphoma	Peripheral T cell lymphoma -NOS
Lymphocyte-rich	Mantle cell lymphoma		Angioimmunoblastic T-cell lymphoma/ follicular T-cell lymphoma
Lymphocyte-depleted	Primary CNS lymphoma		Anaplastic large cell lymphoma
	Follicular lymphoma		Enteropathy-associated T-cell lymphoma
	MALT lymphoma		Adult T cell lymphoma
	Nodal marginal zone lymphoma		Extranodal NK/T-cell lymphoma, nasal type
	Splenic marginal zone lymphoma		Hepatosplenic T-cell lymphoma
			Cutaneous T-cell lymphoma
			Lymphoblastic lymphoma

Table 1.1: Lymphoma subtypes

1.4 ALCL: a rare T-cell lymphoma

First described by Stein et al in 1985 (Stein , et al., 1985), ALCL is a rare CD30-expressing T cell derived NHL, which can be separated into different subtypes

dependent upon disease location: cutaneous, associated with breast implants or systemic. In addition, the absence or presence of the tyrosine kinase Anaplastic Lymphoma kinase (ALK) and associated fusion proteins can further differentiate sub-types of this disease. Therefore, tumours are identified as being either ALK positive (ALK+) or ALK negative (ALK-) with cutaneous ALCL being mainly ALK- and systemic disease largely ALK+. The Prognosis and age of onset of systemic ALCL differs, ALK- disease occurs mainly in 54-61 year olds whereas ALK+ ALCL presents mainly in children, and overall prognosis tends to be better in those with ALK+ ALCL where ALK+ disease has an overall 5 year survival of 70-90% compared to 40-60% observed with the ALK- disease (Savage , et al., 2008) (Ferreri , et al., 2013) (Ferreri , et al., 2012). In patients under the age of 40 the presence of ALK does not have a favourable outcome over ALK negative patients (Sibon , et al., 2012), suggesting that the prognostic impact of ALK expression is age dependent.

1.5 ALK

ALK belongs to a sub-family of the insulin receptor superfamily of tyrosine kinases but its precise role in humans is unknown, other than that its expression is associated with development of the nervous system and that this diminishes in all tissues after birth (Iwahara, et al., 1997)(Hallberg & Palmer , 2013). ALCL, in particular, is associated with the production of ALK translocations with at least 9 fusion proteins (table 1.2). The most predominant of these is Nucleophosmin 1 (NPM1) which with ALK produces (NPM)-ALK first cloned in 1994 by Morris et al, and expressed as a consequence of the t(2;5)(p23;q35) translocation (Morris , et al., 1994). NPM1, a nucleolar phosphoprotein, shuttles between the nucleus and cytoplasm, its oligomerisation motif, within its protein domains, is retained in the fusion protein and

is key to its function. As such, NPM-ALK is capable of forming homodimers, which can auto-phosphorylate and form a signal transduction complex activating multiple signalling pathways (Stein & et al., , 2000) summarised in figure 1.2.

The promotion of survival and proliferation in ALCL is promoted by NPM-ALK via the JAK3/STAT3, p85/PI3K/p110-PIP3-pAKT, GRB2/SHC/IRS1 and PLCg-IP3/DAG/CA2+/PKC pathways all of which converge onto the Ras/MEK/ERK cascade (Zamo , et al., 2002) (Bai, et al., 1998) (Slupianek, et al., 2001). Interruption of STAT3 and ERK, for example, decreases proliferation and cell survival indicating NPM-ALK's reliance on these pathways (Tanizaki , et al., 2012). NPM-ALK can maintain proliferation independent of the RAS/MEK/ERK cascade by the GRB/SHC/IRS1-VAV1-CDC42 pathway (Choudhari, et al., 2016). Loss of SHP-1, which leads to uncontrolled cell growth and development of ALCL, occurs as a result of NPM-ALK activity, linking the role of SHP-1 to that of a tumour suppressor (Buetti-Dinh, et al., 2016) (Khoury , et al., 2004)(Palmer , et al., 2009). Therefore NPM-ALK is highly implicated as the main driver of ALCL although the location of acquisition and the cell from which ALCL arises is still widely debated.

Fusion protein	Genetic Aberration	Frequency	Staining pattern
NPM	t(2;5)	75%	Cytoplasmic and nuclear
TPM3	t(1;2)	10-20%	Cytoplasmic
TFG	t(2;3)	2.5%	Cytoplasmic
CLTC	t(2;22	2.5%	Granular cytoplasmic
ATIC	inv(2)	2.5%	Cytoplasmic
AL017	t(2;17	Rare	Cytoplasmic
TPM4	t(2;19)	Rare	Cytoplasmic
MYH9	t(2;22)	Rare	Cytoplasmic
MSN	t(2;X)	Rare	Membrane

Abbreviations: NPM=Nucleophosmin, TPM3=tropomyosin 3, TFG= tropomyosin receptor kinase-fused gene, CLTC=clathrin heavy chain, ATIC= 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleotide transformylase/inosine monophosphate cyclohydrolase, AL017=ALK lymphoma oligomerization partner on chromosome 17, TPM4=tropomyosin 4, MYH9=myosin heavy chain, MSN=moesin

Table 1.2 NPM-ALK fusions adapted from (Cools , et al., 2002)

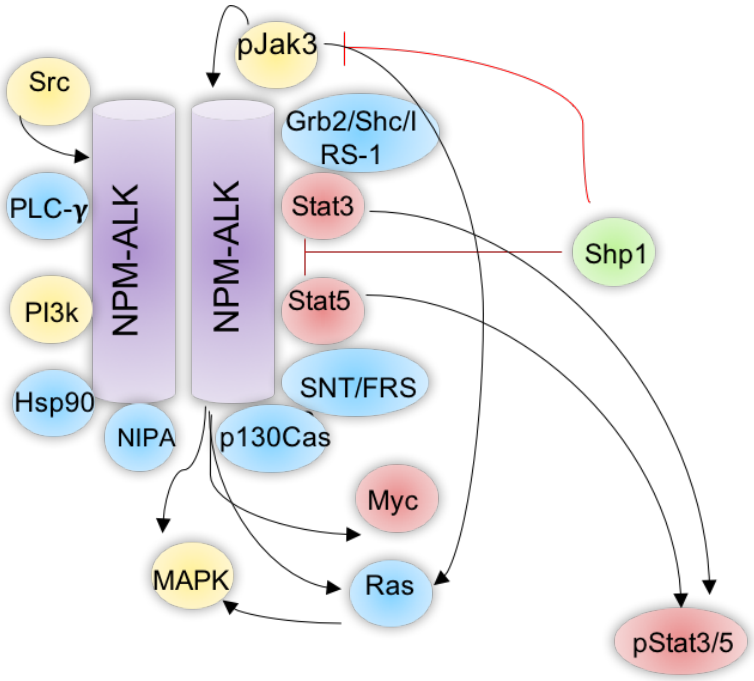


Figure 1.2: NPM-ALK signalling adapted from (Amin & Lai, 2007)
NPM-ALK signals through the PI3K pathways (NPM-ALK and PI3K), JAk3/STAT3, MAPK, PLCγ, GRB2, VAV1 leading to cell survival and proliferation (Ambrogio , et al., 2008), (Chiarle, et al., 2008) (Amin & Lai, 2007) (Han , et al., 2006).

1.6 ALCL: Origins

In systemic ALCL, ALK+ and ALK– cases are immunophenotypically and morphologically indistinguishable with an aberrant T cell phenotype; tumour cells are considered T cells by virtue of molecular TCR rearrangements but in many cases, do not express T cell associated antigens and hence are called null cells (Medeiros & Elenitoba-Johnson, 2007) (Krenacs , et al., 1997). The disease presents within the periphery typically in the skin, bone, lung and soft tissue (Ferreri , et al., 2012) and is typified by Hallmark cells (figure 1.3) which display a kidney or horse shoe-like nucleus, surrounding a clear or more eosinophilic paranuclear Golgi area with abundant basophilic cytoplasm (Ferreri , et al., 2012). Tumour cells typically express CD30 indicating an activated T cell phenotype with effects largely dependent upon the NF-kB pathway (Wright , et al., 2007) (Sabattini, et al., 2013). In addition, cells express CD71 and CD25 along with production of granzyme B and perforin, indicative of a cytotoxic activated T cell as a potential cell of origin, although the WHO classifies ALCL as originating in a cytotoxic αβ T cell potentially of a Th2 type (Turner , et al., 2007) (Campo & et al.,, 2011) (Stein , et al., 1985) (Foss , et al., 1996) (Pearson , et al., 2011). Further evidence suggests a T regulatory (Treg) cell lineage: Expression of fork head box p3 (FOXP3), production of IL-10 and TGFβ have been identified in 4/6 ALK+ALCL cell lines, in addition to expression of Treg cell surface markers (CD25, CD45RO, FAS, CTLA-4 and CD4) although expression varies between samples (Bonzheim & et al .,, 2008). A Th17 origin has also been proposed whereby ALCL cell lines express IL-22R1 and increased levels of circulating IL-22, a cytokine involved in the stimulation of the inflammatory response associated with TH17 cells *in vivo* and production of IL-17 (Savan & et al.,, 2011) (Kasprzycka, et al., 2006) (Matsuyama, et al., 2011). However, many of these T cell

lineage markers have also been shown to be NPM-ALK induced speaking towards the activity of this chimeric protein rather than the cell of origin. In addition, gene expression profiling of isolated ALCL tumour cells showed that ALCL could not be assigned to either a CD4 or CD8 lineage, thereby still leaving the ALCL origin open (Eckerle & et al.,, 2009).

Results from the Turner lab posed a possible and highly probable theory that the cell of origin could be derived from the thymus or earlier hematopoietic progenitors as previous work has identified a cellular sub-set, the so-called cancer stem cell (CSC) which is enriched for a gene expression profile associated with early thymic progenitors (ETP) (Moti , et al., 2014) (Hassler , et al., 2016). In support of these data, *NPM-ALK* transcripts have been identified in 1.95% of cord blood samples indicating not only a primitive cell of origin but also perhaps *in utero* acquisition (Laurent & et al., , 2012). If the cell of origin for ALCL is a primitive progenitor then it is likely that events occurring in the thymus (and subsequently when circulating in the periphery) may play a role in shaping the disease phenotype.

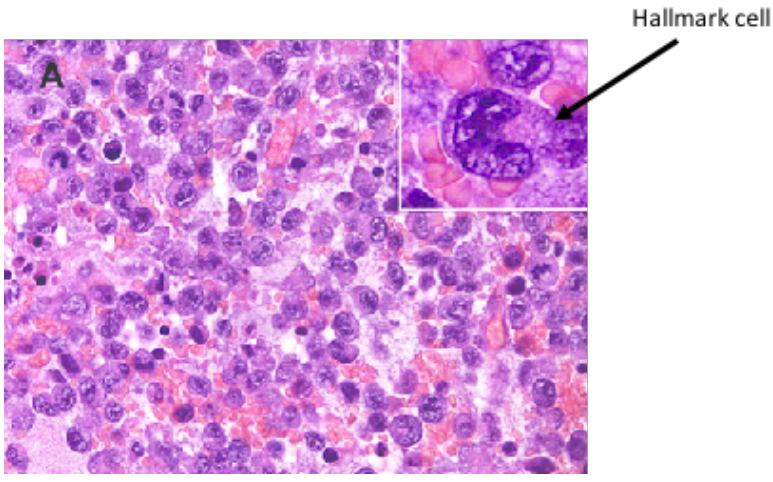


Figure 1.3 Example of ALCL tumour displaying hallmark cells (Bonzheim , et al., 2004)

1.7 T cell development

Early thymic progenitors occupy the endosteal niche within the bone marrow (Ding & Morrison , 2013) and are subsequently seeded in the thymus via either the vascular independent route, mediated by Notch signalling and chemokines CC-chemokine ligand 21 (CCL21) and CCL25 expressed by the foetal thymus primordium (during embryogenesis) or by a vascular-dependent route (Takahama, 2006) (Weber-Arden , et al., 2000). From here, the ETP goes through distinct stages of maturation; at this point cells do not express a T cell receptor (TCR), CD8, CD4 nor CD3 and hence are termed as triple or double negative (DN) T cells. The DN stages are signified by variable expression of CD44 and CD25 expression (in mice) and occurs in the following manner, and as shown in Figure 1, DN1: CD44+/hi, CD25-, DN2: CD44+/hi, CD25+, DN3: CD44-/lo, CD25+ and DN4: CD44-/lo CD25- (Asnafi & et al.,, 2003). Conversely, human DN cells are distinguished by their expression of CD34, CD38 and CD1a (Joachims , et al., 2006). Rearrangements of the TCR chains occur via a process known as V(D)J recombination which commences at the DN2 stage, starting with rearrangements of the TCR δ chain followed by γ and then β by the DN3 stage. If TCR β rearrangements are successful and a β chain is expressed with surface expression in the context of a pre-TCR (pre-Tα and sCD3), cells progress to the double positive (DP) stage. Rearrangement of the β chain at the DN3 stage is termed β selection and is the first major checkpoint in T cell development (Asnafi & et al.,, 2003) (Godfrey, et al., 1994). Following successful β selection, the TCR α chain is rearranged, TCR δ is deleted and the pre-TCR is replaced by a full αβ TCR (Figure 1.4) This process is largely driven by Notch-mediated signalling and the genes involved at each stage are illustrated in (figure 1.5).

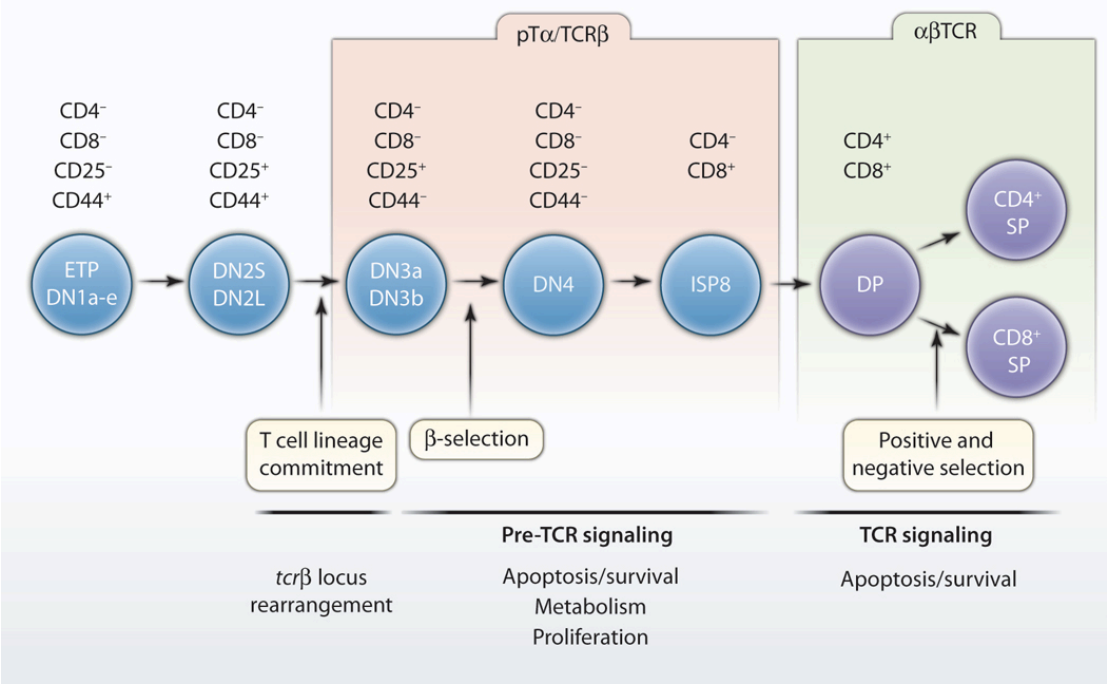


Figure 1.4: T cell development in mice taken from (Germain , 2002)**:** *Thymocyte development has four distinct phases dependent upon the expression of CD44, CD25, CD4 and CD8. Initially cells are negative for both CD8 and CD4 and thus are termed double negative [DN] under this double negative phenotype cells move through three different phenotypical and genetic stages. The DN1 stage cells only express CD44 but at high levels and along with CD117/c-kit; at this point cells are still capable of developing into Natural Killer (NK), dendritic, B and T cells. The next stage, DN2, expresses both CD25 and CD44 and cells can no longer give rise to B and NK cells, here they are restricted to α/β or γ/δ T cells and dendritic cells. Cells then enter the DN3 stage where CD25 is expressed though CD44 levels are low. At this point cells can differentiate into α/β or γ/δ T cells. It is at this specific point where cells lose CD25 expression and gain both CD8 and CD4 becoming a double positive (DP)* (Lind & et al., , 2001) (Germain , 2002). *During the transition from DN4 to DP, proliferation occurs but only in those cells with successful pre-TCR expression. The DP cells expressing CD8, CD4 and TCR go through the positive and negative selection process, whereby cells are exposed to MHC class II and MHC class I, expressing self-antigen, little signalling in response ends up in death by neglect, too much signalling promotes negative selection, whereas intermediate stimulation results in commitment to either a CD4 or CD8 lineage dependent upon the MHC molecule encountered* (Germain , 2002).

1.8 β selection

Driven by the pre-TCR, β selection occurs at the DN3 stage and is dependent upon the expression of a fully functional, rearranged β chain. Only thymocytes expressing a pre-TCR (pre-TCR α chain expressed together with the TCR β chain) are able to progress to the DP stage, cells that fail in this task undergo apoptosis due to a lack of pre-TCR signal transduction (Michie & Zuniga-Pflucker, 2002), Unless they express TCRγδ. The pre-TCR α chain exerts effects via TCR proximal signalling molecules resulting in activation of MAPK and PLCγ which mobilises calcium and activates PKC consequently leading to activation of NFAT and NF-kB, promoting differentiation, proliferation and survival (Aifantis , et al., 2001). Notch plays an important role in this process and is key to maintaining the viability of pre-T-cells promoting survival through regulation of the PTCRA gene via CSL activation (von Boehmer, 2005), or by pre-TCR independent mechanisms through the induction of the PI(3)K-Akt pathway enabling autonomous signalling capacity of the pre-TCR (Ciofani & Zuniga-Pflucker, 2005).

If unsuccessful, rearrangements take place and a successfully rearranged TCRBIgI β chain is not expressed as part of the pre-TCR. The pre-TCR α chain is then no longer expressed thus preventing rescue of DN3 cells from apoptosis. Cells that are successful in surviving this stage of T cell development undergo extensive proliferation and eventually differentiate into mature T cells. Of note, further rearrangement of TCRβ is inhibited due to allelic exclusion whereby *RAG* genes (recombination activation genes) become inactive and the *Trcb* locus becomes inaccessible (Von Boehmer & et al.,, 1998). When cells reach the DP stage, *RAG* is

reactivated and the *Tcra* locus undergoes rearrangement to produce the α chain to form a TCRαβ (Hogquist , et al., 2005). T cells then express a full TCR which allows the cells to undergo the clonal selection process.

T cell developmental processes have been shown to play a role in tumourigenesis through pre-TCR signalling and V(D)J recombination. Signalling via the pre-TCR, for instance, has been linked to T-ALL, where signals derived from pre-TCR activation pathways and Notch3 signalling regulate SCL/TAL-1 and T cell proliferative responses driving T-ALL (Talora , et al., 2006). Pre-TCR signalling inactivates p53 which would otherwise prevent maturation of thymocytes in the absence of a TCR (Haks , et al., 1999) (Jiang , et al., 1996).

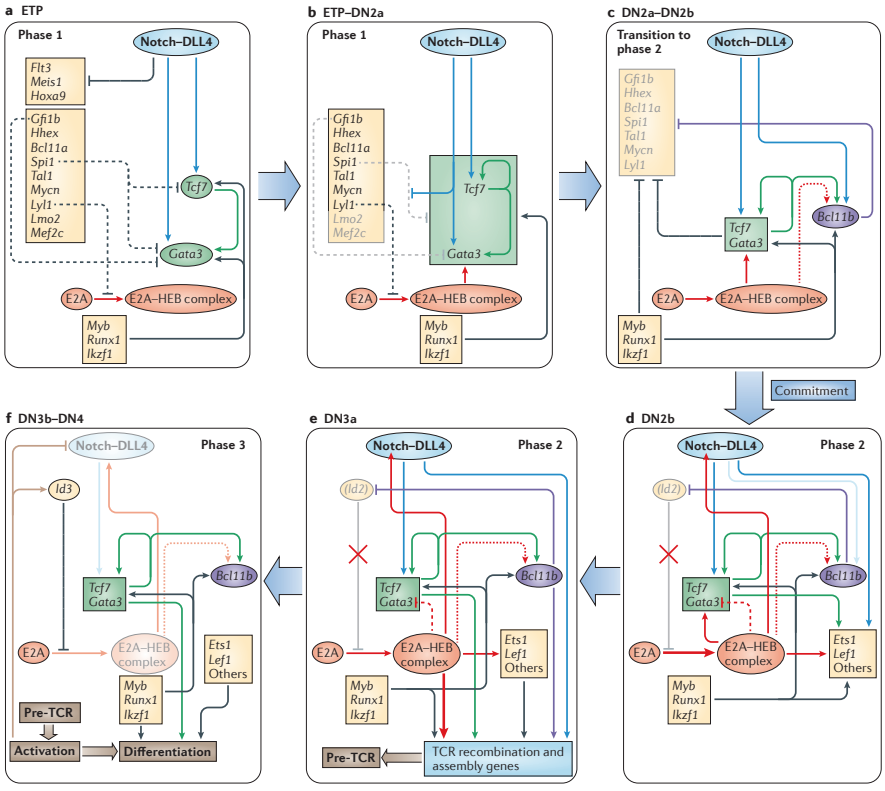


Figure 1.5: T cell development and associated gene networks *Taken from* (Yui & Rothenberg , 2014)*: NOTCH signalling drives the establishment of T cell identity from lymphoid precursors. This is through interaction with notch ligands in the thymic environment up to the DN3 stage of thymic development.* (Yui & Rothenberg , 2014) (Takahama, 2006) *The development and maturation of the T cell is driven by*

developmental gene networks separated into phases 1, 2 and 3. The phase 1 network is thought to support expansion and proliferation of ETPs and DN2a stage cells. Phase 2 gives rise to the T cell identity/ T cell commitment; here TCR gene rearrangement occurs resulting in expression of a Pre TCR or TCR gamma delta. Signal transduction via the TCR promotes the shift to the Phase 3 network, this is also mediated by NOTCH signalling (Yui & Rothenberg , 2014). *Alterations in these particular phases have been associated with early T cell precursor leukaemia / high risk acute lymphoblastic leukaemia.*

1.9 The importance of TCR chain rearrangements: V(D)J recombination

T cells must be able to respond to a vast repertoire of antigens that it may be exposed to in the context of external factors i.e. infectious microorganisms, and not respond to self. A single TCR can respond to greater than one million different peptides, and therefore, in order to recognise this vast array of antigens an extensive library of TCR's must be expressed, this is achieved by different combinations of TCR chain variable regions developed from V, J and occasionally D gene segments (Wooldridge , et al., 2012). This vast array of TCRs arises from the V(D)J recombination process, which is mediated by recombinase activation genes *RAG 1* and *2* gives rise to a number of receptors. RAG expression is therefore essential to the production of mature B and T cells which require V(D)J recombination in order to produce their corresponding antigenic receptors. This is evident in mice null for either of the *RAG* genes (*RAG1/RAG2*) which present with a lack of mature B or T cells (Shinkai, et al., 1992) (Mombaerts, et al., 1992). The process of VDJ recombination extensively reviewed in (Schatz & Yanhong, 2011), is regulated by the level of RAG expression, the accessibility of recombination signal sequences (RSSs) within chromatin and the 3D architecture and position of the antigen receptor loci. Initiation occurs via the RAG complex (RAG1/RAG2) which binds and cleaves DNA, inducing double strand breaks (DSBs) at specific (RSSs) sites. These RSSs flank each of the V, D and J gene

segments exposing DNA. Exposed DNA is then processed and joined by proteins of the non-homologous end joining pathway (NHEJ) via DNA repair enzymes leading to the deletion or inversion of small to large DNA segments (Fugmann, et al., 2000) (Schatz, et al., 1992).

Once TCR chains are rearranged, and β and α chains joined, the 3rd hypervariable region (CD3r) is responsible for peptide sequence recognition, interacting mainly with the peptide of the pMHC complex. The complimentary determining regions (CDR) are important for antigen engagement (CDR1 and CDR2 are germline whereas CDR 3 can be somatically rearranged) with the CDR3 region being important for engagement and recognition of peptides as presented by MHC. Importantly, sequencing of the CDR3 region can be conducted to predict the specificity of any given TCR for antigen (Hughes , et al., 2003) (Borg, et al., 2005).

It is thought that the V(D)J recombination process can give rise to, or contribute towards tumorigenesis by inducing oncogenic rearrangements (Reddy , et al., 2006). RAG itself has also been implicated in contributing to tumourigenesis, infact the recombinase activity of RAG has been shown to be the driver for critical secondary events in the generation of Acute Lymphoblastic Leukaemia (ALL) (Papaemmanuil & et al , 2014). RAG can also induce DNA breaks and chromosomal rearrangements in cryptic RSSs and non-RSS sequences, outside of antigen receptor loci, which again may contribute to tumourigeneis (Gostissa et al., 2011), (Lieber et al., 2006), (Mills et al., 2003). This is demonstrated by the *MCTP1* gene where insertion of the oncogene is mediated by VDJ recombination into the TCRα and TCRβ loci increasing its prevalence in the T cell lineage (Woiciechowsky, et al., 2001).

Though RAG has been implicated in ALL, its role in ALCL development is unclear. Studies have shown the presence of low RAG transcript expression in ALCL cases, potentially indicating a role for RAG in lymphomagenesis (Knecht, et al., 1993) (Malcolm , et al., 2016). In addition, previous research has shown that despite the lack of cell surface expression of a TCR in ALCL, 56% of cases display α and β chain rearrangements at the molecular level indicating a need for RAG during ontogeny. However, CD4/NPM-ALK mice on a RAG knockout background (CD4/NPM-ALK/RAG-/-) still develop thymic lymphomas with a DP phenotype indicating that V(D)J recombination does not contribute greatly to lymphomagenesis in this model, instead its absence only affected tumour phenotype and slightly delayed tumour development. However, these mice did not generate ALCL like disease as tumours were retained within the mediastinum (Malcolm , et al., 2016). Interestingly in the CD4/NPM-ALK/RAG KO model NPM-ALK mediated bypass of the β selection checkpoint via Notch1 (Malcolm , et al., 2016).

1.10 Clonal selection and central tolerance, the CD4/CD8 fate decision

Clonal selection and central tolerance is imperative to the development of T cells, ensuring that T cells responding to self-antigen do not enter into the periphery and are deleted by apoptosis. Lineage commitment towards a CD4 or CD8 SP phenotype occurs during clonal selection in the thymus. Clonal selection is based on self-peptide recognition, via the TCR, expressed in the context of either MHC class I or MHC class II by cortical (cTECs) and medullary thymic epithelial cells (mTECs) (Hogquist & Jameson , 2014) (Labrecque , et al., 2011). Considered as the second major checkpoint in T cell development during the positive and negative selection process

immature DP αβT cells are exposed to self-antigens by thymic antigen presenting cells (APCs), including cTEC's, mTEC's, migratory cDC, resident CDC and pDC as well as thymic B cells, the majority of which express antigens in the context of MHC class II. This exposure initiates at the DN3 stage mediated by antigenic expression via cTEC's. After, at the DP stage, thymocytes are exposed to antigens by dendritic cells, thymic B cells and plasmacytoid dendritic cells (Klein , et al., 2014). At this stage thymocytes that respond with high avidity are deleted via apoptosis also known as negative selection whereby 98% of thymocytes are lost. If the cell does not react to or receive a signal via the TCR, then the cell undergoes apoptosis due to 'neglect' (Takahama, 2006). Cells that respond to antigenic presentation with low avidity receive survival signals allowing for differentiation into a SP (CD4 or CD8) type, this is known as positive selection. Cells down-regulate CD4 if they interact with MHC class I and *vice versa* for MHC class II. Cells then migrate into the medulla via a process mediated by the chemokine CCR7 (Takahama, 2006) whereby SP cells display with a semi-mature cell surface phenotype (Qa-2^{lo}, $CD62L^{lo}$, HSA^{hi}, $CD69^{hi}$). At this point cells are still susceptible to apoptosis and undergo negative selection induced by recognition of self-peptide by mTECs in the medulla (Klein , et al., 2009) (Klein , et al., 2014) (Love & Bhandoola , 2011). As cells mature, mature SP cells (Qa-2hi, CD62Lhi, HAS low, CD69low) proliferate under antigenic stimulation, rather than undergoing apoptosis, and only those SP cells capable of proliferation emigrate into the periphery (McCaughtry , et al., 2007) (Welnreich & Hogquist , 2008) (Germain , 2002).

Eventually, mature, functional T cells exit the thymus in a process that is reliant on S1P1 (Matloubian & et al., , 2004). The S1P1 receptor is expressed on mature SP thymocytes and regulated by the expression of CD69 (a marker of T cell activation);

during development CD69 is expressed on semi-mature thymocytes which lack S1P1 expression (Shiow , et al., 2006) (Dzhagalov & Phee, 2012) (McCaughtry , et al., 2007). Naïve αβ T cells typically do not emigrate from the thymus until 4-5 days after becoming SP cells (McCaughtry , et al., 2007). Once in the periphery, cells migrate to primary and secondary lymphoid tissues and survival is maintained via tonic signalling.

1.11 ALCL and T cell development

Primary patient samples and cell lines developed from ALK- and ALK+ ALCL for the most part do not express a TCR nor CD3 (Ferreri , et al., 2012) (Ferreri , et al., 2013) (Bonzheim , et al., 2004). In addition, many of the proximal TCR signalling proteins are deregulated by epigenetic silencing (Ambrogio & et al.,, 2009). This is at odds with T cell development whereby as mentioned above it is crucial for thymocytes to have a fully functional rearranged TCRβ chain in order to mature and develop, as well as a functional TCR in order to go through positive and negative selection and escape into the periphery. Therefore, signalling downstream of the TCR is crucial to thymocyte development and suggests that if ALCL initiates in the thymus, the cells must in some way compensate for a lack of activity in this regard. Alternatively, transformation (and down-regulation of the TCR and its associated signalling molecules) in ALCL development may occur exclusively in the periphery. In support of the former, recent data acquired from ALCL primary patient samples showed only 56% to have undergone normal αβ TCR rearrangements the remaining 44% showing γδ rearrangements (19%), just γ rearrangements (11%) or germline TCR (14%) (illustrated in (figure 1.6) (Malcolm , et al., 2016)). These data suggest that there must be subversion of TCR rearrangements in the thymus of these patients

and hence is supportive of aberrant activity most likely mediated by NPM-ALK. Hence, cells with abnormal TCR's are escaping into the periphery, possibly promoted by a strong survival signal supplied by NPM-ALK (Herbst , et al., 1989). Indeed, in the absence of RAG activity, NPM-ALK transgenic mice in which the NPM-ALK transgene is expressed throughout thymic development, produce thymic tumours that appear to have derived from mature DP thymocytes despite an absence of TCR rearrangements. This ability to bypass β selection was shown to be mediated by notch upregulation (Malcolm , et al., 2016). However, CD4/NPM-ALK mouse mimics showed that disease was restricted to the mediastinum unless a functional TCR (OTI)

was present (Malcolm , et al., 2016). Indicating that whilst it may be that T cells expressing aberrant TCRs are able to escape into the periphery there is interference with egress by NPM-ALK in the absence of a TCR and that these "aberrant receptors" must closely mimic the function of a fully functional, engaged TCR.

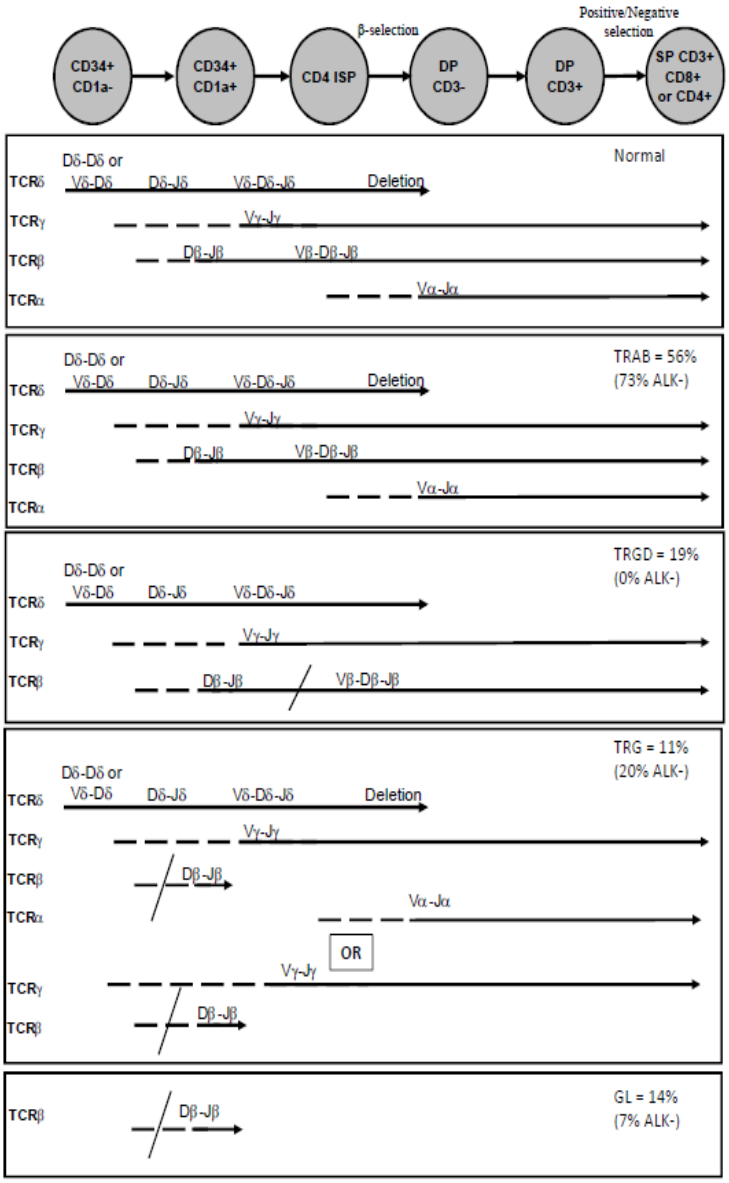


Figure 1.6 TCR chain rearrangements: *During normal T-cell development rearrangement of TR genes occurs in a temporal manner whereby rearrangement of δ precedes γ, which is followed by β and then α, the latter of which coincides with deletion of the d locus. The human ALCL tumours were categorized into 4 distinct immunogenetic profiles with regards to the presence or absence of TR rearrangements. The TRAB category having a normal set of rearrangements consistent with an αβ T-cell, TRGD displaying rearrangements of TRD and G but lacking TRA rearrangements and having partial of full TRB, TRG having a full*

complement of rearrangements including deletion of the TRD locus but having partial or no TRB rearrangement (an alternative explanation is that this category lack TRD, B and A rearrangements with only TRG) and the GL category in which all TR loci are germline with at most some partial TRB rearrangement detected in some cases.

1.12 Signalling through the TCR

The pre-TCR, TCR and its associated signalling is crucial for thymocyte development, egress from the thymus, T cell function and survival, all of which is mediated by antigen recognition. For productive stimulation and activation of the T cell to take place, a 3-step process is required: (1) antigen recognition (via MHC), (2) co-stimulation (via CD28), (3) autocrine cytokine-mediated differentiation, proliferation and expansion (via, IL-2, IL-6, IL-12, TGF beta and IL-4). Out of sequence signalling from cytokines can lead to cessation of CD4+ mediated immunity, or the presence of signal one alone can lead to T cell inactivation and deletion (Sckisel , et al., 2015). Cell surface expression of the TCR is dependent upon its association with CD3γε, δε and ζζ chains which anchor the αβ heterodimer into the plasma membrane (Kuhns, et al., 2006). Once activated by a ligand or peptide recognition, a conformational change in the TCR occurs prompting signal transduction (Kuhns, et al., 2006).

Stimulation of the TCR/CD3 complex results in the activation of lymphocyte specific protein tyrosine (lck) and Fyn, (Src family tyrosine kinases) which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMS) on the CD3 chains. SH2 domains of spleen tyrosine kinase (Syk) and ZAP-70 recognise the phosphorylated ITAM domains, which are then activated by Lck phosphorylation. Syk family kinases then activate downstream targets such as Linker of activated T cells (LAT) (Burbach ,

et al., 2007) (Figure 1.7) transducing signals towards increasing calcium levels, activation of ERK and transcription of the IL-2 promoter with up-regulation of CD25 providing the ability for autocrine signalling via IL2. The T cell proliferates, undergoes clonal expansion and differentiation into effector or memory T cells. Effector cytotoxic T cells exert their effects through cytotoxic molecules such as: perforin, granzymes, granulysin, Fas ligand along with IFN-γ, LT-α, TNF-α cytokines which induce apoptosis in target cells (Anderson , et al., 2006).

There is some limited evidence that T cell lymphomas might be driven by TCR signalling. For example, a mouse model of PTCL, has been shown to be dependent upon an intact TCR for cellular transformation and tumour development via proximal signalling activity (Wang & et al, 2011). Indeed, the majority of PTCLs maintain TCR expression along with proximal signalling molecules including LCK, ZAP-70, LAT and ITK suggesting that the outcomes of TCR-dependent signalling provide growth and survival signals for these cases (Wilcox, 2016).

Activation of T cells via the TCR leads to internalisation of the receptor mediated by LCK and PKC. This internalisation can also occur in resting naïve T cells resulting in two separate pathways for TCR endocytosis: the ligand-induced pathway (antigen engagement) and the constitutive pathway (resting naïve T cells). In resting T cells the TCR cycles between the plasma membrane and intercellular compartments with 70-85% of this pool being on the cell surface where as 15-30% is located within the cell. Internalisation upon antigenic engagement not only removes the TCR from the cell surface but also maintains continuously signalling during the activation process (Dietrich , et al., 2002) (Minami, et al., 1987) (Willinger, et al., 2015). In the periphery, naïve T cells require a basal level of signalling in order to remain viable, referred to as tonic signalling. Tonic signalling is essential for naïve T cell survival

and function mediated by signals received from self- peptide presented by MHC molecules, encountered during positive selection in the thymus and the production of IL-7. Key to maintaining this are Lck and FYN in which Lck in particular is continuously active via interactions with the TCR ζ chain (Brownlie & Zamoyska, 2013), (Stefano , et al., 2002) (Tanchot , et al., 1997) (Surh & Sprent , 2008) (Seddon & Zamoyska, 2003) (Seddon & Zamoyska, 2002).

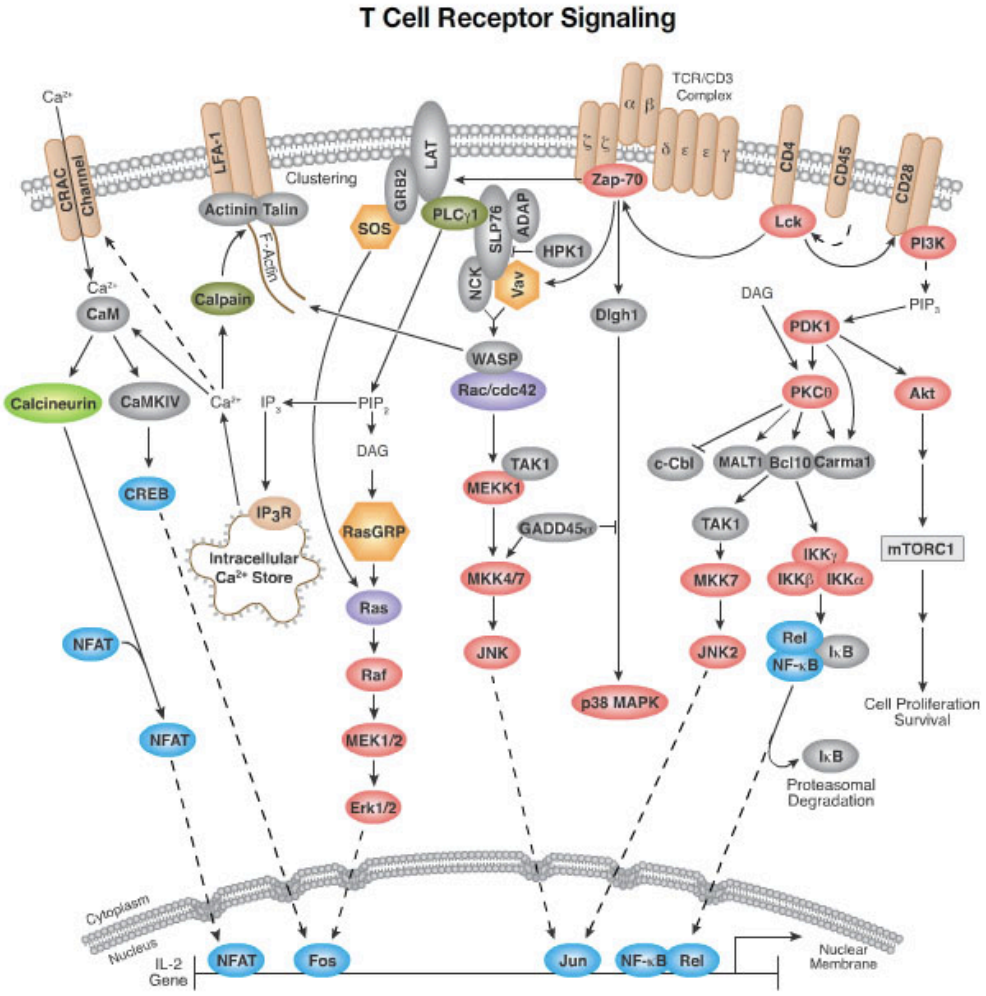


Figure 1.7: TCR receptor-signalling pathways. *TCR associates with the CD3 complex and either CD4 or CD8 (dependent upon the cell and the class of MHC presented by the APC) and accessory proteins are recruited. ITAMs on the CD3 ζ and ε chains are phosphorylated by LCK. ZAP70 binds to these sites, becomes active and phosphorylates LAT. This leads to the recruitment and activation of mitogen activated protein (MAP) kinases, ERK and p38 as well as PI-3 Kinase pathways and ultimately*

transcription factors including nuclear factor kappa B (NFκB) and nuclear factor of activated T cells (NFAT) (Smith Garvin , et al., 2009).

1.13 Regulation of TCR signalling

The degree of signalling mediated via the TCR is essential for maintenance, selection and function. Therefore, regulation of TCR signalling is an essential cellular requirement. Regulation can occur via signals transduced from cell surface molecules such as CD5 or direct regulation through proximal signalling or components further downstream (Perez-Villar, et al., 1999). It is also a requirement to ensure that cells are not overstimulated and that the TCR can distinguish between antigens of varying avidity for which recognition is mediated via the CDR3 region subsequently dictating the level of activation (Hofmann , et al., 2004). Activation is rapid, whereby phosphorylation occurs in seconds to minutes but sustained signalling is required to induce effector functions such as proliferation and differentiation (Poltorak , et al., 2013).

Responses to ligands of different stimulatory strengths is dependent upon affinity, the rates of association and disassociation from the pMHC complex and the half-life of TCR-MHC interactions. Encounters with strong ligands has a rapid association–disassociation rate, with a short dwell time allowing for binding of multiple TCRs. However, for weak stimuli, association-disassociation rates are slower with a longer half life (Huang , et al., 2010). Signalling patterns of the TCR also vary dependent upon the ligand strength, for instance, weak ligands alter ζ chain phosphorylation impacting ZAP70 phosphorylation and activation affecting the ability of ZAP70 to associate with the TCR complex (Sloan-Lancaster , et al., 1994) (Madrenas, et al., 1995).

The majority of negative regulation occurs through interference with proximal signalling. Negative regulators of TCR signalling include SHP-1, DOK1 and DOK2, HPK1, STS1 and STS2, and PTPN22 all of which exert their effects mainly via inactivating/dephosphorylating ZAP-70 and LCK and other components of the signalosome (Acuto , et al., 2008); PTPN22, for example, dephosphorylates the activating TYR394 residue of LCK (Acuto , et al., 2008) (Cloutier & Veillette , 1999). Cell surface regulators of TCR signalling, such as CD5, also function via SHP-1 in response to TCR signalling (Perez-Villar, et al., 1999).

Patient tumours, cells lines and tumours derived from NPM-ALK mouse models all lack expression of the cell surface TCR complex, even in models forcibly expressing TCRs via transgenesis. (Malcolm , et al., 2016) (Chiarle , et al., 2003) (Alexander & Turner , 2005) Cell lines and patient samples lose the expression of proximal signalling molecules, although the TCR signalling cascade is upregulated more distally by NPM-ALK (Bonzheim , et al., 2004) (Turner , et al., 2007), thus linking a potential incompatibility between NPM-ALK and a functional TCR. The mechanisms of receptor loss are unknown and may be due to interferences with receptor recycling and internalisation or the promotion of negative regulatory molecules of proximal signalling. Or death by negative selection of cells which signal through TCR and NPM-ALK.

1.14 ALCL and the TCR

As described above, ALCL cases rarely express a TCR and lack expression of TCR proximal signalling proteins as a consequence of epigenetic silencing, this however is largely in cells lines and may be an *in vitro* artefact. However, it is also known that

NPM-ALK can compensate for this by mimicking TCR-induced signalling through activation of the RAS-MAP Kinase pathway inducing AP-1/NFAT proteins (Warner , et al., 2013) (Turner 2007) as well as promoting signalling through PI3-K (Bai & et al., , 2000). However, previous data also show that TCR rearrangement is abnormal in approximately 42% of primary tumours and that NPM-ALK can compensate for this by driving apparent T cell development (Malcolm , et al., 2016). Regardless, in murine models, peripheral T cell lymphomas do not develop unless a transgenic TCR is expressed, suggesting that a TCR of some form is required for thymic exit. However, in this model system (absence of a tg TCR), tumours are restricted almost exclusively to the thymus in contradiction to the peripheral presentation of human ALCL tumours. This tumour phenotype can be overcome if NPM-ALK is expressed throughout thymic development with co-expression of a transgenic TCR, namely the OTI TCR via back-crossing NPM-ALK to OTI transgenic mice (Malcolm et al.,).

Therefore, it could be hypothesised that NPM-ALK expression initiates in a primitive haematopoietic cell, most likely an ETP, enabling cells with aberrant TCR to survive, as well as mimicking TCR signalling pathways, accounting for thymic emigration and the development of a peripheral disease. However, it is not clear if stimulation through the TCR prior to down-regulation also contributes to disease pathogenesis, for example via an infectious agent. It should also be noted that 14% of ALCL cases analysed where shown to have germ line TCR chain rearrangements with some partial TCRB chain rearrangements, suggesting that the TCR may be not play a role in all ALCL cases where disease may have arisen from earlier stages of T cell development, However the majority of ALCL cases analysed (86%) did show evidence of TCR loci rearrangements (Malcolm , et al., 2016).

1.15 Infectious aetiologies of lymphomas

The association of infectious agents and the increased risk of cancer is not a new concept although proving certain cancers to have an infectious aetiology, or linking specific infectious agents to cancer type has evolved slowly (known organisms are summarised in table 1.3). The hypotheses by Kinlen and Greaves have linked or attempted to link an infectious aetiology to childhood leukaemia (Greaves , 1999) (Hausen , 2009). Kinlen's hypothesis states that: when widespread infections from urban areas are carried to less exposed rural areas this sudden exposure could elevate the risk of leukaemia (Zur Hausen , 2009). Greave's Hypothesis, states that the combination of a failed response of an immature immune system contributes along with the presence of an acquired chromosomal translocation, possibly from the prenatal phase (Greaves , 1999). It has been suggested that *in utero* exposure to viral infections can play a role in increasing the probability or incidence of leukaemia and NHL. In evidence, prolonged maternal infection with influenza and varicella viruses during pregnancy have been associated with 4 cases of leukaemia and 2 of NHL (Roman, et al., 1997) .

Studies have shown B cell lymphomas to be driven by extensive stimuli and activation (e.g. MALT lymphoma) (Van Vilerberghe & Ferrando, 2012). The association of an infectious aetiology to ALCL is yet to be proven and the WHO classification of tumours of haematopoietic and lymphoid tissue 2008 states that ALCL is "consistently negative for Epstein-Barr virus (EBV)". However, there have been some cases reported whereby an ALCL patient has shown strong positivity for EBV; 64 cases of ALK-ve T or null cell phenotype demonstrated CD30 expression along with positivity for EBV (Ma & et al.,, 2010). In addition, there is evidence pointing towards antigenic stimuli in ALK negative cases for instance there have been

correlations with breast implants (Aladily & et al.,, 2012) HIV infection (Perez, et al., 2010) and autoimmune disease (Smedby & et al.,, 2008). In addition, ALK+ cutaneous ALCL cases have been linked to insect bites (Piccaluga, et al., 2000) (Lamant & et al.,, 2010). However, there are still no known aetiological risk factors associated with ALK+ ALCL, although the same disease presentation to ALK- cases (i.e. an activated T cell immunophenotype) suggests that antigenic stimulation may play a role.

Type	Organism	Cancer
Virus	Human Herpes virus 8	Kaposi's sarcoma, primary effusion lymphoma and related DLBCL, MCF-associated plasmablastic NHL
	Hepatitis B	Hepatocellular carcinoma
	Hepatitis C	Hepatocellular carcinoma
	Human papilloma virus; Types 16 and 18	Cervical carcinoma
	Epstein Barr virus	Hodgkin's lymphoma
		Burkitt's lymphoma
		Nasopharyngeal Carcinoma, Burkitt's lymphoma, AIDS-associated NHLs post-transplant, Lymphoproliferative disorder, extra nodal natural killer/ T-cell NHL
	Human immunodeficiency virus	AIDs associated NHL
	Human T cell leukaemia virus	Adult T-Cell leukaemia/lymphoma
Bacteria	*Helicobacter pylori*	Gastric cancer, gastric MALT NHL
	Salmonella typhi	Gall bladder cancer
	Chlamydia psittaci	Lung cancer
		Ovarian cancer
		Ocular lymphoma, MALT NHL
	Mycobacterium tuberculosis	Lung carcinoma, Kaposi's sarcoma
	Tropheryma Whippelii	Lymphoma
	Borrelia afzelli	Cutaneous MALT NHL
Parasites	*Schistosoma spp*	Bladder, colorectal, hepatocellular carcinoma
	Liver flukes	Cholangiocarcinoma (cancer of the bile duct)
	Plasmodium Falciparum	Burkitt lymphoma

Table 1.3: Summary of infectious agents implicated in tumorigenesis: adapted from (Engles , 2007)

NHL= Non-Hodgkin lymphoma, DLBCL=diffuse large B cell lymphoma.

1.16 The Hygiene hypothesis

It is evident that direct and indirect effects from infections can increase the risk of cancer contributing to tumorigenesis. It has been hypothesised for some time that early exposure to infections/ microorganisms, during the early stages of life, may in fact be protective against tumorigenesis. The Hygiene hypothesis revolves around the idea that in today's environment, especially in more developed and westernised cultures, the increase in allergies, autoimmunity and cancer prevalence are due, in part, to a lack of exposure to infectious agents thus linking an inverse association with socioeconomic status and cancer development (Oikonomopoulou, et al., 2013). This lack of exposure is postulated to be due to a limited interaction with others during the early stages life resulting in a lack of exposure to microorganisms and antigens, in a way an immunological isolation. A key study that supports this theory is the incidence of B cell precursor acute lymphoblastic leukaemia (ALL) and association with children attending day care centres. This study showed a reduced incidence of ALL developing in children which attended day care in the early years of life (Gilham , et al., 2005) (da Rocha Pavia Maia & Filho , 2013). The explanation for this was thought to be that children are more likely to be exposed to a variety of infectious agents and microorganisms which may present with asymptomatic responses and therefore are not noted. Greaves postulates this lack of exposure and a consequent lack of "protection" against ALL development comes first from the inadequate priming of the naïve immune system and then continuous susceptibility to infections, leading to immune response dysregulation due to the lack of initial "priming" to common agents (Greaves , 2006).

1.17 Modelling ALCL

Mouse mimics of human cancer have been pivotal to gaining greater insight into the genetic influences of disease. The development of a mouse model representing ALK+ ALCL has been problematic. Primary attempts to model ALCL in mice focused upon the generation of chimeras whereby bone marrow was transduced to express NPM-ALK before adoptive transfer into irradiated recipient mice. However, this approach gave rise to B cell lymphomas, malignancies of myeloid origin and plasmocytomas but did prove the oncogenic potential of NPM-ALK *in vivo* (Kuefer , et al., 1997) (Miething , et al., 2003). It was not until NPM-ALK bone marrow cells were transduced into an IL-9 deficient mouse that lymphoblastic T cells were observed in the mediastinum (Lange , et al., 2003). These observations indicated a role for the T cell developmental compartment itself and not the haematopoietic compartment to give rise to T cell lymphomas driven by NPM-ALK. A number of transgenic mice have also been developed driven by the vav and CD2 promoters in mice which developed B cell lymphomas (Turner , et al., 2003) (Turner , et al., 20006). A cre-lck promoter chimeric model produced thymic T cell lymphomas with CD30 expression (Illert , et al., 2017). Chiarle et al developed a murine model which expressed NPM-ALK under the CD4 promoter, therefore restricting NPM-ALK to T cells. This model still did not fully mimic ALK positive ALCL, with largely thymic-restricted tumours although cells did express CD30 (Chiarle , et al., 2003). However, this model represents the closet mimic to date (at that time) of ALCL and indicated that NPM-ALK had to be expressed in cells which were already committed to the T cell lineage in order to develop T cell over B cell lymphoma. In our hands, Chiarle's CD4/NPM-ALK model produces thymic tumours of a DP or CD4SP phenotype and do not express a TCR nor CD30, a slight change from the observations of Chiarle et al,

although this may be due to a number of factors including the status of housing facilities and their cleanliness. When this model was placed onto a RAG knockout background minimal alterations in disease phenotype and progression were observed with a slight delay in disease generation and a DP phenotype. Due to the restriction of disease to the thymus, in both models, it was though that there was a requirement for the expression of a functional TCR to permit thymic egress and establish peripheral disease. By backcrossing the CD4/NPM-ALK model onto a TCR tg OTI mouse model, whereby CD4/NPM-ALK is expressed on a MHC class I restricted TCR tg mouse background. This gave rise to peripheral T cell lymphomas of a null cell phenotype (CD8-/CD4-/TCR complex-) with markers of activation (i.e. CD25) and hallmark cells indicating that a functional TCR was required for thymic egress.

1.18 What is the ALCL origin story so far?

So far only a few pieces of the ALCL origins puzzle have been identified and the postulated theory of lymphomagenesis in ALK+ALCL is displayed in Figure 1.8 and can be summarised as follows: 1) The t(2;5) translocation is acquired *in utero*, or in the thymus, 2) an ETP signature has been identified pinpointing the seeds of ALCL to be sown within the thymus, 3) mouse models have shown that NPM-ALK is capable of bypassing the β selection checkpoint, 4) despite this a fully functional αβ TCR is still required for egress, 5) human cases of ALCL show 'mature' T cells with aberrant TCR expression. 6) Tumours form in the periphery but lose expression of cell surface TCR and remove or silence proximal signalling molecules, potentially providing a link towards stimulation. This thesis aims to address the role of a CD4 skew and presence of the TCR (Chapter 3), to investigate a potential role for RAG in

lymphomagenesis (Chapter 4), to study the interplay between the TCR and NPM-ALK and finally, whether there is a role for infection in disease pathogenesis (Chapter 5). All in all, to determine potential mechanisms contributing to lymphomagenesis in peripheral ALK+ ALCL lymphomagenesis.

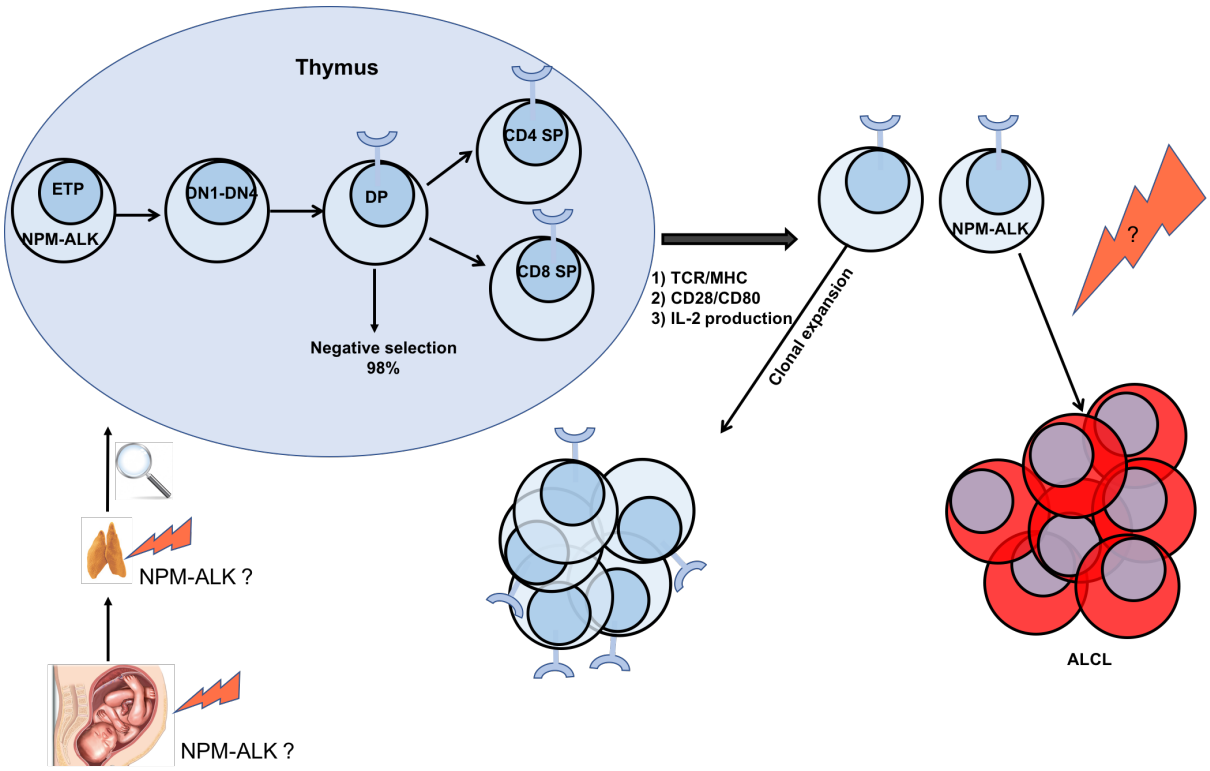


Figure 1.8: Proposed theory of ALCL development

2 Materials and Methods

2.1 Generation of transgenic mice

CD4/NPM-ALK mice (C57BL/6 45.1 or 45.2) were provided by Prof. G. Inghirami (University of Turin) as described in (Chiarle , et al., 2003). The OTII line was provided by Dr Michelle Lintermann, The Babraham Institute, the OTI line by Prof. Chris Rudd, University of Cambridge and the $RAG^{-/-}$ line by Prof. Gillian Griffiths, Cambridge Institute for Medical Research. The Marilyn line was provided by Dr Elizabeth Macintyre, The Necker institute, Paris. All mice were maintained on a C57BL/6 background and were backcrossed onto the appropriate backgrounds mostly $RAG^{-/-}$ and CD4/NPM-ALK. The OTI mouse was backcrossed onto the $RAG^{-/-}$ and CD4/NPM-ALK producing CD4/NPM-ALK/OTI and CD4/NPM-ALK/OTI $RAG^{-/-}$, respectively. OTII mice were backcrossed onto the CD4/NPM-ALK and $RAG^{-/-}$ background producing CD4/NPM-ALK/OTII and CD4/NPM-ALK/OTII/$RAG^{-/-}$, respectively. Marilyn mice were backcrossed onto the CD4/NPM-ALK background producing CD4/NPM-ALK/Marilyn mice. Mice were housed at the University of Cambridge under spf conditions under Home Office project license 80/2630.

2.2 Genotyping of mouse lines

Genotyping was performed using a proteinase K based digest solution [10% Chelex 100 w/v (Bio-Rad Laboratories, Hertfordshire, UK), 0.1% Tween-20 v/v (Sigma-Aldrich, Dorset, UK) and 100µg/ml Proteinase K (Sigma-Aldrich); samples were incubated with 50µl of this solution for 3 hours or overnight at 55°C. Lysed tissue was further disaggregated via pulse vortexing and then centrifuged. 1µl of the supernatant was used for PCR: DNA template, 1x DreamTaq PCR Buffer (Fermentas,

York, UK), 10mM mixture of deoxyribonucleotide triphosphates (dNTPs) (Fermentas), 10μM of both forward and reverse primers and one unit of DreamTaq DNA polymerase (Fermentas) to a total volume of 25μl. All PCR reactions were run using the Eppendorf Mastercycler epigradient 5 PCR machine (Eppendorf, Cambridge, UK). Tables 2.1 and 2.2 shows the programs and primers used.

Gene	Primer sequence
OTII α chain Forward	5'AAAGGGAGAAAAAGCTCTCC'3
OTII α chain Reverse	5'ACACAGCAGGTTCTGGGTTC'3
OTII β chain forward	5'GCTGCTGCACAGACCTACT-3'
OTII β chain reverse	5'-CAG CTCACCTAACACGAGGA-3'
Band size:	α chain: 160bp, β chain: 500bp
NPM (Forward of NPM-ALK)	5'-TCCCTTGGGGGCTTTGAAATA-3'
ALK (Reverse of NPM-ALK)	5'-CGAGGTGCGGAGCTTGCTCAG-3'
Band size:	120bp
Vβ2 (forward for the V segment of the TCRβ chain)	5'-GTAGGCACCTGTGGGGAAGAAACT-3'
Dβ2 (forward for the D segment of the TCRβ chain)	5'-GGGTCACTGATACGGAGCTG-3'
Jβ2 (reverse for the J segment of the TCRβ chain)	5'- TGAGAGCTGTCTCCTACTATCGATT-3'
18S (F)	GTAACCCGTTGAACCCCATT
18S (R)	CCATCCAATCGGTAGTAGCG
Band size	800bp
Marilyn F	3'TGCTGTCTGTACCACCAGAAATAC5'
Marilyn R	3'GCAGAGGAACCTGGGAGCTGT5'
Band size	350bp
OTI F	5′- ACGTGTATTCCCATCTCTGG -3′
OTI R	5′- CTGTTCATAATTGGCCCGA -3′
Band size :	238 bp
Rag 2,1	3'CCTTAATTCAACCAGGCTTCTCACTT 5'
Rag B 2,3	3'TAGCCTGCTTATTGTCTCCTGGTATG 5'
Neo A	3'CCAACGCTATGTCCTGATAGCGGT5'
Band size:	WT 970bp, KO 1100bp

Table 2.1: Primer sequences

OT1	95°C 2mins 95°C 1 min 57°C 1 min — x35 cycles 72°C 1 min 72°C 5 mins
OTII	94°C 1min 94°C 30s 55°C 1min — x35 cycles 72°C 1min 72°C 1min
NPM-ALK	94°C 5min 94°C 30s 56°C 30s — x35 cycles 72°C 30s 72°C 5min
RAG 2	94°C 5 min 94°C 30 s 60°C 30 s — x35 cycles 72°C 1 min 72°C 10 min
TCRβ rearrangements	95°C 7 min 95°C 30s 60°C 30s — x34 cycles 72°C 30s 72°C 10min
18s	94°C 2min 94°C 30s 59°C 30s — x 23 cycles 68°C 30s 72°C 2 min
Marilyn	94°C 5min 94°C 30s 60°C 30s — x 35 cycles 72°C 1min 72°C 10min

Table 2.2: PCR programs for genotyping and experiments

2.3 MHV-OVA infection

CD4/NPM-ALK/OTI mice were infected with a Murine gamma herpes virus expressing the ovalbumin peptide (SIINFEKL) for which the OTI receptor is specific (Smith , et al., 2007). Virus was administered to pregnant and pre-tumorigenic mice at a concentration of 4E8 pfu/ml via intranasal (10µl/nostril) and I.P administration (10µl of virus per 100µl of PBS). The virus administration protocol is illustrated in figure 1.

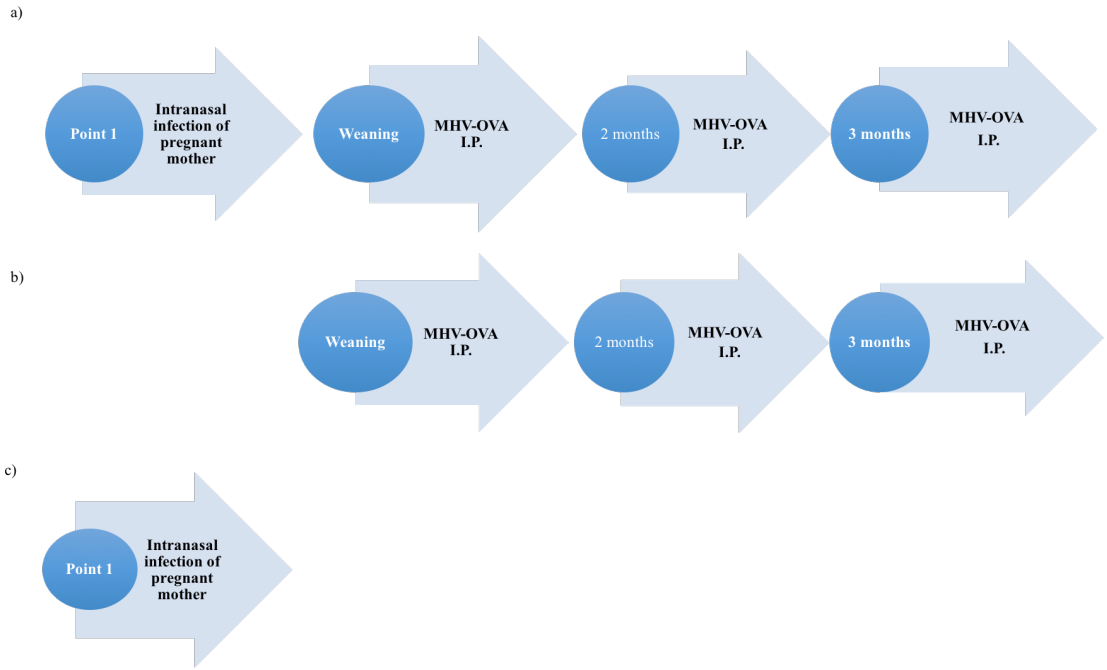


Figure 2.1: Administration of MHV-OVA to CD4/NPM-ALK/OTI mice

Surface Marker	Clone	Isotype control
CD4 PE	RM4-5	Rat IgG2a PE
CD30 PE	mCD30.1	Hamster IgG1 PE
CD25 PE	PC61 / 3c7 (DC)	Rat IgG1 PE
CD8 PE	53-6.7 (DC)	Rat IgG1 PE
CD3 FITC	145-2c11	Hamster IgG1 FITC
CD4 FITC	RM4-5	Rat IgG2a FITC
CD8 FITC	53-6.7	Rat IgG2a FITC
CD117 FITC	2B8 or ACK2	Rat IgG2b FITC
CD44 APC	IM7	Rat IgG2b APC
CD8 APC	53-6.7	Rat IgG2a APC
TCRβ FITC	H57-597	Hamster IgG FITC
Vα2 PE	B20.1	Rat IgG2a PE
Vα2 PerCP-Cy5.5	B20.1	Rat IgG 2a PerCP-Cy 5.5
TCRβ PerCP-Cy5.5	H57-597	Armenian Hamster IgG PerCP-Cy5.5
CD3 APC	145-2C11	Rat IgG2b k
CD25 FITC	PC61	Rat IgG1 FITC
Notch 1 PE	HMN1-12	Armenain Hamster IgG PE
CD71 PE	RI7217	IgG2a PE
CD98 PE	RL388	IgG2a PE
CD8 PE/Cy 7	53-6.7	Rat IgG2a pecy7
CD4 PE/Cy 7	RM4-5	Rat IgG2b pecy7
CD28 FITC	E18	Rat IgG2b k FITC
CD69 PE	H1.2F3	Armenian hamster IgG PE
CD5 APC	53-7.3	Rat IgG2a APC
NOTCH PE	HMN1-12	Armenian Hamster IgG PE
CD62L brilliant violet 421 ™	MEL-14	Rat IgG2a k brilliant violet 421™
CD62L PE	MEL-14	Rat IgG2a k PE
Vβ6 PE	RR4-7	Rat IgG2a PE
CD25 APC	PC16	Rat IgG1 APC
B220 PE	RA3-6B2	Rat IgG21 PE
CD19 APC	6D5	Rat IgG2a APC

Table 2.3 Primary antibodies used in FACS analysis of mouse tissue

2.4 Fluorescent-activated cell sorting (FACS) analysis

Single cell suspensions were generated from the acquired tissue by passing the sample through a 70μM cell strainer. The cell count was determined following trypan blue exclusion and cells re-suspended at a concentration of $1x10^7$ cells/ml in FACS buffer (Phosphate Buffered Saline (PBS) with 0.1% Bovine Serum Albumin (BSA) (w/v)). Cells were incubated on ice, in the dark for 45 minutes, with rat and hamster anti-mouse antibodies conjugated to phycoerythrin (PE), Flourescein isothiocyanate (FITC), Allophycocyanin (APC), PerCP-Cy5.5, PE/Cy7, specific for various T cell markers as listed in table 2.3. Concentrations used were determined by antibody titration with their corresponding isotype controls. Typically, 1μl of antibody was used per sample ($1x10^6$ cells in 100ul FACS buffer) apart from CD25 (2μl) and TCRβ (0.5μl) depending on the specific antibody titration. Antibodies were purchased from BD biosciences (Oxford, UK) and Biolegend (San Diego, US). After incubation cells were washed in FACS buffer and samples collected by centrifugation at 1200rpm for 5 mins then re-suspended in 300μl of FACS buffer. Samples were analysed recording 10,000 - 20,000 events on the BD accuri C6 flow cytometer (BD Biosciences). Data was interpreted using FlowJo® software (LLR) where gates were drawn in accordance with isotype controls. Samples were gated according to their corresponding isotype control.

2.5 Intracellular staining

For intracellular staining, cells were fixed in 0.01% formaldehyde for 15 min, then membranes disrupted using 100µl of 0.5% Tween-20 v/v in PBS and incubated in the dark at RT for 15 min. Cells were then washed in 2mls of 0.1% triton in PBS and washed at 2,000rpm for 5 min and then pellets were re-suspended in 100µl PBS containing 0.1% triton X-100. Cells were incubated with the appropriate antibody for 45 minutes in PBS containing 0.1% triton, cells were washed in FACS buffer and then re-suspended in 200µl of FACS buffer and analysed via the C6 accuri (BD biosciences) collecting 10,000 events and data were interpreted using FlowJo® software (LLR) where gates were drawn in accordance with isotype controls.

2.6 Analysis of thymocyte development in pre-tumour mice

Thymi from 5-week-old mice were taken and analysed via flow cytometry looking at the effects of NPM-ALK during thymocyte development, in pre-tumour mice. Cells were prepared and stained as previously mentioned. CD8, CD4 negative populations were gated on and from here thymocyte development was observed by distinguishing the individual developmental stages (DN1-DN4) using CD44 and CD25. From here cells were stained with markers for the OTII receptor Vα2 and TCRβ to look at the expression of the receptor during the developmental stages along with activation indicated by CD69. Compensation was determined using OneComp™ ebeads (eBiosciences) stained with each antibody used. Samples were then analysed using the BD Fortessa (BD Biosciences) gating on live cells recording 100,000 events. Results were analysed using FlowJo® software (LLR).

2.7 Detection of TCR protein production

RNA was extracted from $5X10^6$ tumour cells using the Qiagen RNAeasy mini kit (Qiagen) according to manufacturer's instructions. RNA was used to make cDNA using Superscript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. This included DNTPs (10mM) (New England Biolab) (RNase OUT (ThermoFisher) and random hexamer primers (100μM) (ThermoFisher), including a DNase step: Turbo DNase (Invitrogen). PCR was conducted on the resulting cDNA utilizing programs and primers corresponding to the protein being assessed (for this thesis OTI, OTII and RAG) using PCR programs listed in (Table 2.2, Table 2.1). 18s controls and PCR for the target of interest were performed on cDNA from both the RT+ and RT- reactions. PCR products were separated by electrophoresis on a 1.5% (w/v) agarose/TAE (Roche) gel containing 0.5mg/ml ethidium bromide (Sigma-Aldrich).

2.8 TCRβ rearrangements

DNA was extracted from $5x10^6$ tumour cells or thymocytes using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. DNA (100ng) was added to PCR reactions that consisted of 1x DreamTaq PCR buffer (Fermentas), 10mM mixture of dNTPs (Fermentas), 10 μM each of either Vβ2 or Dβ2 forward primers and Jβ2 reverse primer (for the V-J and D-J reactions respectively) (see Table 2.1), and one unit of DreamTaq DNA polymerase (Fermentas) in a final volume of 25μl. The PCR reactions were performed in an Eppendorf Mastercycler epgradient 5 PCR machine (Eppendorf). The PCR products were separated by electrophoresis on a

1% (w/v) agarose/TAE (Roche) gel containing 0.5mg/ml ethidium bromide (Sigma-Aldrich).

2.9 Mouse Stimulation assay: MTT

96 well plates were coated (in the appropriate wells) with LEAF™ anti-mouse CD3e (145-2C11) (Biolegend) or NA/LE anti mouse CD3e (BD biosciences) at a concentration of 10μg/ml in PBS for which a 96 well plate was coated with 200μl per well of the CD3e PBS solution and left overnight at 4°C then plates were washed with PBS before seeding. Lymph nodes and spleen were extracted from pre-tumour mice, a single cell suspension was made and red blood cells lysed using RBC lysis buffer (420301) (Biolegend). Cells were re-suspended at $1x10^{6}$cells/ml in cell culture media (RPMI supplemented with 10% FBS, 1% antibiotics and 2-mercaptaethanol (0.01mM)) and cells were seeded at $5x10^{5}$cells/well. LEAF™ Anti-mouse CD28 (biolegend) or NA/LE anti-mouse CD28 (BD) was added to wells coated with CD3 at a final concentration of 2μ/ml or OVA peptide at a final concentration of 5μ/ml and incubated for 24 hours at 37°C, 5% CO2. MTT reagent (Sigma) was prepared at 5mg/ml and protected from light in the fridge. 10μl of MTT was added to each well to achieve a final concentration of 0.45mg/ml and incubated at 37°C for 4 hours before 100μl of solubilisation solution was added (1:1 ratio of DMF to dH20, 20% SDS PH 4.7) and left at 37°C, 5% CO_2 overnight. Absorbance at 570nm was measured using a SpectramaxI3 (Molecular devices).

2.10 Proliferation assay: CFSE

Coated plates were prepared as described for the MTT assay. A single cell suspension was made from lymph nodes and spleens and $5x10^7$cells were re-suspended in 10 mls of pre-warmed 37°C sterile 0.1% BSA in PBS. 5µl of CFSE Cell trace (Invitrogen) stock solution (powder in 18µl of DMSO) was placed on the side of a 50ml falcon and vortexed into 10ml of cells to a final concentration of 0.5µg/ml. Cells were incubated at 37°C for 15 mins and then 20ml of cold 0.1%BSA/PBS was added before incubating for 5 mins on ice to inhibit staining. Cells were washed twice with RPMI 1640 (10% FBS+ 1% P/S + 0.01mM 2-ME Sigma). Cells were re-suspended at a concentration of $4x10^6$cells/ml and seeded at $2x10^5$ cells/well in 50µl of media. 50ul of media or media containing CD28 (2µg/ml), OVA (5µg/ml) and OVA peptide epitopes were added to the corresponding well OVA 5µg/ml (Invivogen), G4 5µg/ml (SIIGFEKL, Anaspec), E1 5µg/ml (EIINFEKL, Sigma) and incubated for 72 hours at 37°C in 5% CO_2. Cells were washed and re-suspended in FACS buffer and stained for CD8 (APC) and CD25 (APC) as previously described for flow cytometry analysis.

2.11 Proximal signalling analysis

Plates were prepared for stimulation as mentioned in 2.9. Cells were seeded at $2x10^6$/well in a 96 well plate, in RPMI supplemented with 10% FBS 1% P/S. Anti-CD28 (2µg/ml), OVA (5µg/ml) and OVA peptide epitopes were added to the appropriate sample (OVA 5µg/ml (Invivogen) G4 5µg/ml (SIIGFEKL, Anaspec) E1 5µg/ml (EIINFEKL, Sigma)). Cells were collected by centrifugation for 5 mins at 37°C @1400rpm and then incubated at 37°C for 25mins. Cells were then removed, collected by centrifugation and lysates made by incubating pellets in RIPA lysis

buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS and 4mM EDTA) with a protease inhibitor cocktail (P8340 Sigma) for 10 minutes on ice. Samples were then subject to centrifugation at 12,000rpm for 10 minutes and lysates removed and stored at -80°C, ready for western blot analysis.

2.12 Western Blot

Lysates were thawed on ice and re-suspended in an equal volume of 2X SDS loading buffer (Laemmli's loading buffer 62.5mM Tris-HCL pH6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue) containing 2-Mercaptoethanol (5% (1.43 M)) and boiled at 100°C for 5 mins. Samples were loaded into a 10% polyacrylamide SDS-PAGE gel with precision plus dual colour protein marker (Bio-rad) and run in SDS running buffer (250mM Tris, 1.92M Glycine, 1% SDS) at 100v for around 2 hours.

Protein was then transferred onto Immobilon-P membrane PVDF, 0.45um (Merck) (activated in MeOH for 5 minutes prior to transfer) using a wet apparatus (Bio-rad) in transfer buffer (1.92mM Glycine, 25mM Tris, 0.01% SDS), for 1 hour at 100 volts. Membranes were washed in TBST (TBS (10X solution, 0.5M Tris, 1.5M NaCl pH 7.5) TBST (1X TBS with 0.1% Tween)) for 3x5 minutes and blocked in blocking buffer (5% BSA in TBST). After blocking blots were washed 3x in TBST and incubated over night at 4°C in blocking buffer with either pALK (Y1604, 3341) (Cell Signalling), pZAP70 (Tyr 319/syk tyr 352) (2701) (Cell Signalling), pLAT (Y132) (ab4476 Abcam) or pERK (44/42, Thr 202/Tyr 204) (Cell Signaling) rabbit antibodies. Blots were washed 3x5 mins in TBST and incubated with secondary antibody anti-rabbit HRP Mouse Immunoglobulins (polyclonal swine anti-rabbit immunoglobulin HRP PO21702-2 Agilent technologies), for 1 hour at RT, then

washed 3x5min in TBST and exposed to Immobilon western chemiluminescent HRP substrate (Merck) and analysed using a Fujifilm LAS-4000 imager and Aida image analyser (Raytest). Membranes were washed in TBST and then stripped for 30minutes at 60°C with rocking with stripper buffer (pH 6.8, Tris (62.5 mM), SDS (2%) 2-ME 70µl in 10ml). Blots were washed 3x 5 min in TBST then blocked with 5% BSA TBST for 1 hour before incubating with unphosphorylated antibodies, in blocking buffer at 4°C overnight, for ALK (D5F3), ZAP70 (DICI0E), LAT (9166) ERK (9102) all Cell Signaling. Blots were additionally stained for β-actin (AC-15 ThermoFisher) as a loading control. Followed by secondary anti-mouse HRP (Rabbit Anti-mouse, Polyclonal, HRP. Ig fraction, Agilent Technologies, P016102-2) (Agilent Technologies).

2.13 Histopathology analysis and slide preparation of mouse tissue

Sections were made from mouse samples by Helen Skelton in the Histopathology department (University of Cambridge). Immunohistochemistry and analysis was performed by Prof. Lukas Kenner, Dr Simone Roos (Medical University Vienna) and Dr Katherine Hughes (University of Cambridge, Department of Veterinary Medicine).

2.14 Statistics

Data were analysed using a two-tailed T test (assuming equal variance) or log-rank test, as indicated. All tests were performed using Prism 7 (GraphPad Software, La Jolla).

3 Dissecting the phenotype of ALCL mouse models via MHC class II restricted TCR transgenic mice

3.1 Introduction

Anaplastic Large Cell Lymphoma (ALCL) is classified as a peripheral T cell lymphoma yet the specific T cell subset from which it arises is unknown. Cases often have a 'null' cell surface phenotype lacking expression of T cell-identifying cell surface proteins (yet displaying molecular TCR rearrangements and often produce cytotoxic proteins such as perforin and Granzyme B) although CD4 expression has been documented in some cases (Campo & et al., 2011). It has been speculated that ALCL tumour cells are transformed T helper (Th) cells of either a Th2, Th17 or even a Treg origin, although gene expression studies have been unable to assign them to any particular T cell lineage (Bonzheim, et al., 2004) (Eckerle, et al., 2009) (Kasprzycka, et al., 2006) (Matsuyama, et al., 2011). Expression of NPM-ALK in mice from the T cell specific CD4 promoter (which drives expression throughout thymic T cell development) gives rise to thymic lymphomas not mimicking human ALCL (Malcolm, et al., 2016) (Chiarle & et al., 2003). In this model, NPM-ALK is expressed at all stages of thymocyte development and is therefore not exclusive to CD4 single positive T cells (Malcolm, et al., 2016). However, as in human ALCL, tumours arising in these mice lack cell surface expression of the TCR complex but variably express CD4, CD8 or CD4 in combination with CD8 (Malcolm, et al., 2016) (Bonzheim, et al., 2004). As such, this is not a good model of human ALCL which, as mentioned previously is a peripheral T cell lymphoma. However, the appearance of tumours in the CD4/NPM-ALK transgenic mouse line better mimics human ALCL when a transgenic TCR is introduced, specifically when back-crossing this strain to the OTI TCR transgenic mouse.

OTI mice express a transgenic TCR specific for ovalbumin that is MHC class I restricted, thus giving rise to a skew towards CD8 T cell development, particularly when expressed on a RAG knockout background (Hogquist , et al., 1994). Interestingly, CD4/NPM-ALK transgenic mice expressing the OTI TCR generate peripheral lymphomas with hallmark cells of ALCL histologically mimicking human ALCL: Tumours lack expression of T cell specific cell surface markers such as CD8, CD4 and the TCR complex (OTI, TCRβ and CD3), but show high expression of CD25 the latter perhaps indicative of an activated T cell phenotype. As these mice develop peripheral disease, similar to that of ALCL, indicated by the presence of hallmark cells, the origin of ALCL may be a thymic T cell with aberrant TCR expression. An early thymic progenitor (ETP) signature was identified in tumour propagating cells of ALCL cell lines indicating a primitive cell of origin arising within the thymus (Moti , et al., 2015). This in combination with the detection of NPM-ALK in cord blood (1.95%) hints towards an origin within the thymus or earlier. Attempts to model ALCL in mice, by expressing NPM-ALK in the bone marrow, did not lead to T cell lymphoma generation unless NPM-ALK was restricted to the T cell lineage supporting the idea that the "seeds are sown" within the thymus. From the CD4/NPM-ALK models to date there is strong evidence to suggest that a functional TCR is required for thymic egress and peripheral disease (Malcolm, et al., 2016).

Whilst ALCL tumour cells have an ambiguous cell surface profile, they more often express CD4 than cytotoxic T cell marker proteins such as CD8. Therefore, to assess whether MHC class II restriction inducing a CD4, helper T cell skew influences the phenotype of the CD4/NPM-ALK model, CD4/NPM-ALK mice were backcrossed onto two MHC class II restricted TCR transgenic backgrounds: OTII (Tg(TcraTcrb)425Cbn) and Marilyn (Tg(TcraH-Y, TcrbH-Y)1Pas). The OTII transgenic mouse was developed by Francis

Carbone and is MHC class II restricted in the context of H2-Ab1 (I-Ab), skewing T cell development to the CD4 positive helper T cell lineage. Like the OTI model, the OTII receptor is also specific for ovalbumin (SIINFEKL, OVA 323-339) (Barnden, et al., 1998). The OTII receptor is forcibly expressed during the early stages of thymocyte development, as early as DN1 and then throughout T cell development, both chains are expressed early on but can vary slightly depending on promoters or enhancers used (Hogquist, et al., 2005). The Marilyn transgenic model again has this feature, although the TCR in this model recognises the male specific H-Y antigen in the context of the MHC class II I-A^b29 protein (Lantz, et al., 2000). Therefore, male mice of this strain respond to the H-Y antigen during development with developing thymocytes undergoing negative selection resulting in a reduced number of SP T cells within the thymus and very few peripheral T cells on a RAG knockout background (Holst, et al., 2006). It could be assumed that on a RAG competent background there is a chance that this would give rise to additional endogenous TCR's in which cells would not undergo negative selection and thus the percentage of SP cells would be greater but would not carry the Marilyn receptor. As the role of Rag was to be addressed, all mouse models for these studies were placed on RAG competent backgrounds. The influence of RAG will be discussed in the next chapter.

This chapter aims to address the effects of a CD4 skew in ALCL development using the CD4/NPM-ALK mouse model. The main aims are as follows:

- Address the role of the TCR in ALCL development further
- The influence of the T cell phenotypic skew upon disease development.
- The effect of NPM-ALK on negative selection within the thymus. NPM-ALK can bypass β selection, does it affect central tolerance?

3.2 Results

3.2.1 CD4/NPM-ALK/OTII mice develop cortical thymic lymphomas that phenocopy the parental CD4/NPM-ALK transgenic line

Disease presented within the mediastinum, in the CD4/NPM-ALK/OTII model, producing thymic lymphomas, by which mice were sacrificed once they showed signs of illness (Table 3.1). The survival recorded was 98 days compared to 101 days for the parental CD4/NPM-ALK mice, this slight increase was not significant (p>0.05) (figure 3.1a). The majority of CD4/NPM-ALK/OTII mice developed lymphomas within the mediastinum (14/15) with one case presenting with a peripheral lymphoma in the spleen (1/15; 6%) (Table 3.1). Immunohistochemistry shows evidence of ALK expression within tumours which were diagnosed as lymphoma (figure 3.1b and c). These data are comparable with those obtained from the parental CD4/NPM-ALK transgenic mouse line which generated thymic CD4SP tumours (Malcolm, et al., 2016).

Mouse	Location	AGE	Diagnosis
1	Thymus	83	Lymphoma
2	Thymus	140	Lymphoma
3	Thymus	63	Lymphoma
4	Thymus	63	ND
5	Thymus	65	Lymphoma
6	Thymus	79	Lymphoma
7	Thymus	102	Lymphoma
8	Thymus	72	Lymphoma
9	Thymus	117	Lymphoma
10	Thymus	98	Lymphoma
11	Spleen	98	Lymphoma
12	Thymus	83	Lymphoma
13	Thymus	104	Lymphoma
14	Thymus	108	Lymphoma
15	Thymus	111	Lymphoma

Table 3.1: Table shows summary of disease onset and location

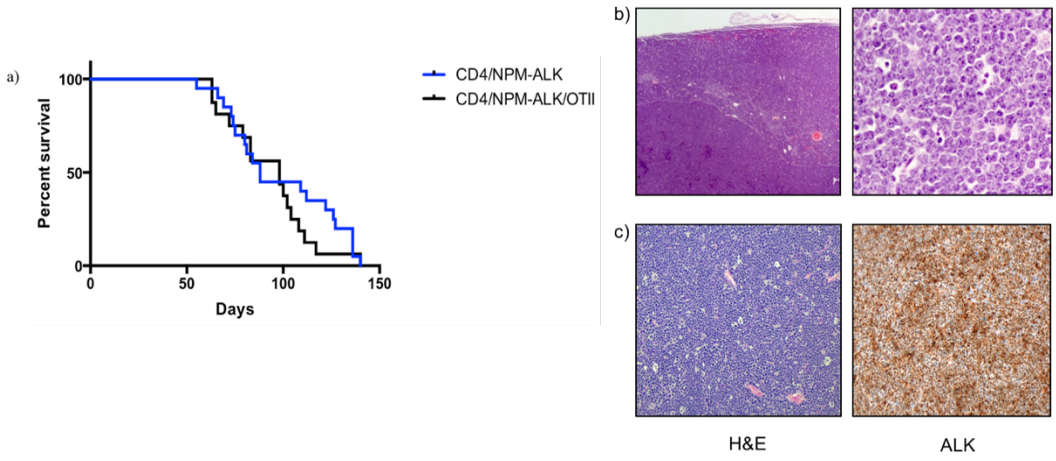


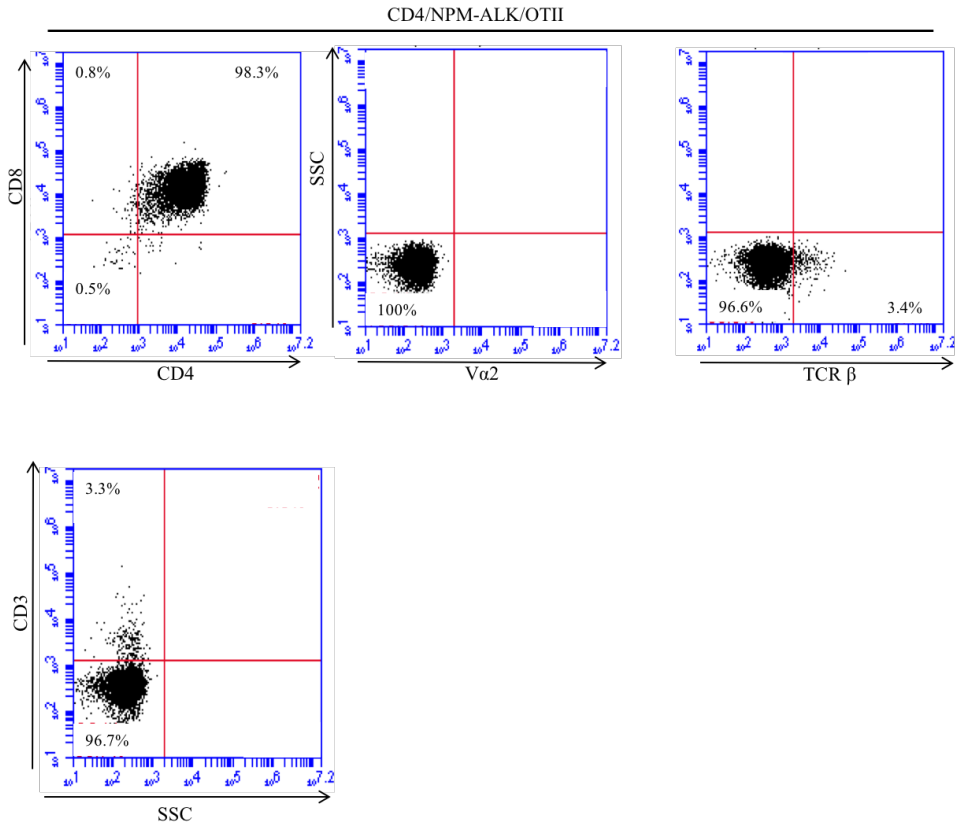
Figure 3.1: CD4/NPM-ALK/OTII mice develop cortical thymic lymphomas similar to the parental CD4/NPM-ALK transgenic line: *a) KAPLAN MEIR curve showing overall survival: CD4/NPM-ALK/OTII (n=16) and CD4/NPM-ALK (n=20) transgenic mice. CD4/NPM-ALK/OTII median survival (101 days), CD4/NPM-ALK median survival (88 days), p=0.6126 (via log rank test and a G-B-W test). (b) H&E staining of a representative CD4/NPM-ALK/OTII thymic tumour at 200X (left) and 400X (right) magnification, the latter showing many mitotic bodies indicative of rapid cell proliferation. (c) A Haematoxylin and eosin (left) and ALK antibody (right) stained section of a representative tumour at 200X magnification.*

3.2.2 Characterization of tumours derived from CD4/NPM-ALK/OTII transgenic mice

CD4/NPM-ALK/OTII tumours resembled those derived from the parental line (CD4/NPM-ALK) with regards to time of onset, location and histology. Tumours were also assessed for their cell surface expression profile by flow cytometry, staining for an extensive list of lymphoid cell lineage and differentiation status-defining proteins (Table 3.2). The predominant phenotype observed was that of a CD4SP T cell (55.56%) followed by DP (CD8+/CD4+) T cells (33.33%) and one CD8SP (Table 3.2). Tumour cells also expressed high levels of CD44, although no expression of CD30 was recorded (Figure 3.2, Table 3.2).

Tumour	CD4	CD8	CD44	CD25	Vα2	TCR β	CD30	CD3	CD4 CD8	CD44 CD25	CD117	Cell type
1	85.7	9.2	90.7	10.7	14.5	7.8	0	17.3	7.5	8.8	2.1	CD4 SP
2	61.3	25.4	85.2	1.6	80.9	24.8	0	55.7	30.3	1.9	6	DP
3	80.3	22.1	69.7	0	68.4	31.6	0	56	20.7	ND	ND	CD4 SP
4	71.1	50.3	93.3	6.7	4.4	10	10	2.2	28.6	0	76.5	CD4 SP
5	89	92.6	88.2	22.1	9.9	23.3	11.6	0	92.7	16.9	20.7	DP
6	75.5	90.1	70.4	7.6	0	5.6	0	5.6	93	5.2	11.2	DP
7	24.3	75.3	95.4	6.6	26	ND	25	20.3	23.1	12	70.4	CD8 SP
8	12.6	4.3	93	ND	14.5	ND	ND	17.4	ND	ND	ND	CD4 SP
9	69.1	20.4	96.8	0	4	ND	0	4.1	23.5	0	34.4	CD4 SP
10	89.6	60.3	94.8	4.2	15	35.2	ND	14.7	58	2.4	64.1	CD4 SP
11	9.8	0	32.9	0	3.9	43.1	0	10	5.9	0	14.4	CD4SP

Table 3.2 Expression profile of tumour cells isolated from CD4/NPM-ALK/OTII mice: *Numbers represent the percentage level of expression in 10,000 recorded events corrected by their corresponding isotype controls, gated on the tumour population. The main cell type was derived from the majority cell surface phenotype, i.e. DP = CD8 and CD4 co-expression on the majority of cells (>30%) ND=not determined.*



3.2 Flow cytometry representation of the phenotype of tumours derived from the CD4/NPM-ALK/OTII model:

(a) Flow cytometry representation of a DP tumour gated on the tumour population based on 10,000 events. Vα2 represents the α chain of the OTII receptor.

3.2.3 Pre-Tumour thymocyte development displays with a transient block at the DN3 stage

5-week-old CD4/NPM-ALK/OTII mice show an accumulation of thymocytes at the DN3 stage in comparison to NPM-ALK negative, OTII+ littermates, suggesting a delay in

development at this stage CD4/NPM-ALK/OTII DN3(22.5%+/-7.042) opposed to OTII+ DN3 (12.7% +/- 1.017) (Figure 3.3) although this is observation is not significant p=0.215.

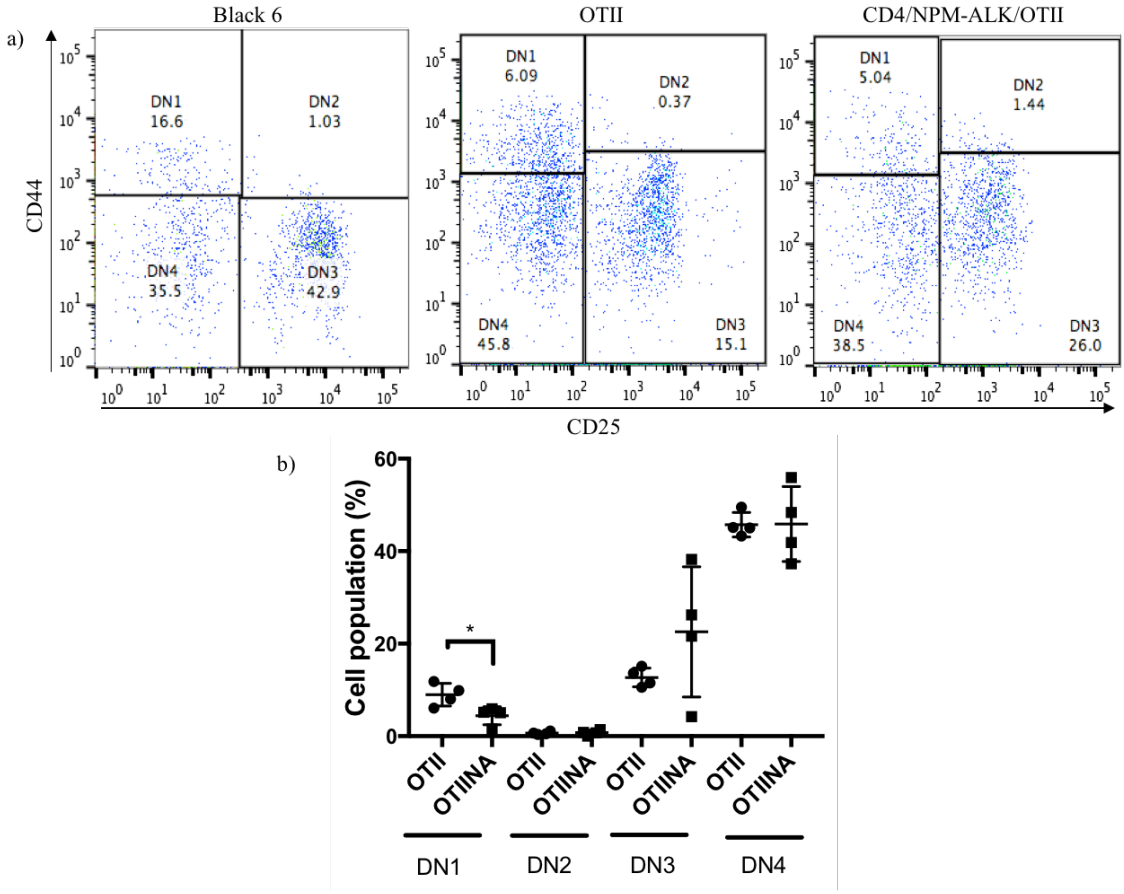


Figure 3.3: CD4/NPM-ALK/OTII mice display with a partial block at the DN3 stage
*Thymi were isolated from pre-tumour 5-week-old CD4/NPM-ALK/OTII mice and NPM-ALK negative, OTII+ littermates. DN stages were defined by CD44 and CD25 expression on gated CD4/CD8 negative cells: DN1 (CD44+, CD25-), DN2 (CD44+, CD25+), DN3 (CD44-, CD25+), DN4 (CD44-, CD25-). (a) FACS plot representation of thymocyte development representing 100,000 events collected. (b) Graph showing thymocyte development, *=0.0276 CD4/NPM-ALK/OTII vs OTII (n=4).*

3.2.4 Tumours arising in CD4/NPM-ALK/OTII mice lack cell surface OTII receptor expression but intracellular protein and OTII transcripts can be detected

Cells from established tumours in the CD4/NPM-ALK/OTII model displayed with minimal Vα2 expression (α chain of the OTII receptor). This was indicated by a low percentage of cells within the tumour, expressing this chain (4-15% (21.94%+/- 25.64)) although 2 tumours

did display with high levels (Vα2 expression at 68% and 80%) of the OTII receptor. A similar pattern was observed for other components of the TCR complex as shown by a loss of cell surface expression of TCRβ (22% +/-16.33) and CD3 (18.28% +/- 18.7) (Table 3.3). In order to determine whether this effect was tumour intrinsic peripheral T cells were also analysed. Peripheral T cell populations were determined via analysis of lymphocytes derived from spleens of tumour mice (Table 3.3). Peripheral T cells of mice that had developed mediastinal tumours showed low expression of the OTII receptor, as indicated by Vα2 (14.06% +/- 10.11), TCRβ (30.1% +/-24), CD3 (16.48%+/- 9.74). This indicates a potential loss of the receptor within the thymus, perhaps during development, which may account for the lack of peripheral disease.

To assess whether cells were still producing the OTII receptor, RT-PCR was performed using RNA derived from established tumour cells. Transcripts were detected for the OTII receptor in established tumour cells (figure 3.4a), therefore cells expressing NPM-ALK do not lose the ability to transcribe the OTII receptor. To complement this, flow cytometry was used to detect the intracellular presence of the TCR whereby the OTII receptor (Vα2) was detected within tumour cells indicated by Vα2 expression (39.6% +/-7.816, p=0.0193) and CD3 (77.5% +/- 0.306, p=<0.0001) (figure 3.4b). The OTII, receptor though not expressed on the tumour cell surface, is still produced as a protein suggesting potential interference with cell surface expression.

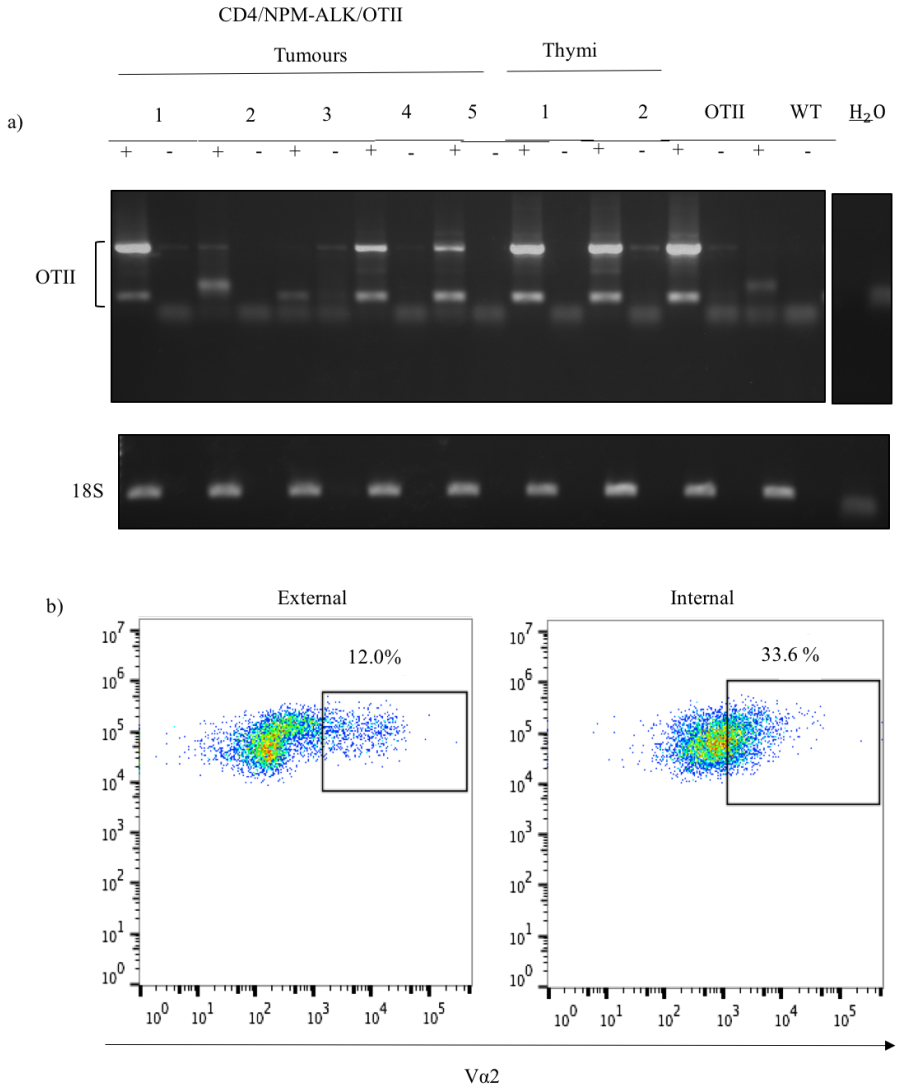
Tumour	Vα2	TCRβ	CD3
1	14.5	7.8	17.3
2	80.9	24.8	55.7
3	68.4	31.6	56
4	4.4	10	2.2
5	9.9	23.3	0
6	0	5.6	5.6
7	26	ND	20.3
8	14.5	ND	17.4
9	4	ND	4.1
10	15	35.2	14.7
11	13.7	6.3	14.1
12	12	53.6	12

Spleen	Vα2	TCRβ	CD3
2	10.7	18	4.5
6	2.8	33.6	17.4
7	11.1	ND	15.4
9	15.5	ND	13.6
10	30.2	62.2	31.5
12	ND	6.6	ND

Table 3.3:CD4/NPM-ALK/OTII tumours lack cell surface expression of the OTII TCR complex: *(a) Summary of TCR complex % cell surface expression in CD4/NPM-ALK/OTII tumours and peripheral T cells, all indicated by Vα2(OTII), TCRβ and CD3. Cell surface analysis of tumour cells showed lack of the OTII TCR complex as indicated by Vα2 (OTII), TCRβ and CD3 in the majority of samples.*

Tumour	Vα2	TCRβ	CD3
Mean	21.94	22	18.28
SD	25.64	16.33	18.7

Spleen	Vα2	TCRβ	CD3
Mean	14.06	30.1	16.48
SD	10.11	24	9.74



Tumour phenotype	Vα2 external (%)	CD3 external (%)	Vα2 internal (%)	CD3 internal (%)
CD4SP	4.4	2.2	55.1	77.7
DP	9.9	0	19.1	76.8
DP	12.0	0	33.6	78

Figure 3.4 CD4/NPM-ALK/OTII tumours lack cell surface OTII receptor expression but intracellular protein and OTII transcripts can be detected:
Summary of TCR complex cell surface expression in CD4/NPM-ALK/OTII tumours. a) OTII transcripts are detectable in tumour samples (1-4) and pre-tumour thymi (6-7) of CD4NA/OTII transgenic mice, WT= cDNA from wild type mouse thymi, OTII= cDNA extracted from OTII thymi. +/- refers to RT positive and RT negative (b) The OTII receptor is not detectable on the tumour cell surface but do express it intracellularly as determined by flow cytometry of a representative tumour n=3. All flow cytometry plots are corrected for isotype controls.

	Vα2 external (%)	CD3 external (%)	Vα2 Internal (%)	CD3 Internal (%)
Mean	8.767	0.733	39.6	77.5
SD	2.266	0.733	7.816	0.306
P			*0.0193*	*<0.0001*

3.2.5 CD4/NPM-ALK/OTII mice lose the OTII receptor during thymic T cell development

Established tumours of the CD4/NPM-ALK/OTII model presented mainly with a CD4SP phenotype and lack cell surface expression of the OTII receptor. To obtain a greater insight as to the fate of the OTII receptor during development, pre-tumourigenic thymi were obtained from CD4/NPM-ALK/OTII mice and were analysed and compared to pre-tumourigneic OTII+ NPM-ALK negative littermates. Cells of the individual developmental stages (DN1-DN4) were analysed for the cell surface expression of OTII (Vα2, TCRβ) complex expression throughout T cell development and compared to NPM-ALK negative, OTII+ littermates (Figure 3.5a, b). In wild type OTII mice the receptor is expressed as early as DN1 (63.6% +/- 9.792), expression is reduced during the DN3 (15.99% +/- 9.157) stage but recovered to the same or generally a higher level at DN4 (78.1% +/- 8.215) p= 0.0012. However, in the presence of NPM-ALK, the percentage of cells expressing the OTII receptor is decreased in comparison DN1(30.47%+/-1.576) *p=0.0301* DN2 (39.6% +/- 8.782) *p=0.3759*, DN3 (15.99% +/- 9.157) p=NS, DN4(78.1%+/-8.215) *p=0.0012*. In addition, CD4/NPM-ALK/OTII pre-tumour thymocytes display with an overall loss of the OTII receptor (DN1-DN4 p=0.0003 (figure 3.5b)) where loss of receptor is not observed in the OTII NPM-ALK negative control (figure 3.5b). For comparison OTI receptor expression was analysed on pre-tumour thymi from the CD4/NPM-ALK/OTI model, which generates peripheral lymphomas with hallmark cells. In this model pre-tumour DN cells did not lose the receptor during development instead expression was increased between DN1-DN4, p=0.0002(figure 3.5c). These results along with the detection of OTII transcripts and internal expression suggest that NPM-ALK is actively down regulating the OTII receptor during thymocyte development.

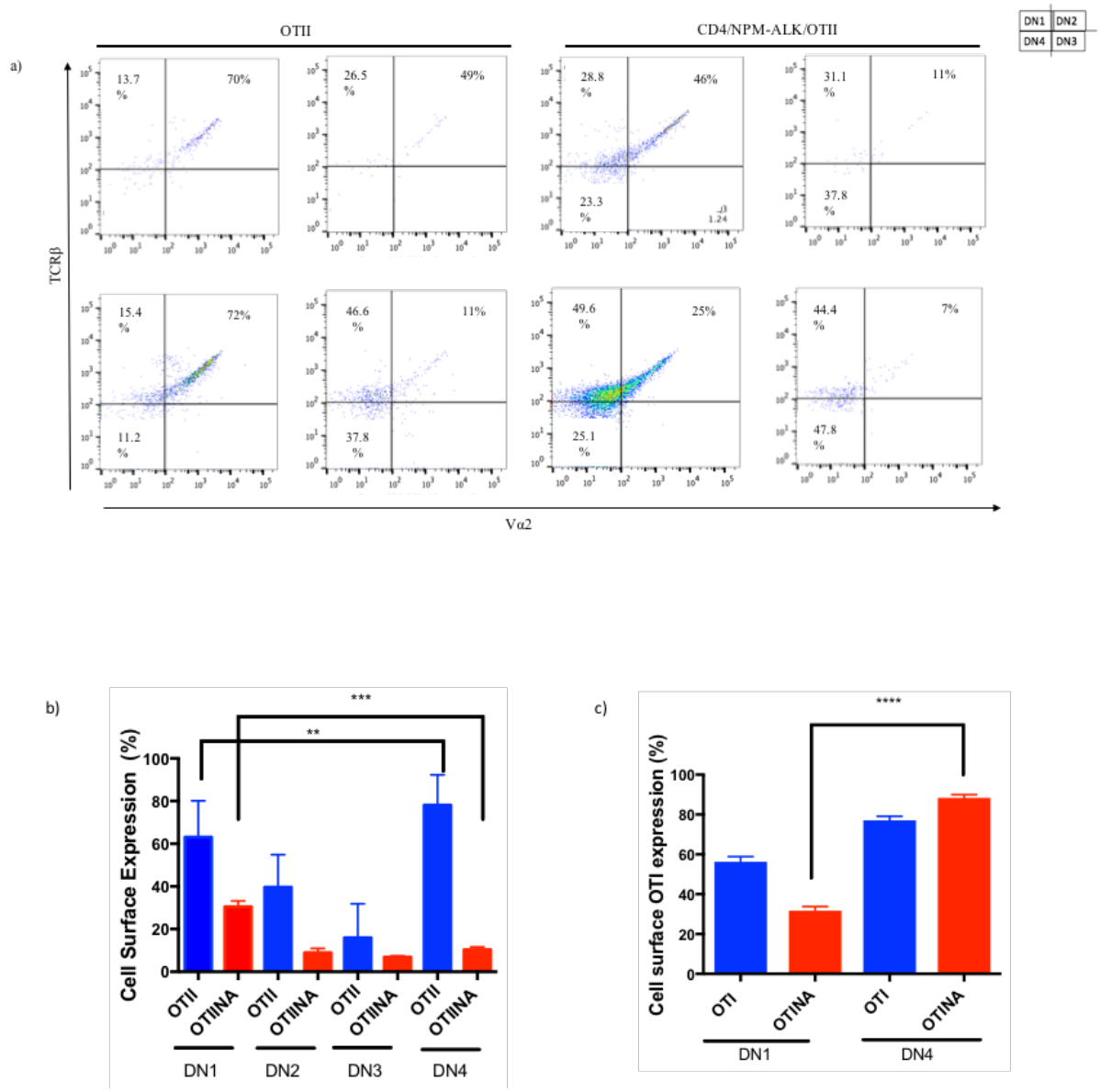


Figure 3.5: The OTII receptor is down-regulated during thymocyte development:
*(a) FACS plots showing a representation of cell surface OTII (Va2) and TCRβ expression on gated DN1 to DN4 thymic populations of CD4/NPM-ALK/OTII and their NPM-ALK negative, OTII+ littermates, n=4. (b) Percentage of DNI-DN4 thymocytes expressing Va2 in CD4/NPM-ALK/OTII (OTIINA) and OTII mice p values illustrate the loss/increase of OTII expression between DN1 and DN4 (OTII TCR P values ** 0.0012, *** = 0.0003) (c) shows OTI expression on CD4/NPM-ALK/OTI (OTINA) pre-tumour thymi. In NPM-ALK OTI mice the transgenic receptor is expressed at the DN1 stage, and expressed to a similar or higher level at DN4, p****= <0.0001 (indicates reduced expression at DN1 in comparison to DN4).*

3.2.6 CD4/NPM-ALK/OTII tumour cells are capable of endogenous TCR rearrangements

As the CD4/NPM-ALK/OTII model is on a RAG competent background, T cells are capable of V(D)J recombination and thus have the potential to produce additional TCR's other than the forcibly expressed OTII receptor. Tumours developing in the CD4/NPM-ALK parental model show clonal and oligoclonal rearrangements for the TCR β chain whereas tumours derived from the CD4/NPM-ALK/OTI model show germline TCR β chain rearrangements suggesting that the presence of the OTI TCR inhibits endogenous TCR rearrangements or that tumours only arise from cells expressing the transgenic TCR (Malcolm, et al., 2016). In contrast, tumours arising in the CD4/NPM-ALK/OTII transgenic line show germline, clonal and oligoclonal TCRβ rearrangements (Figure 3.6). Oligoclonal rearrangements were observed in a CD4SP tumour expressing 14.5% Vα2 (5), clonal rearrangements were observed in a CD4SP with 80% Vα2 expression (6), a CD4SP with no Vα2 expression was oligoclonal (7), and a CD8SP tumour with 26% Vα2 expression was clonal for TCRβ rearrangements.

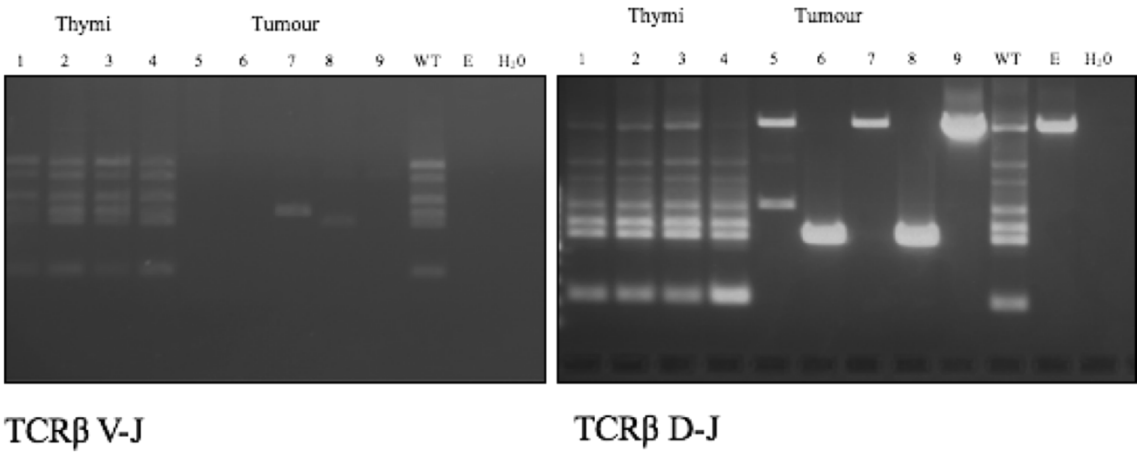


Figure 3.6: Tumours isolated from CD4/NPM-ALK/OTII mice show endogenous TCRβ rearrangements: *DNA was extracted from tumour cells and TCRβ V-J or D-J rearrangements were analysed via PCR. Lanes 1-4 represent pre-tumour thymi = CD4/NPM-ALK/OTII pre-tumour thymi (1 and 4) and (2-3) NPM-ALK negative, OTII+ littermate control thymic DNA (2 and 3), lanes 5-8 = DNA from CD4/NPM-ALK/OTII tumours, 9 = DNA from a CD4/NPM-ALK/RAG−/− mouse, E= DNA extracted from a OTII mouse ear biopsy and WT = DNA extracted from a C57BL/6 mouse thymus. H_2O = water control.* 5= *CD4SP with 14.5% Va2 expression, 6= CD4SP with 80% Va2 expression, 7= CD4SP with no Va2 expression, 8=CD8SP with 26% Va2 expression.*

In conclusion, the CD4/NPM-ALK/OTII model develops thymic lymphomas yet the CD8 skewed CD4/NPM-ALK/OTI model develop peripheral disease with hallmark cells mimicking human ALCL, as previously shown. Is this alteration in tumour phenotype due to a skew to either the CD4 or CD8 T cell phenotype? To determine the potential influence of a CD4 skew on lymphomagenesis the NPM-ALK model was backcrossed onto another MHC class II restricted model, Marilyn.

3.2.7 Female CD4/NPM-ALK/Marilyn mice develop thymic lymphomas

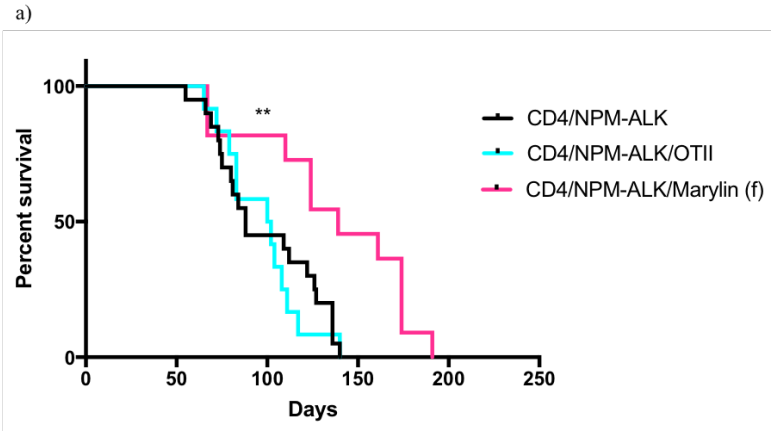
Marilyn TCR transgenic mice expresses a TCR specific for the male H-Y antigen presented in the context of MHC class II I-A^b29 and therefore have a T cell developmental skew towards a predominant CD4 T cell phenotype (Lantz , et al., 2000). Thymic T cells of male mice, of this strain, undergo negative selection in the thymus during development, due to presentation and recognition of the H-Y antigen, whereas female counterparts are unaffected as the H-Y antigen is not expressed. Both female and male mice were backcrossed onto the RAG competent CD4/NPM-ALK background. Female CD4/NPM-ALK/Marilyn(f) mice developed thymic lymphomas with a heterogeneous phenotype (table 3.4). Of the 12 mice in this cohort, 9 developed thymic tumours and 3 died as a result of pneumonia like symptoms. The presence of the Marilyn TCR significantly increased overall survival of female mice to 139 days in comparison to 101 days for the CD4/NPM-ALK/OTII line (p=0.0049) (figure 3.7a).

Analysis of the cell surface phenotype via flow cytometry showed the majority of tumours to express CD4 alone or CD4 in combination with CD8 (DP) (table 3.5) but in the majority of cases tumours displayed minimal to no expression of the Marilyn TCR indicated by Vβ6 (15%+/-34.2) (figure 3.8, table 3.5). CD30 expression, characteristic of human ALCL, was also undetectable. Notch expression was also detectable within tumours along with expression of CD69. CD69 is indicative of signalling via the TCR and thus can identify cells which have just undergone positive selection. After positive selection, CD69+ cells migrate to the medulla to undergo central tolerance. Here thymocytes positive for CD69 can either be defined as TCR^{lo} CD4+ CD8+ (DP), TCR^{hi} CD4+, CD8- (CD4SP) or TCR^{hi} CD4- CD8+ (CD8SP) representing a cellular population between immature DP and mature SP cells (Hu , et al., 2015), (Yamashita , et al., 1993).

Tumours derived from CD4/NPM-ALK/Marilyn(F) were either CD4SP or DP. In DP tumours, expression of CD69 was (35.82% +/- 39.71) and (55.2% +/- 18.67) in CD4SP tumours (table 3.5). Why tumours are restricted to the thymus may be down to an interference with thymic egress, perhaps via CD69 expression. Notch expression was also recorded in CD4SP and DP tumours, (56.2%+/- 36.90).

	Survival (Days)	Location	Pathology	Phenotype
1	110	Thymus	Lymphoma	CD4SP
2	191	Thymus	Lymphoma	CD4SP
3	161	Thymus	Lymphoma	DP
4	139	Thymus	Lymphoma	DN
5	174	Thymus	Lymphoma	DP
6	124	Thymus	Lymphoma	DP
7	67	Lungs	Pneumonia	ND
8	67	Lungs	Pneumonia	ND
9	174	Thymus	Lymphoma	CD4SP
10	124	NA	No tumour	NA
11	174	Thymus	Lymphoma	DP
12	139	Thymus	Lymphoma	ND

Table 3.4: Summary of disease location and phenotype of female (f) CD4/NPM-ALK/Marilyn mice



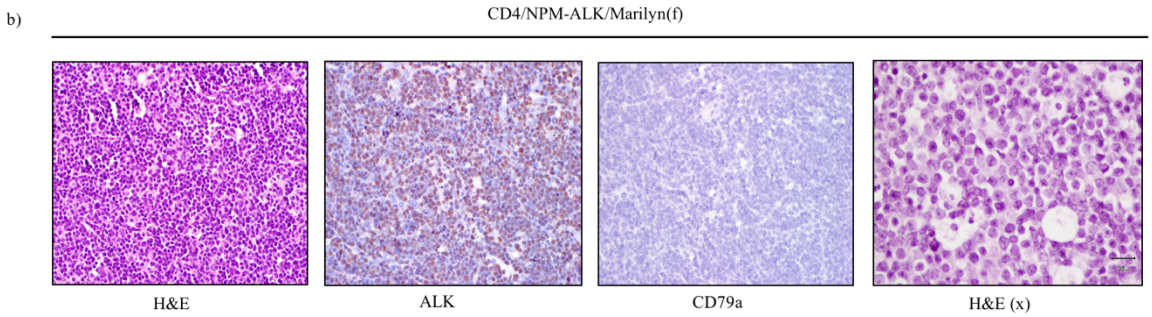


Figure 3.7 Female CD4/NPM-ALK/Marilyn mice develop thymic tumours

*(a)KAPLAN Meir survival curve shows overall survival of CD4/NPM-ALK (n=20), CD4/NPM-ALK/OTII (n=16) and female CD4/NPM-ALK/Marilyn (n=13) mice. Median survival CD4/NPM-ALK (88 days), CD4/NPM-ALK/OTII (101 days), female CD4/NPM-ALK/Marilyn (139 days). **p= 0.0049 for CD4/NPM-ALK/Marilyn(f) in comparison to previous thymic lymphoma mice the CD4/NPM-ALK/OTII and CD4/NPM-ALK. (b) Representative histology of a thymic lymphoma showing immunohistochemistry for ALK and CD79a (indicative of a B cell origin) at 200x magnification and a H and E stain at 400x.*

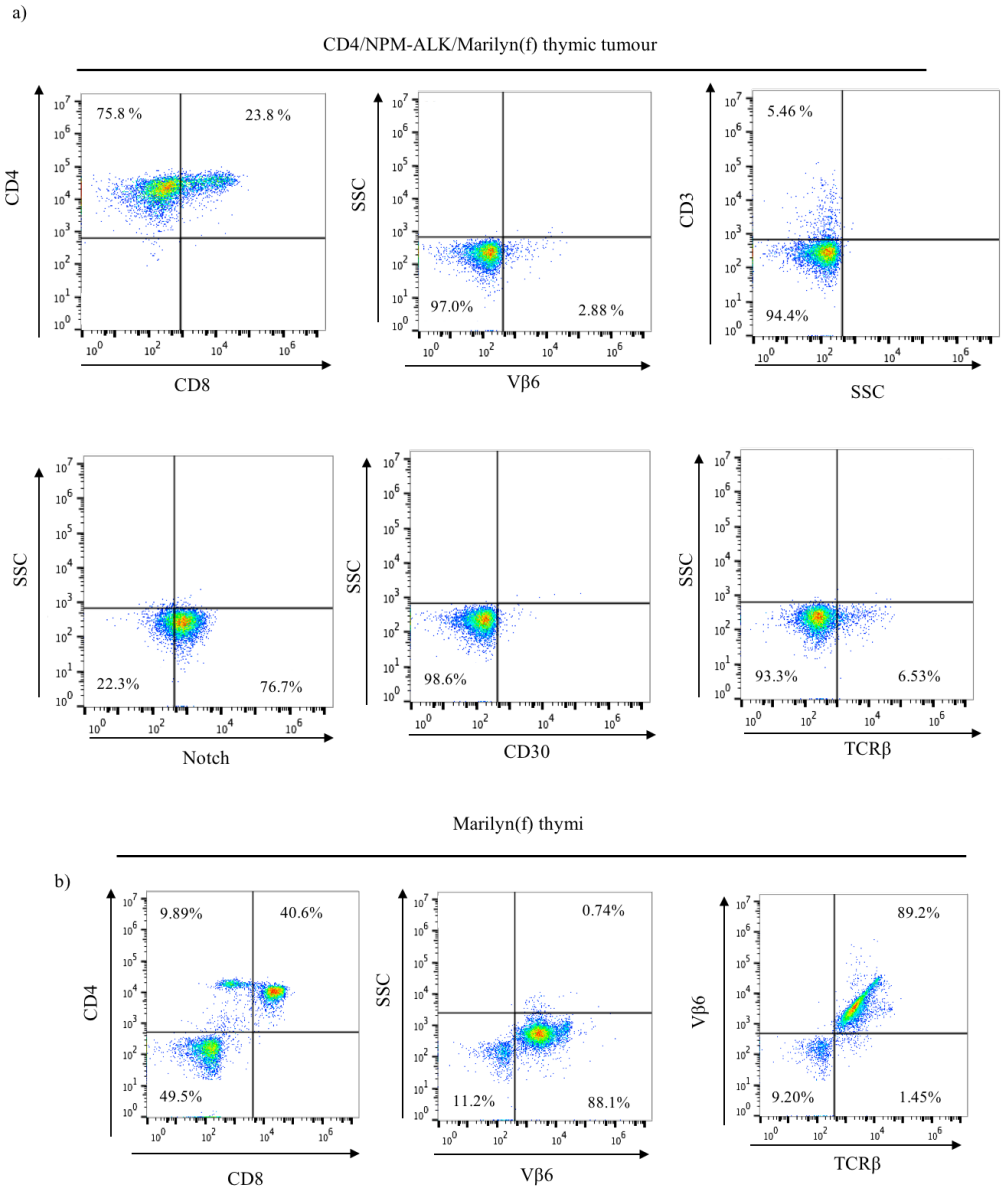


Figure 3.8: Female CD4/NPM-ALK/Marilyn mice develop CD4SP or CD4/CD8 DP tumours and lack surface expression of the Marilyn TCR:
Tumour phenotype was determined as previously described. (a) Represents a summary of key cell surface markers analysed on tumours. (b) Representative analysis of thymic content of Marilyn transgenic littermates negative for NPM-ALK expression.

Thymic tumours	CD4	CD8	DP	TCRβ	Vβ6	CD3	CD30	CD25 CD44	CD69	CD62L	Notch	Cell type
1	29	27.7	26	52.7	12.7	61.5	0	16.2	8.49	6.67	3.57	DN
2	66.8	12.5	14.2	91.9	91.9	91.9	0	78.5	42	88.8	22.7	CD4SP
3	4.81	4.18	59.6	12.5	0	4.47	4	96.1	63.9	94.4 hi	95.7	DP
4	62.7	0.14	35.2	1.76	0	1.64	0	98	68.4	ND	50.2	CD4SP
5	0.063	1.46	59.2	20	0	13.6	0	38.2	ND	ND	ND	DP
6	0.14	0.22	99.6	24.8	0	19	0	63.1	7.74	ND	ND	DP
7	83.5	55.9	63.9	12.2	0.66	5.45	3.33	96	ND	ND	ND	CD4SP

Table 3.5 Key markers of CD4/NPM-ALK/Marilyn(f) thymic tumours
Table represents key markers used to determine the phenotype of CD4/NPM-ALK/Marilyn(f) tumours. Tumour phenotype (CD4SP, DP and DN) was determined by expression of CD8 and CD4 on tumours, i.e. DP = CD8 and CD4 co-expression on the majority of cells (>30%) ND=not determine.

3.2.8 The Marilyn receptor speeds up thymocyte development

In order to assess the effects of NPM-ALK on thymic development and expression of the transgenic TCR in the Marilyn transgenic mouse, pre-tumourigenic thymi were taken from 5-week-old mice and analysed (Figure 3.9a,b). As previously observed with other TCR transgenic strains in the presence of NPM-ALK, there is an accumulation of cells at DN3 stage and a decrease in DN4 cells indicative of a transient block in thymic development at the in comparison to Marilyn NPM-ALK negative mice, CD4/NPM-ALK/Marilyn(f) DN3 (19.23%+/- 6.838), Marilyn DN3 (6.1%+/-1.71) (p=0.1710 NS), CD4/NPM-ALK/Marilyn(f) DN4 (57.8%+/-5.854) and Marilyn DN4 (74.27%+/-1.378) p=0.0660). Unlike the CD4/NPM-ALK/OTII mice there does not seem to be evidence indicating towards the loss of receptor expression during development at the DN4 stage when compared to NPM-ALK negative littermates (figure 3.9c). An interesting observation was the decreased cell surface expression of the Marilyn receptor (TCRβ/Vβ6) in CD4/NPM-ALK/Marilyn(f) at the DN3 stage (24.38%+/-4.498) in comparison to Marilyn DN3(52.47+/-4.776) p=0.0083. An increased presence of endogenous TCRβ expression (not associated with the Marilyn receptor) was reported to be increased in the CD4/NPM-ALK/Marilyn(f) (47.1%+/-0.3) in comparison to the Marilyn control (32%+/-0.3) at the DN3 stage, p=0.0008 (figure 3.9d).

Profiles of CD8 vs CD4 in the thymi showed that CD4/NPM-ALK/Marilyn(f) had a greater proportion of DP (77.4%+/-2.261) in comparison to the Marilyn control (60.5%+/-4.5) p=0.0320 cells and reduced CD4SP population (NS) (Figure 3.10a). There was also a non-significant reduction in Vβ6 expression, in CD4/NPM-ALK/Marilyn on SP cells and DP cells in comparison to Marilyn mice (figure 3.10b).

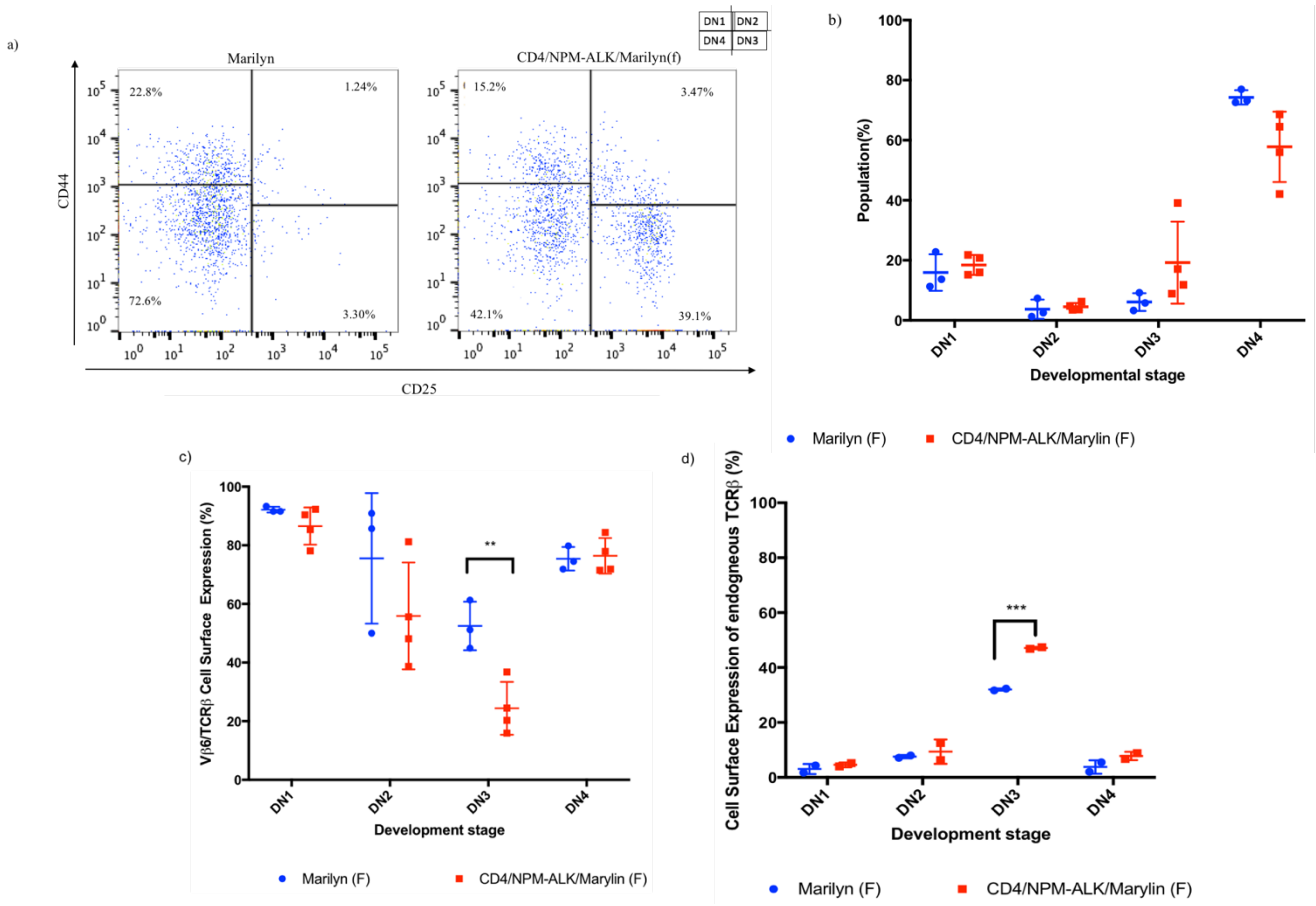


Figure 3.9: Developing thymocytes in CD4/NPM-ALK/Marilyn(f) accumulate at the DN3 stage and express endogenous TCRβ chain:
Thymi were taken from 5week old CD4/NPM-ALK/Marilyn pre-tumour mice and NPM-ALK negative littermates and thymocyte development analysed via flow cytometry. TCR expression during development was also assessed staining for TCRβ and Vβ6 (Marilyn β chain) (a) FACs plot representation of thymocyte development of CD4/NPM-ALK/Marilyn (f) and Marilyn mice showing DN stages 1-4 with corresponding summary graph below. (b) Graph summarising Marilyn TCR (Vβ6) expression during development, p= 0.0083 for Vβ6/TCRβ (in combination) reduction at DN3 in NPM-ALK positive mice (c)Graph summarising expression of endogenous TCRβ chain during development. For DN3, p=0.00078

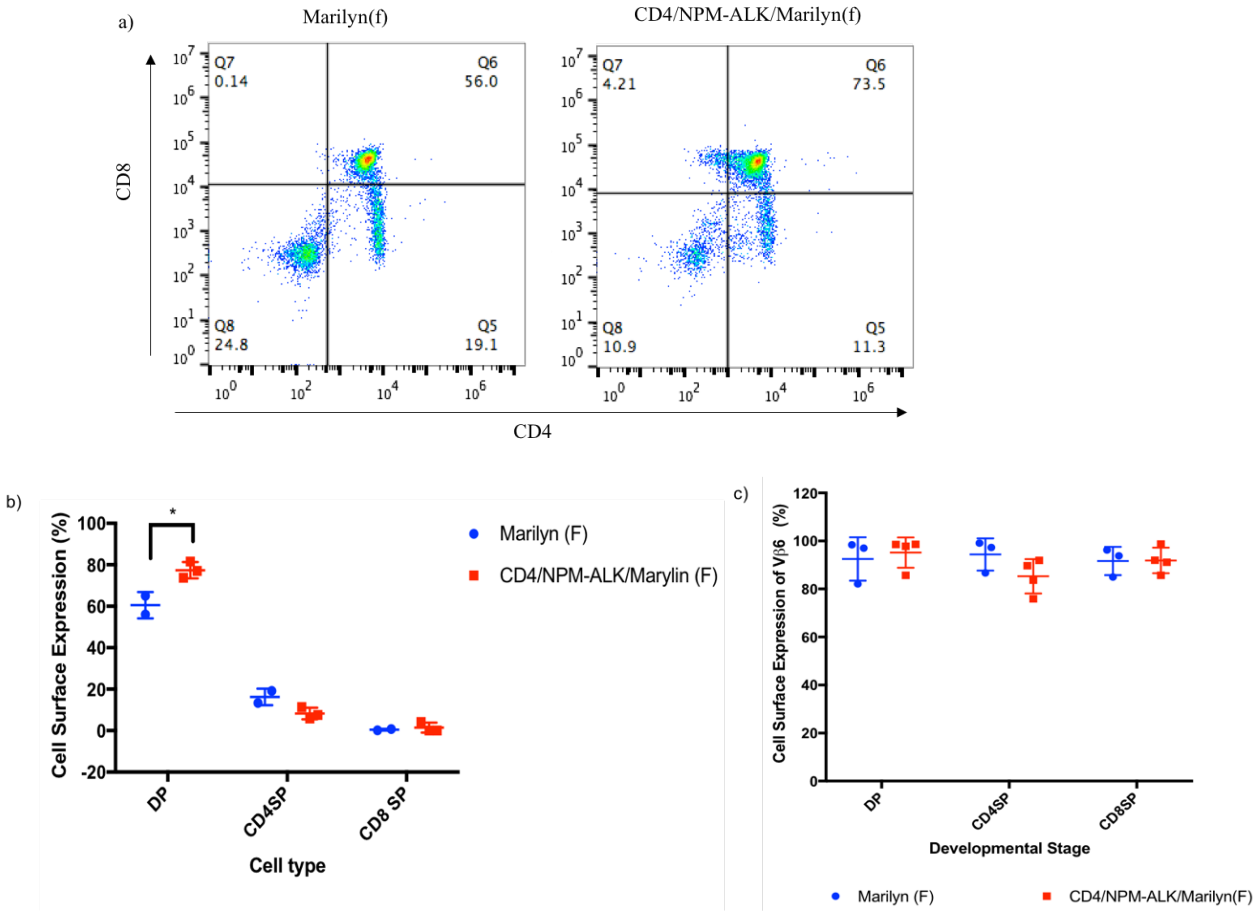


Figure 3.10 Proportion of DP and SP cells expressing the Marilyn receptor in pre-tumour thymi derived from CD4/NPM-ALK/Marilyn mice:
Pre-tumour thymi were assessed for the proportion of cells expressing both CD8 and CD4 (DP), CD4 (CD4SP) and CD8 (CD8SP). (a) FACs plot representing pre-tumour thymi from CD4/NPM-ALK/Marilyn(f) and Marilyn(f) mice. (b) Graph shows summary of CD4, CD8 and DP populations in thymi from pre-tumour mice=0.0320. (c) graph shows summary of expression of the Marilyn receptor on SP and DP cells, NS.*

3.2.9 NPM-ALK is incapable of bypassing negative selection

The previous two models discussed within this chapter looked at the influence of a CD4 skew, upon tumour phenotype and progression. The CD4/NPM-ALK/Marilyn(f) and

CD4/NPM-ALK/OTII mimics produced thymic lymphomas which lost their corresponding tg TCR receptors and hinted towards cells successfully undergoing positive selection. NPM-ALK is capable of bypassing β selection (Malcolm, et al., 2016), whether NPM-ALK was capable of bypassing negative selection was assessed in the CD4/NPM-ALK/Marilyn(m) mimic. The Marilyn receptor is specific for the H-Y antigen which is only expressed in male mice and therefore cells undergo excessive negative selection in the thymus (Hoist , et al., 2006).

Male CD4/NPM-ALK/Marilyn(m) mice presented with a variety of disease phenotypes which were categorised into ALK negative and ALK positive (table 3.6). ALK positive disease presented as thymic lymphomas 4/12 (33.33%). Those that developed thymic lymphomas had a similar survival to the parental CD4/NPM-ALK transgenic mimic line which likewise develop thymic lymphoma (107 days) (table 3.6). Again, similar to the parental line, the mice developing thymic lymphomas produced tumours expressing CD4 or CD4 with CD8 (double positive; DP tumours) with no evidence of cell surface transgenic TCR expression in spleens or tumours of these mice (3.11c, 3.12).

ALK negative (7/12 (58.3%)) presented with suspected myeloid/hematopoietic malignancy in the spleen and liver (4/7 (57.1%)) (table 3.6, figure 3.11b). 2/4 presented with CD3+ thymic lymphomas and a splenic B cell lymphoma (table 3.6, figure 3.11a).

Flow cytometry analysis of splenic tumours showed evidence of activation markers CD25 and CD30 and there was still expression of the Marilyn receptor along with CD8 and CD4 within the tumour population with $CD5^{hi}$, CD69, CD25, CD30 expression which could be infiltrating activated T cells which are negative for the Marilyn receptor (Vβ6) (3.2.14a).

The final mouse 1/12 (8%) presented with lethal myocarditis (Table 3.2.6). Mice that developed haematopoietic malignancies had a long median survival of 201 days in comparison to previous CD4/NPM-ALK mice (table 3.6).

Mouse	Days	Disease location	Histology	FACS/Immunohistochemistry
1	182	Thymus	Lymphoma	ALK-
2	252	Spleen and liver	Lymphoma in the liver and suspected myeloid malignancy	Spleen ALK-, CD79a-, CD3-
3	266	Spleen	Hematopoietic tissue suspect of myeloid malignancy, LN lymphoma	ALK- disease in the spleen LN had ALK + lymphoma
4	160	Spleen	Spleen Haematopoietic tumour	ALK- CD79a-
5	126	Thymus and spleen	Both Lymphoma	ALK – lymphomas, thymic lymphoma: CD3+, splenic lymphoma: CD79a+
6	82	Thymus	Lymphoma	ALK+ CD3+ DP
7	102	Thymus	Lymphoma	ALK+ CD4SP
8	34	Unknown	Hematopoietic tumour suspected myeloid with 70% acidophilic macrophage pneumonia	ALK-
9	36	Liver	Hematopoietic tumour suspicious of myeloid neoplasia	ALK-
10	99	Thymic	Lymphoma	ALK+
11	71	Thymic	Lymphoma	ALK+
12	126	Heart	Degeneration of the myocardium	ND

***Table 3.6: Survival and disease in CD4/NPM-ALK/Marilyn(m) mice*:**
Summary of disease location, age of onset, and phenotype of the 12 mice analysed.

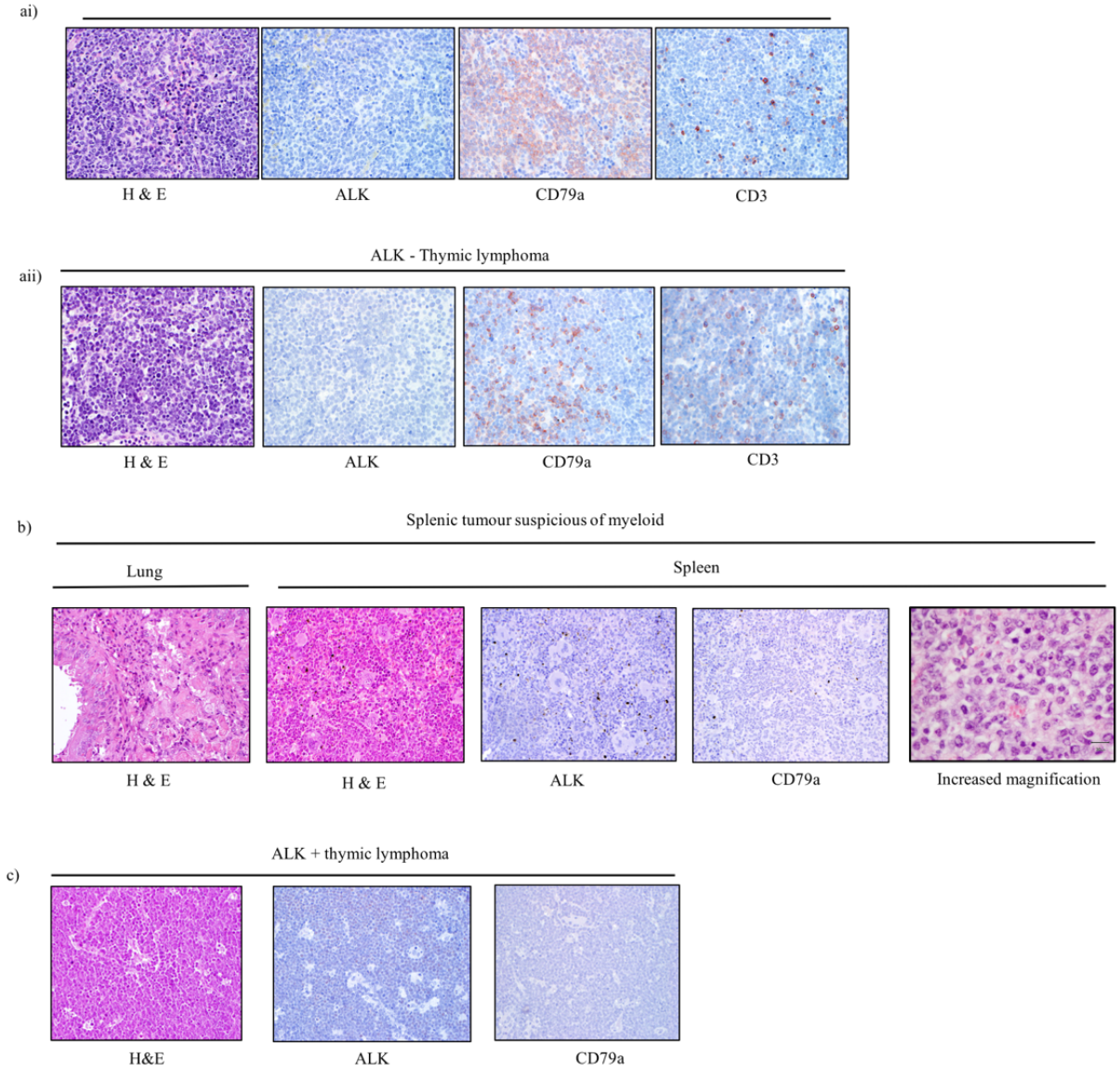


Figure 3.11: CD4/NPM-ALK/Marilyn(mice) develop a range of malignancies
Histology and immunohistochemistry analysis of tumours derived from CD4/NPM-ALK/Marilyn(m) mice staining for ALK, CD79a (B cell marker) and CD3 at 400X magnification. (a) Displays an ALK- B cell lymphoma present in the spleen and thymus, (b) Splenic tumour suspected to be of myeloid origin, ALK-. (c) Example of an ALK+ thymic lymphoma.

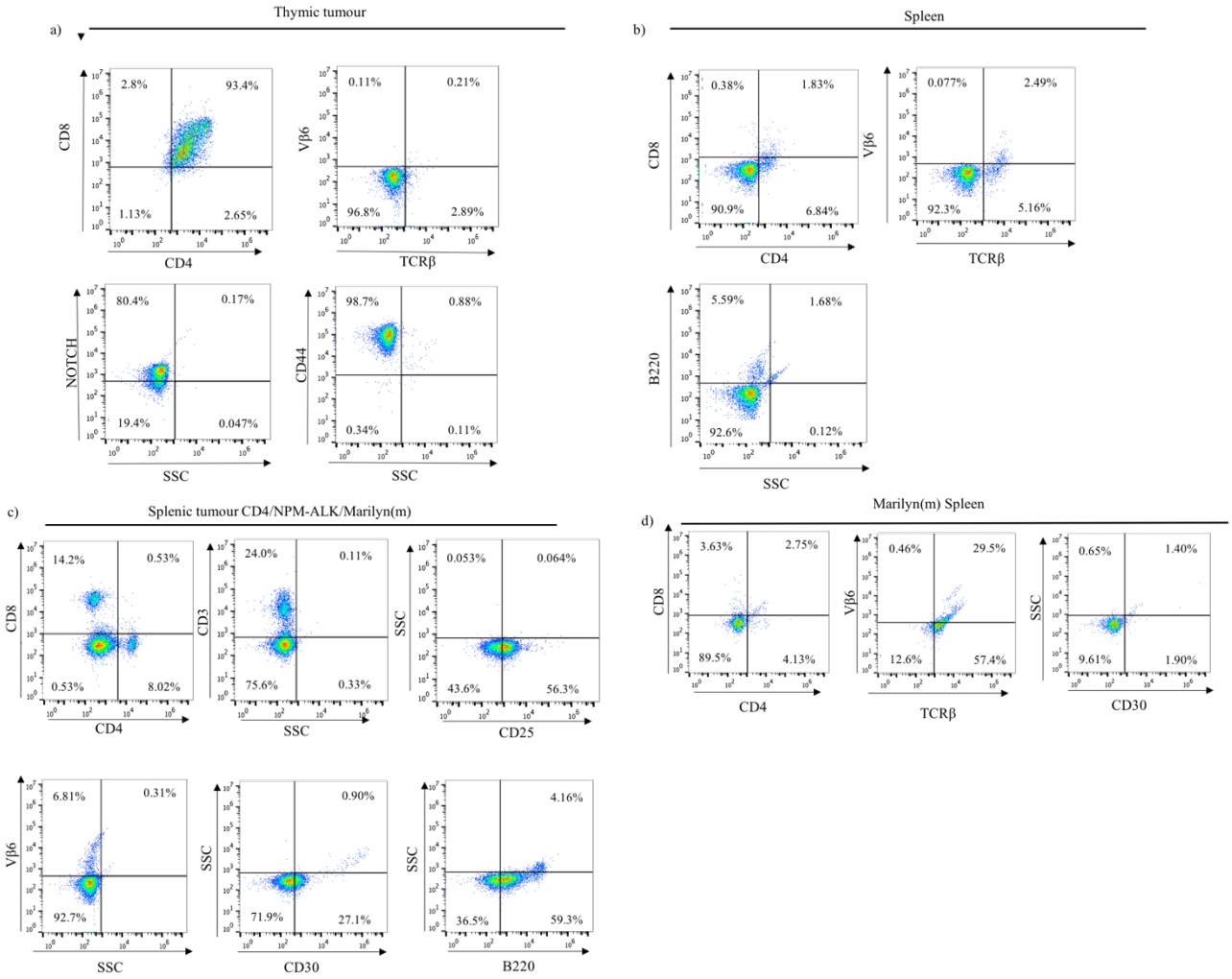
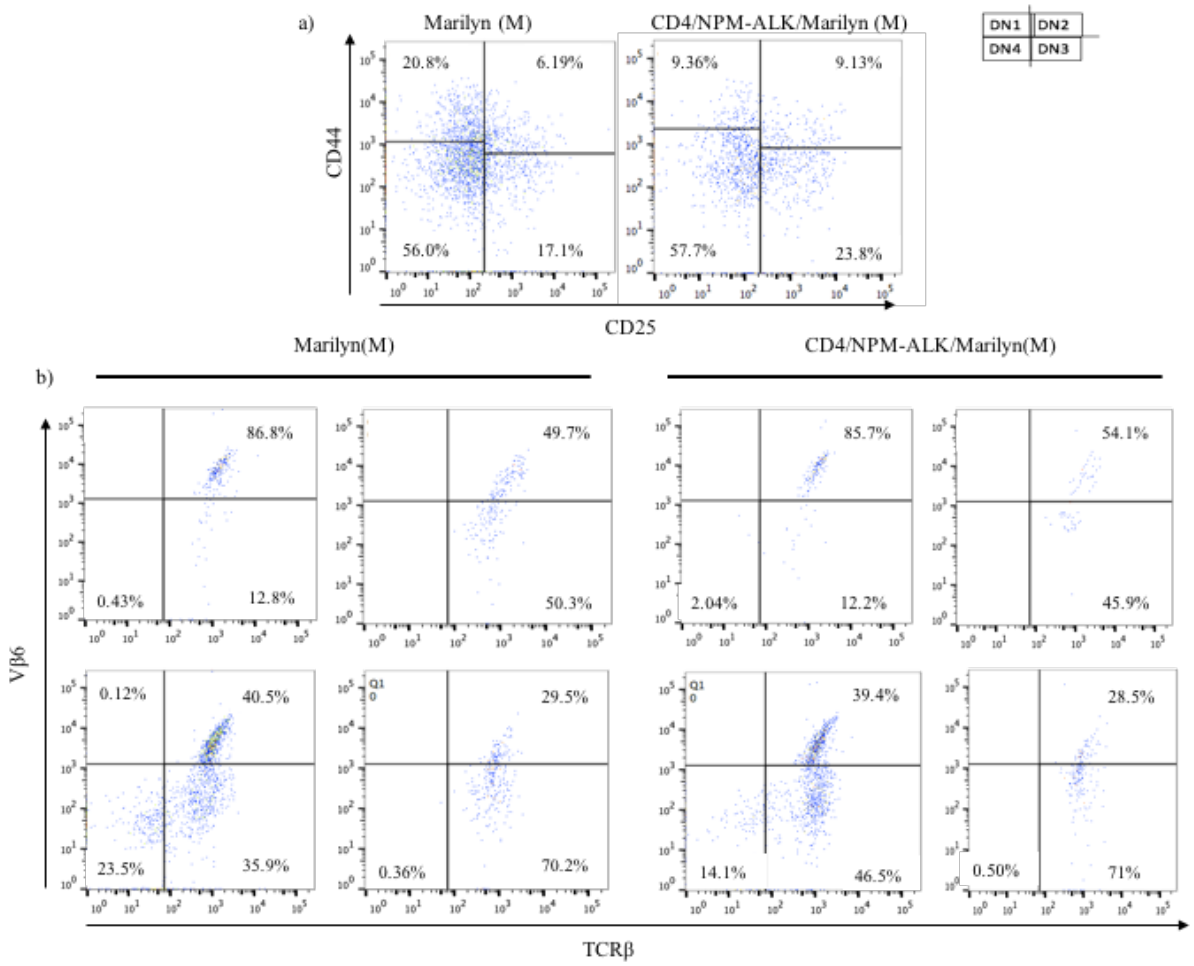


Figure 3.12: CD4/NPM-ALK/Marilyn(m) mice develop thymic and splenic malignancies: *Representative cellular phenotypes of splenic and thymic tumours. (a) Analysis of an ALK- splenic tumour (gated on the tumour population) and (b) spleen of an NPM-ALK negative littermate. (c) FACs representation of an ALK+ thymic tumour (gated on the tumour population) (d) analysis of the spleen from the same mouse.*

3.2.10 Pre-tumour development

Pre-tumour thymi development shows an increased accumulation at the DN3 and DN4 stages though this was not significant (figures 3.13 and 3.13c). Marilyn TCR (Vβ6) expression during development reduces in the presence and absence of NPM-ALK, as expected (figures 3.13b and 3.13d). Pre- tumour thymi have a greater population of DP cells (2/3) and CD4SP

cells (1/3) in the 3 pre-tumour thymi analysed (figure 3.14), suggesting that perhaps this would be the tumour phenotype of the lymphoma, of this mouse, which would develop. Marilyn mice on NPM-ALK and NPM-ALK null backgrounds are capable of producing endogenous TCRβ, in which there is increased expression at the DN3 stage of development (figure 3.13e). This increased additional expression of endogenous TCRβ could indicate a potential for tumours to arise from cells expressing endogenous TCRs.



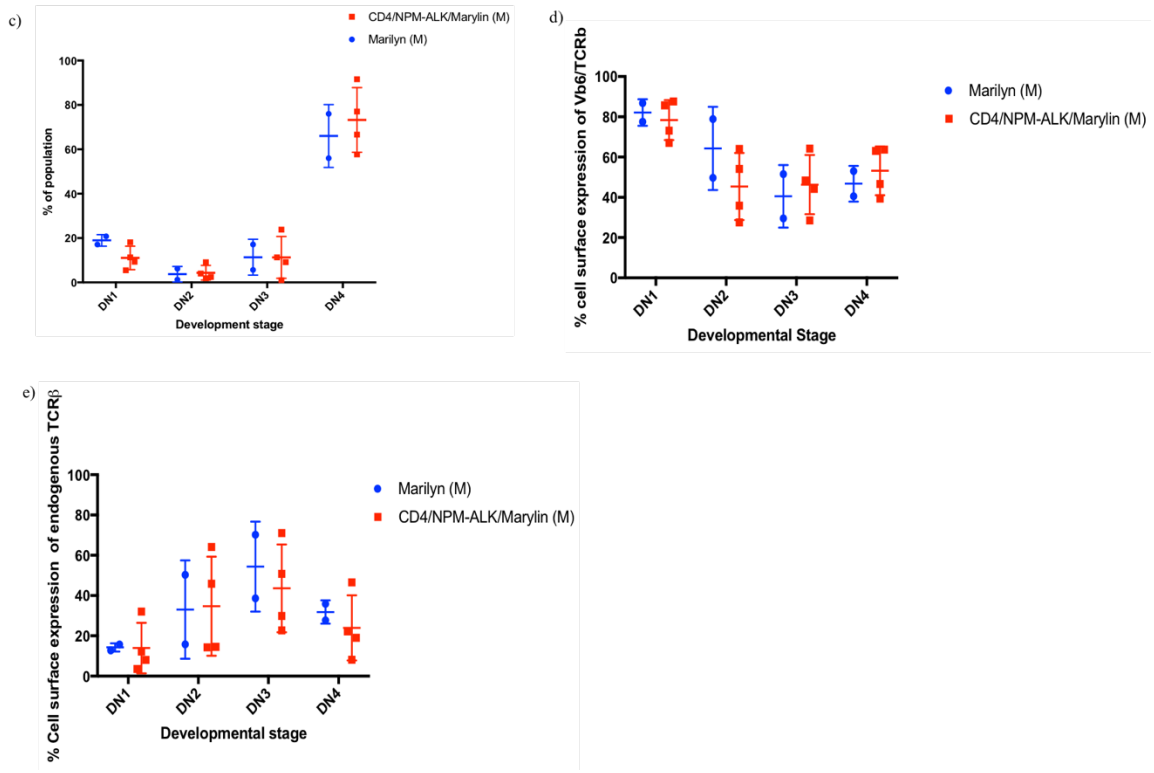


Fig: 3.13 Analyses of pre-tumour thymocyte development in CD4/NPM-ALK/Marilyn(M) mice:

Pre-tumour thymocyte development was analysed from 5-week-old as previously described. (a) pre-tumour thymocyte development in CD4/NPM-ALK/Marilyn(m) mice, (b) NPM-ALK negative littermates, (c) FACs representation of Marilyn receptor expression during development represented by Vβ6 (d) FACs representation of SP cells in CD4/NPM-ALK/Marilyn(M). (e) NPM-ALK negative littermates.

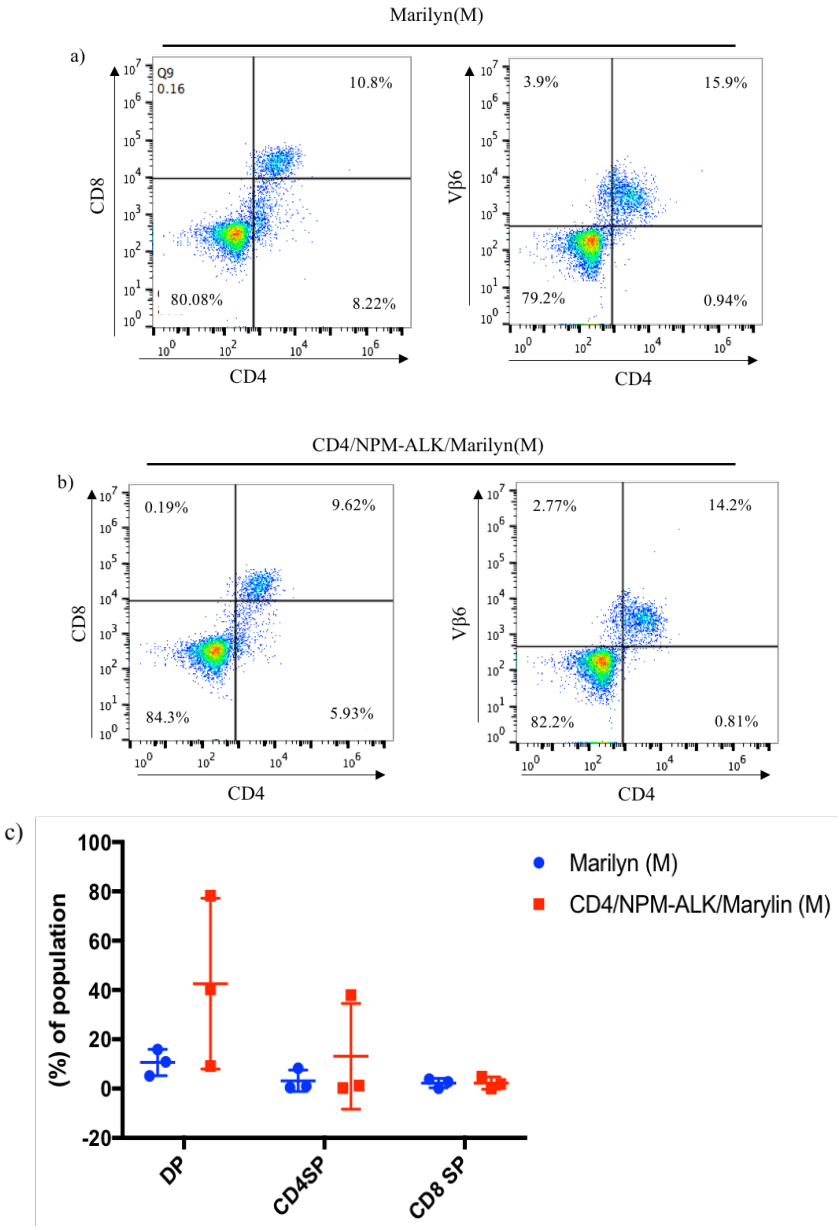


Figure 3.14 Percentage of CD4 and CD8 positive cells in pre tumourigenic thymi: *Cells were isolated from 5-week-old mice and proportions of CD4 SP, CD8SP and DP cells were determined as previously described. a) flow cytometry representation of pre- tumour Marilyn+ NPM-ALK- cells, (b) Flow cytometry plot representing pre-tumour CD4/NPM-ALK/Marilyn(m) cells, (c) graphical summary of DP and SP populations n=3 p=NS.*

3.2.11 CD4/NPM-ALK/Marilyn TCR rearrangements

To determine whether the cells from which CD4/NPM-ALK/Marilyn tumours were derived, cells from established tumours were assessed for rearrangements of the TCR β chain. Both models were on a RAG competent background allowing for endogenous TCRβ rearrangements (figure 3.15). The majority of tumours derived from CD4/NPM-

ALK/Marilyn(f) are germline with one clonal tumour suggesting that the majority of tumours come directly from cells expressing the Marilyn receptor. Whereas CD4/NPM-ALK/Marilyn(m) displayed TCRβ rearrangements within a thymic and splenic tumour indicating that the male strain produces tumours arising from T cells expressing endogenous receptor in the case of the thymic tumours observed.

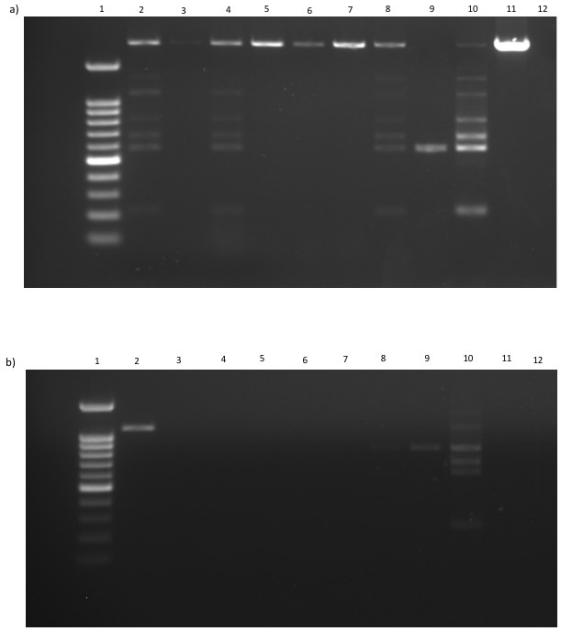


Figure 3.15: TCR β chain rearrangements in tumours derived from CD4/NPM-ALK/Marilyn (m):
Shows rearrangements of the TCRβ chain D-J (a), V-J (b) from cells derived from CD4/NPM-ALK/Marilyn tumours and NPM-ALK negative littermates. (2= Male normal thymi ,3= female spleen (from tumour female), 4=Male splenic tumour, 5=female thymic tumour, 6=female thymic tumour, 7=CD4/NPM-ALK/OTII/Rag-/- thymic tumour, 8=female spleen from tumour mouse, 9=female tumour, 10=Wild type (C57BL/6J) 11= ear,12=water

CD4/NPM-ALK/Marilyn(m) mice provide evidence that NPM-ALK cannot bypass the negative selection process and the CD4/NPM-ALK/Marilyn(f) provides evidence hinting towards a role for positive selection. In addition, the differences between overall survival in the OTII and Marilyn models hints towards an incompatibility between a functional TCR and NPM-ALK and that perhaps the signalling strength of the receptor may contribute to disease progression.

3.3 Discussion

To date, the best murine mimic of ALCL is the CD4/NPM-ALK/OTI transgenic mouse previously described (Malcolm , et al., 2016). These mice develop peripheral lymphomas, with hallmark cells and an activated, null cell phenotype. Interestingly, established tumour cells, from this model, like human ALCL, lack expression of the forcibly expressed TCR (Malcolm, et al., 2016). This model indicates two things, firstly a link between CD8 pathways being permissive towards transformation of T cells and ALCL and secondly that the TCR may be prohibitive of cellular transformation. The identification of an ETP signature, in ALCL, indicates towards the cell of origin as an immature T cell arising within the thymus (Malcolm, et al., 2016) (Moti , et al., 2015) (Hassler , et al., 2016). This could suggest that developmental pathways or specific signalling pathways associated with a CD8 or a CD4 lineages shapes the disease phenotype.

ALCL are largely considered CD4 SP T cells despite the presence and production of cytotoxic proteins such as perforin. To address whether a forced CD4SP T cell developmental skew affected disease phenotype in this model, the CD4/NPM-ALK mice were backcrossed to the OTII (CD4/NPM-ALK/OTII) and Marilyn (CD4/NPM-ALK/Marilyn(f)) MHC class II restricted TCR transgenic strains. In this chapter mice developed thymic lymphomas not displaying ALCL like disease and a consistent loss of the Tg TCR.

3.3.1 MHC class II restricted TCR transgenic mice develop thymic lymphomas in the presence of NPM-ALK

Contrary to expectations, both CD4/NPM-ALK/Marilyn(f) and CD4/NPM-ALK/OTII mice developed thymic lymphomas with the same mixed CD4SP/DP phenotypes, as seen in the parental CD4/NPM-ALK strain (tables 3.1, 3.2, 3.4, 3.5). The presence of Tg TCRs were expected to allow for peripheral disease generation, as observed with the CD4/NPM-ALK/OTI mimic. However, this was not the case suggesting that perhaps NPM-ALK has a preference for CD8 related signalling pathways and that to elucidate this further the CD4/NPM-ALK mimic should be backcrossed onto additional MHC class I restricted TCR transgenic mice such as P14 (Brandle , et al., 1995). Or that CD4+ NA+ cells are deleted in the periphery.

Though a similar phenotype and disease location was observed for the NPM-ALK MHC class II restricted TCR tg models, CD4/NPM-ALK/Marilyn(f) mice tended to generate less CD4SP tumours in comparison to the CD4SP majority observed in CD4/NPM-ALK/OTII. In both models, tumour populations were heterogeneous and varied between mice, although the presence of CD4SP tumours, would suggest that cells have the characteristics of mature cells hinting towards an ability for thymic egress. Interestingly the majority of tumours from both models lacked cell surface expression of their corresponding TCR tg receptors. Apart from two CD4/NPM-ALK/OTII tumours which expressed a high percentage of Vα2 (80.9 and 68.4% (table 3.3)) and one CD4/NPM-ALK/Marilyn(f) tumour (91.9% (table 3.5)). These results are in line with what has previously been observed in murine mimics and ALCL cases which lack the expression of a cell surface TCR.

Cells from established CD4/NPM-ALK/OTII tumours showed that the loss of the receptor was not due to a lack of receptor production as indicated by the detection of transcripts for the OTII receptor and intracellular protein corresponding to the α chain of the receptor (figure 3.4). This suggests that NPM-ALK may either be actively down regulating the

receptor and/or interfering with expression, perhaps via post translational mechanisms or an interference with receptor recycling. The TCR is internalised and continuously recycled upon ligand induced activation and in naïve resting T cells, via LCK and PKC mediated endocytosis (Crotzer, et al., 2004). Therefore, there could be a mechanism by which NPM-ALK interference with expression or recycling. Therefore, this potential for NPM-ALK interfere with receptor recycling should be addressed by measuring the role of internalisation or the effect upon stimulation of T cells in the presence of NPM-ALK.

3.3.2 Transgenic TCR's speed up thymocyte development in NPM-ALK negative mice and create a transient block at DN3 in the presence of NPM-ALK, during development

The DN3 stage is particularly important in thymocyte development as this is where β selection takes place, the first major checkpoint in development. At this point the TCRβ chain is rearranged and expressed in the context of the pre-TCR and cells are selected via pre-TCR engagement. Cells unable to undergo TCRβ chain rearrangement are unable to progress to the next stage of development. It has been shown in the CD4/NPM-ALK model that NPM-ALK expression can be detected as early as the DN1 and DN2 stage of development. In addition, pre-tumour thymi analysis of these mice showed a transient block at the DN3 stage when compared to NPM-ALK negative littermates. When this model was placed on a $RAG^{-/-}$ background, pre-tumour thymi analysis showed that NPM-ALK was capable of bypassing the β selection process and proceeded to produce tumours with a DP phenotype although SP tumours were rare (Malcolm, et al., 2016). In contrast, β-selection is not required in TCR tg mice as cells already express a full TCR. However, in both the OTII and Marilyn(f) models the process of thymocyte development seemed to be increased by the transgenic TCR's alone, in NPM-ALK negative TCR transgenic mice, as indicated by the accumulation of the majority of cells at DN4 stage in comparison to DN2 and DN1 (figures

3.3a, 3.9b). Though not directly comparable from results obtained it may be concluded that the Marilyn receptor pushes T cells through development at a much faster rate than the OTII. In the presence of NPM-ALK, cells in both mimics accumulate at the DN3 stage in comparison to the NPM-ALK negative littermates, although this was not significant, due to outliers (figures 3.3b, 3.9b). Suggesting that there was an interference with the β selection process which may be due to an interference with the Tg TCR receptors and associated signalling, as signalling via the pre-TCR is crucial to the β selection process. This may be due to an inability to signal via the TCR if NPM-ALK has silenced proximal signalling (Ambrogio , et al., 2009).

Analysis of DP and SP cells in the CD4/NPM-ALK/Marilyn(f) showed that these mice had an increase in DP positive cells which could explain the common DP phenotype observed. Thus indicating towards and importance for the DN3 stage in ALCL lymphomagenesis.

3.3.3 Pre-tumour NPM-ALK interferes with thymic egress by downregulating the OTII receptor during development

Both the CD4/NPM-ALK Marilyn and CD4/NPM-ALK/OTII tumours display with phenotypes suggestive of successful positive selection, the expression of a predominant CD4SP phenotype in the CD4/NPM-ALK/OTII tumours and CD4SP and CD69 expression observed in DP CD4/NPM-ALK/Marilyn(f) tumours (tables 3.2, 3.5).

The presence of a forcibly expressed receptor, differentiation into a CD4SP cell and a mature quiescent phenotype suggests that cells have successfully undergone positive selection, developed to the mature SP phenotype and should in theory be capable of leaving the thymus. Despite this, neither model was able to produce peripheral disease, suggesting an interference with egress mechanisms, unlike the peripheral lymphomas observed in the CD4/NPM-ALK/OTI mimic, which suggested that a functional TCR was required for thymic egress and development of peripheral disease. The difference in disease location between the OTI and

OTII/Marilyn TCR expressing NPM-ALK transgenic mice is not immediately apparent but could be related to expression levels of the transgenic TCR at key stages in thymic development. In particular, pre-tumour cells were shown to downregulate the tg TCR in the CD4/NPM-ALK/OTII model during development, displaying a reduction at the DN4 stage in comparison to DN1 (figure3.5). As NPM-ALK positive cells lose tg TCR expression during development and potentially proximal signalling this may cause a lack of egress due to a potential interference with (S1P1) expression with the CD4/NPM-ALK/OTII mouse.

Cells need to proliferate, under antigenic stimulation, in order to egress from the thymus in which NPM-ALK is capable of mimicking signalling downstream of the TCR cascade despite silencing proximal signalling of the TCR (Ambrogio , et al., 2009) (Turner , et al., 2007). Timing of export from the thymus, is regulated by TCR signalling and the sphingosine-1-phosphate receptor (S1P1). Therefore, signalling through the TCR dictates a) how quickly thymocytes move through the thymus and b) when and if cells are allowed to be exported into the periphery (Matloubian , et al., 2004). Lack of S1P1r expression on mature SP thymocytes, does not permit for T cell egress from the thymus (Dzhagalov & Hyewon , 2012).

In the case of CD4/NPM-ALK/Marilyn(f) pre-tumour T cell development, reduction of the Tg TCR was observed at the DN3 stage but was recovered at DN4. This was also observed in CD4/NPM-ALK/OTI pre-tumour cells hinting that there was not an overall loss of tg TCR receptor during development in these two mimics (figures 3.5, 3.9). More specifically the CD4/NPM-ALK/Marilyn(f) displayed reduced expression of the Marilyn receptor at the DN3 stage and an increase in endogenous TCRβ expression (figure 3.9) along with expression of Notch-1 on tumour cells. This hints that whilst loss of the TCR during thymocyte development in the CD4/NPM-ALK/OTII mimic may be the cause for lack of egress, this may not be the case for CD4/NPM-ALK/Marilyn(f) cells. Marilyn mice display a reduction

of the receptor at the DN3 stage but do not lose expression on SP or DP cells, posing the question as to why Marilyn cells are not capable of thymic egress. This must be another mechanism other than loss of TCR. It should be noted that Marilyn develop diseases at a much later time frame than the OTII mouse suggesting that the presence of the Marilyn receptor may lead to transformation at a later time point, in development.

Further analysis of tumours derived from CD4/NPM-ALK/Marilyn(f) and staining for CD69, allowed for a greater insight into the developmental stage of these tumours. This staining was introduced after analysis of CD4/NPM-ALK/OTII mice was completed and therefore only CD4/NPM-ALK/Marilyn(f) were analysed for CD69 expression. CD69 is indicative of signalling via the TCR and thus can identify cells which have just undergone positive selection. The CD4/NPM-ALK/Marilyn(f) CD4SP and DP tumours cells expressed CD69 which suggests that tumours may derive from an intermediate between DP mature, and SP mature cells (table 3.5).

Expression of CD69 was also examined in pre-tumour CD4/NPM-ALK/Marilyn thymocytes. Increased expression of CD69 was observed in DP, CD4SP and CD8SP cells in comparison to NPM-ALK negative littermates (appendix 1 p.197), though again it is to be noted that, due to lack of breeders and adverse disease in the housing facility, the number of mice assessed was limited, therefore, these results are in the appendix and to be used as preliminary data. Expression of CD69 as a consequence of TCR signalling also downregulates cell surface S1P1 (Love & Bhandoola , 2011) Studies have shown that a block in CD69 expression results in a reduction of SP cells and increase in DP populations, whereas if CD69 is overexpressed this increases thymic SP cells numbers but reduces peripheral T cell presence (Nakayama , et al., 2002). Therefore, CD69 expression could be a contributing factor towards a lack of thymic egress and peripheral disease. As these phenotypes are observed it could also be suggested that the point of transformation or trigger for that matter is positive

selection in the CD4/NPM-ALK/Marilyn(f) mice which may also be the case for OTII mice and potentially CD4/NPM-ALK mice. ALCL tumours and cell lines and CD4SP tumours do not express CD69 which perhaps suggest that expression is only important during development and that it may result from the initial stimulation of the cell rather than upregulation via NPM-ALK (Dorfman & Shahsafaei, 2002).

Additional mechanisms controlling thymic egress may be affected by NPM-ALK. For instance, ALK activates STAT3 which in turn enhances NPM expression leading to further STAT3 activation (Ren , et al., 2015). STAT3 raises S1P1 levels which is crucial in turn for STAT3 activation (Lee , et al., 2010). This increased expression or ability to increase, S1P1 levels in theory should increase migration and egress in thymocytes expressing NPM-ALK. For instance NPM-ALK upregulates the PI3K pathway which has a negative effect upon the transcription factor Kruppel lung factor 2 (KLF2), in which KLF2 downregulation has a negative impact upon thymic egress due to its regulation of S1P1, CD62L and beta-7-integrin (Carlson , et al., 2006) (Barbee & Alberola-lla, 2005) (Slupianek , et al., 2001). Studies have shown that in L-Plastin null mice where S1P1 expression is not affected, CD4SP cells accumulated within the thymus and interference with CXCL12 expression also displayed with this accumulation. (Dzhagalov & Hyewon , 2012) It may be suggestive that further studies addressing NPM-ALK in terms of migration through and out of the thymus need, to be investigated. Though reasons for lack of egress could purely be down to cells transforming after the positive selection point and the active downregulation of the TCR in response to perhaps, TCR stimulation. Or due to NPM-ALK silencing proximal signalling which would not allow for antigenic stimulation leading to egress, or a combination of the two. Successful positive selection but a lack of egress may hint towards a role for the positive selection process in ALCL lymphomagenesis.

3.3.4 CD4/NPM-ALK/Marilyn(f) have a greater overall survival compared to CD4/NPM-ALK/OTII mice

Though both the MHC class II restricted TCR tg mice had a similar tumour phenotype and disease presentation, mice differed in age of onset and overall survival. The CD4/NPM-ALK/Marilyn (F) mice had a much greater survival than the CD4/NPM-ALK/OTII, median 139 to median of 101 respectively (figures 3.1,3.7). If mice developing pneumonia were excluded from this analysis the median survival for disease generation would be 166 days. In fact, overall survival of the CD4/NPM-ALK/Marilyn(f) was much greater than previous mimics which generated thymic lymphomas (88 and 128 days) (Malcolm , et al., 2016). Signalling strength via the Marilyn TCR tg may be different to that of the OTII receptor resulting in this prolonged duration to disease onset. Avidity/ signalling strength via the TCR varies between TCR tg models within and outside of their MHC class skew category. Mouse T cells display with varying levels of CD5 expression indicative of varying levels of TCR signalling strength (Azzam , et al., 2001), (Orta-Mascaro, et al., 2016) (Azzam , et al., 1998). These differences in tg TCR avidity could also be an explanation as to why the CD4/NPM-ALK/OTI mice produced peripheral lymphomas. Therefore, to address a potential role for signalling strength differences via the tg TCRs would be to look at CD5 expression during thymocyte development in all CD4/NPM-ALK TCR tg mimics discussed and presented within this thesis.

It was speculated that perhaps if CD4/NPM-ALK mice were able to live to an older age before generating thymic lymphomas, which dramatically impeded health and mice had to be sacrificed early, they would have developed peripheral lymphomas. Those that did generate peripheral lymphomas in the spleen still resembled cells derived from the thymus suggesting that this was due to metastases from the thymus. However, the CD4/NPM-ALK/Marilyn(F) did have this greater degree of survival suggesting that these cells would have been retained

within the thymus for a much greater period and allowed for the ability for thymic egress and consequently peripheral disease. Yet this model still developed thymic lymphomas. Thus, differences between the TCR tg models discussed here may be due to signalling strengths of the receptors and their role in regulating S1P1 levels. Where levels of signalling can also affect the speed by which they migrate through the thymus and perhaps the ability for NPM-ALK to transform the cell.

3.3.5 NPM-ALK positive cells do not bypass negative selection

So far in this chapter it has been hypothesised that the process of central tolerance/ clonal selection could play a role in cellular transformation. Therefore, the effect of negative selection was addressed in the males of the CD4/NPM-ALK/Marilyn mimic. In the CD4/NPM-ALK/ Marilyn(m) mice, NPM-ALK positive cells carry the Marilyn receptor specific for the endogenously expressed H-Y antigen, here T cells undergo mass negative selection in NPM-ALK negative mice leading to a lack of SP cells in the thymus and periphery (Hoist , et al., 2006). Female Marilyn mice, CD4/NPM-ALK/Marilyn(f), developed thymic lymphomas, with a DP or CD4 SP phenotype with a greater overall survival to previous NPM-ALK mimics developing mediastinum restricted tumours. CD4/NPM-ALK/Marilyn(m) Mice displayed with a variable selection of disease the majority of which were ALK- tumours (7/12) consisting of B cell lymphoma, CD3+ thymic lymphoma and haematopoietic malignancies which could not be fully confirmed as bone marrow was not sampled and analysed from these mice (Table 3.6) (Figure 3.11). The presence of ALK-ve disease could suggest that NPM-ALK is incapable of bypassing negative selection rendering all ALK+ cells, expressing the Marilyn receptor, to undergo deletion. As this model is on a Rag competent background and is capable of producing endogenous TCRs

it could be suggested that the ALK + thymic lymphomas recorded, arose from cells expressing endogenous TCRs (4/12).

Flow cytometry analysis of the splenic lymphomas displayed with CD30 expression, though as histology revealed these tumours to be of ALK-ve haematopoietic tumours, potentially myeloid, this suggested that the presence of CD30 indicated towards activated, infiltrating T cells (figure 3.12). Thymic tumours that developed also varied in phenotype, as seen previously in mice developing mediastinal lymphomas, DP and CD4SP, and lost expression of the Marilyn receptor as seen before in the females of this strain that did not express the H-Y antigen. If anything, due to mass negative selection elicited by the presence of the H-Y antigen, tumours should arise from DP cells. More data needs to be collected for these mice to see if this trend lies more towards thymic lymphoma generation. Though restrictions due to autoimmunity within this line greatly affected breeding, thereby limiting this study.

Analysis of pre-tumour thymocyte development showed a transient block at the DN3 stage and DN4. CD4 SP and DP populations were greater in NPM-ALK +ve mice in comparison to NPM-ALK negative (2 cases showed greater DP expression and 1 case greater CD4 SP expression). In the absence of NPM-ALK CD4SP and CD8SP levels are normally very low suggesting that these pre-tumour results link towards CD4SP and DP cells bypassing β selection as seen in CD4/NPM-ALK/RAG$^{-/-}$ mice (Malcolm , et al., 2016). Not via negative selection but maybe loss of Vβ6 or disease arising from Vβ6 negative cells.

This model shows that NPM-ALK is not capable of bypassing negative selection and that NPM-ALK positive thymic tumours may have arisen from T cells expressing endogenous TCRs.

3.3.6 Does expression of endogenous TCRs contribute to disease generation

RAG permits V(D)J recombination of TCR chains therefore TCR tg mice can have additional non tg TCRs in their TCR pool. However previous studies have shown that tumours derived from the CD4/NPM-ALK/OTI RAG competent model were germline for TCR rearrangements, indicating that tumours had formed from OTI cells (Malcolm , et al., 2016). This was also evident in CD4/NPM-ALK/Marilyn(f) mice (figure 3.15). Tumours derived from the CD4/NPM-ALK/OTII mainly displayed with clonal and oligo clonal rearrangements of the TCR β chain, with one germline, where almost all rearrangements were D-J (figure 3.6). Pre-tumour thymi displayed with oligo clonal and clonal rearrangements for the TCRβ. So, unlike the OTI receptor OTII seems incapable of suppressing the generation of additional TCRs when placed onto a RAG competent background. Pre-tumour thymi analysis of the CD4/NPM-ALK/Marilyn(f) mice showed expression of TCRβ chains not associated with Vβ6 in both NPM-ALK positive and NPM-ALK negative littermates which was much greater in NPM-ALK positive mice (figure 3.9). It could suggest that perhaps tumours may have arisen from this endogenous TCR pool, which could also explain a greater survival. However, analysis of TCRβ rearrangements showed tumours to be germline for TCRβ with one clonal rearrangement (figure 3.15). To elucidate further whether T cells expressing endogenous TCRs contribute to disease, mouse models mentioned in this chapter, should be backcrossed onto $RAG^{-/-}$ mice to prohibit endogenous TCR expression.

MHC class II restricted mice develop thymic lymphomas with CD4SP/DP phenotypes and are not representative of ALCL like disease. As ALCL can be linked to an early thymic progenitor these results could suggest that NPM-ALK maybe more permissive towards CD8 related signalling pathways in shaping peripheral ALCL like disease. Cell surface expression of tg TCR's is downregulated in established tumours and during thymocyte development

though transcripts and intracellular protein can still be detected, thus hinting towards an incompatibility between NPM-ALK and a functional TCR. Analysis of TCRβ chain rearrangements show that even with the commitment of a clonally expressed t cell receptor NPM-ALK seems to be able to bypass OTII and Marilyn expression permitting additional TCRβ rearrangements. Therefore, these models, on a RAG knockout background would help to rule this out. Time taken for disease generation seems to be affected by the transgenic receptor expressed and therefore signalling via the TCR may play a role in lymphomagenesis.

3.4 Main Conclusions

- Skewing the T cell phenotype to a CD4 phenotype only permits development of thymic lymphomas not resembling ALCL like disease.
- The presence of a functional TCR is not a requirement to establish peripheral ALCL like disease.
- TCR expression is lost in tumours and during thymocyte development. This indicates towards an incompatibility between NPM-ALK and a functional TCR.
- Mediastinum derived lymphomas are derived from a mature CD4SP or mature DP cell, both providing evidence that tumours are derived from cells that have undergone or just undergone positive selection. Indicating an intermediate signal via the TCR.
- Duration in the thymus is not a limiting factor to tumour development as CD4/NPM-ALK/Marilyn(f) have a similar survival age to CD4/NPM-ALK/OT1 mice yet still only develop thymic lymphomas.
- Potential role for the strength of the TCR signal and duration of time for disease generation.

- Tg TCR NPM-ALK models hint towards a CD8 phenotype as the cell of origin due to mice producing tumours closest to the human disease. Alternatively, NPM-ALK could have a preference for CD4 leading to quicker disease onset.

4 The role of RAG in ALCL Lymphomagenesis

4.1 Introduction

In order for our adaptive immune system to respond to a vast array of potential threats, diverse recognition mechanisms are required. T and B cells elicit these effects through peptide recognition by their corresponding antigen receptors, the TCR and BCR. These receptors need to be able to recognise a diverse range of antigens made possible by V(D)J recombination. The TCR comprises of either α and β, or γ and δ chains, expressed as a consequence of somatic recombination of the Variable (V), Diverse (D) and Joining (J) genes (Von Boehmer , 1990) (Schatz, et al., 1992) (Market & Papavasiliou, 2003). V(D)J recombination, therefore, allows for the generation of an extensive and diverse library of TCRs arising from the rearrangement of TCR chains. This process is driven and regulated by the Recombination Activation Genes (RAGs) 1 and 2 which are essential to the formation of the adaptive immune system (Nishana & Raghavan, 2012). This is illustrated in mice deficient in either RAG 1 or RAG 2 where lack of expression of either of the *RAG* genes leads to an absence of mature B and T cells in these mice (Shinkai, et al., 1992) (Mombaerts, et al., 1992). In the context of T cells, RAG expression initially occurs in the thymus during early developmental stages. Rearrangement of the TCR chains starts with γ and δ before the DN3 stage. At DN3, rearrangement of the β chain occurs with the β selection point being a crucial stage in thymocyte development (Livak , et al., 1999). Cells incapable of rearranging

their β chain successfully, do not pass this point and undergo apoptosis unless they have a γδTCR. Following successful β selection signalling via the pre-TCR complex, composed of the newly expressed β chain, pre-Tα and CD3, RAG expression is terminated and cells undergo proliferation in a ligand-independent manner. RAG is then re-expressed at the DP stage, a point at which α chain rearrangement occurs producing a mature αβ TCR T cells expressing this TCR consequently enter the clonal selection process. Successful interaction between the TCR and MHC complexes during positive selection terminates the expression of RAG via TCR signalling. This suppression of Rag is maintained by tonic signalling in the periphery with the exception of TCR immunoediting whereby RAG is re-expressed in autoreactive T cells responding to self-antigen to "edit" the autoreactive TCR (Wagner , 2007).

The process of V(D)J recombination is considered to be a cause of oncogenic rearrangements (Reddy , et al., 2006). In fact, aberrant RAG recombinase activity has been shown to be the driver for critical secondary events in the generation of Acute Lymphoblastic Leukaemia (ALL) (Papaemmanuil & et al , 2014). RAG can also induce DNA breaks and chromosomal rearrangements at cryptic RSSs and non-RSS sequences outside of antigen receptor loci (Karo , et al., 2014) (Shimazaki, et al., 2009). Studies have shown the presence of low RAG transcript expression in ALCL cases, potentially indicating a role for RAG in lymphomagenesis (Knecht, et al., 1993) (Malcolm , et al., 2016). In addition, previous research has also shown that despite the lack of cell surface expression of a TCR in ALCL cases, 56% display α and β chain rearrangements at the molecular level indicating a need for RAG during ontogeny. However, CD4/NPM-ALK mice on a RAG knockout background (CD4/NPM-ALK/RAG$^{-/-}$) still develop thymic lymphomas with a DP phenotype indicating that V(D)J recombination does not contribute greatly to lymphomagenesis in this model; its

absence only affected tumour phenotype and slightly delayed tumour development. However, these mice did not generate ALCL-like peripheral disease with tumours retained within the mediastinum (Malcolm , et al., 2016).

TCR transgenic mice express a fully functional TCR during the early stages of thymic T cell development and therefore RAG is not required for production of TCRs. However, RAG competent TCR transgenic mice are still capable of V(D)J recombination and therefore can generate additional endogenous TCRs other than the specific transgenic TCR. Analysis of TCRβ rearrangements within peripherally-located tumours developing in CD4/NPM-ALK/OTI mice showed germline TCR genes, suggesting that these tumours derived from cells that had not generated additional TCR through VDJ recombination (Malcolm , et al., 2016). In contrast, thymic-located CD4/NPM-ALK/OTII-derived tumours, carrying the tg OTII TCR, like tumours arising in the CD4/NPM-ALK mice display clonal and oligoclonal rearrangements of the TCRβ chain indicating that some tumours may arise from T cells expressing endogenous TCRs. Hence, to assess the role of the transgenic versus endogenous TCR in tumour development, CD4/NPM-ALK/OTI and CD4/NPM-ALK/OTII mice were backcrossed onto a $RAG2^{-/-}$ background.

4.2 Results

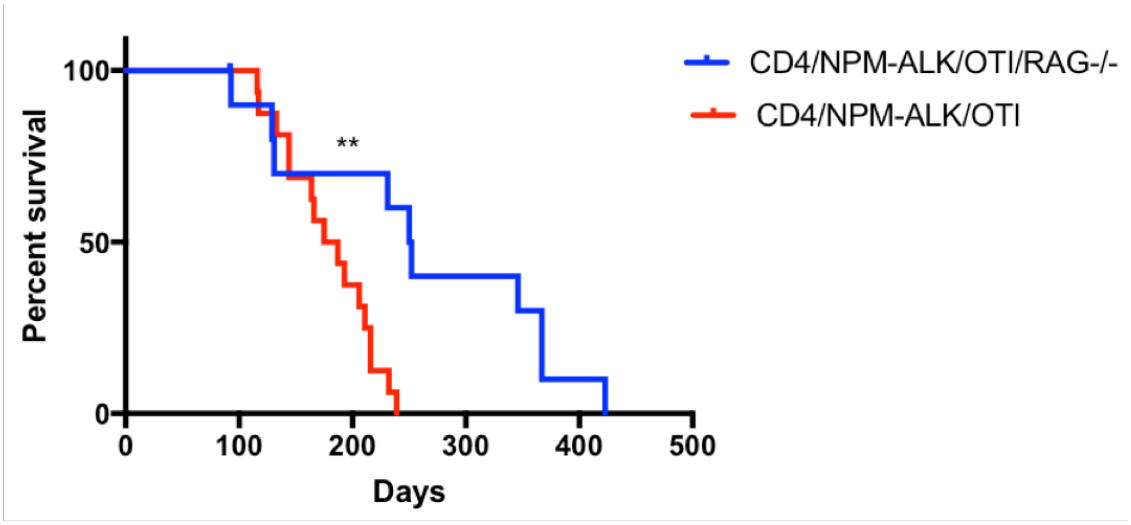
4.2.1 CD4/NPM-ALK/OTI/$Rag2^{-/-}$ mice do not generate ALCL-like disease

CD4/OTI/NPM-ALK mice were backcrossed onto a $RAG2^{-/-}$ background (CD4/NPM-ALK/OTI/$RAG2^{-/-}$) and aged to monitor tumour development. Kaplan-Meier analysis shows

a significant increase in overall survival for CD4/NPM-ALK/OTI/RAG2$^{-/-}$ mice (median 251 days) compared to their RAG competent littermates (median 181 days) (figure 4.1(a)). CD4/NPM-ALK/OTI/RAG2$^{-/-}$ mice did not generate ALCL-like disease as did their RAG-competent littermates nor thymic lymphoma and instead presented with sarcomas, hepatocellular carcinomas (HCC) and gastrointestinal stromal tumours (GIST) (table 4.1). As aged mice are prone to spontaneous tumour development, immunohistochemistry was performed to assess whether the tumours express NPM-ALK (Figure 4.1b). NPM-ALK expression was detectable in all tumours analysed suggesting that leaky, low level expression (from the CD4 promoter) in other tissues is able to drive tumour development presumably only when T cell lymphomagenesis is inhibited.

The reasons for the disparity in tumour phenotype presenting in these mice, in the presence and absence of RAG are not immediately clear. What can be established is that in the absence of RAG, there are no B cells which may contribute to tumour growth. Alternatively, RAG may induce further tumour-promoting mutations, through recognition of cryptic RSS sites in the CD4/NPM-ALK/OTI mouse contributing to T cell lymphomageneis although, CD4/NPM-ALK/RAG2$^{-/-}$ mice develop thymic T cell tumours albeit at a delayed latency. Another possibility is that endogenously rearranged TCRs contribute to tumour development.

a)



b)

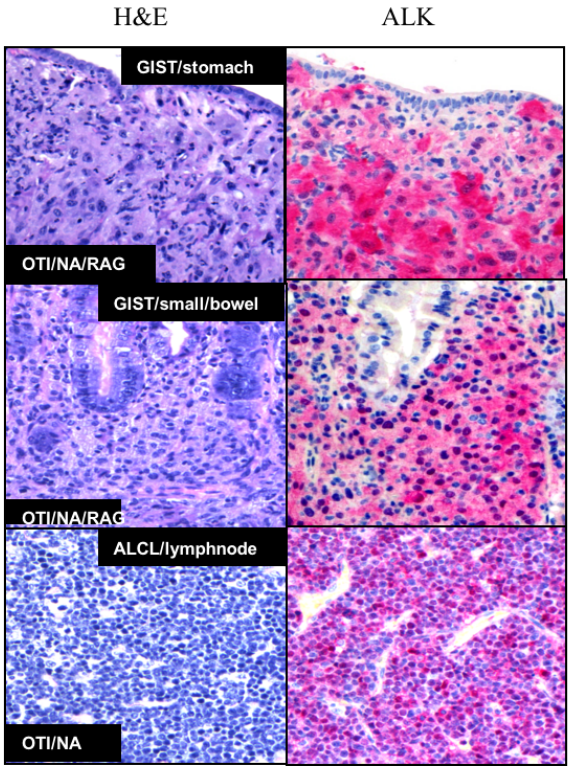


Figure 4.1 CD4/NPM-ALK/OTI/RAG2$^{-/-}$ do not develop ALCL like disease:
(A) Kaplan Meir analysis of overall survival of CD4/NPM-ALK/OTI (n=10) and CD4/NPM-ALK/OT1/RAG-/- mice (n=16), p=0.0052. (b) Haematoxylin and eosin, and ALK staining of representative tumour sections. For comparison, a representative tumour derived from a CD4/NPM-ALK/OTI mouse is also shown. Magnification is 400x

Mouse	Survival (days)	Diagnosis
1	231	Hepatocellular carcinoma
2	250	GIST
3	252	GIST
4	131	Sarcoma
5	346	Leiomyosarcoma and Hepatocellular carcinoma
6	93	GIST
7	129	Hepatocellular carcinoma
8	367	Sarcoma
9	367	Sarcoma
10	423	Hepatocellular carcinoma

Table 4.1: CD4/NPM-ALK/OTI/RAG$^{-/-}$ mice do not develop ALCL like disease:
Table shows summary of disease and age of onset for 10 mice.

4.2.2 B cells are not prevalent in tumours derived from CD4/NPM-ALK/OTI mice

Mice deficient in either RAG 1 or RAG 2 lack mature B and T cells and therefore, to assess whether mature B cells are a contributory factor towards lymphomagenesis in CD4/NPM-ALK/OTI mice, tumours from this model were assessed for the presence of B cells. Immunohistochemistry was performed on lymphoma sections derived CD4/NPM-ALK/OTI mice and B cells were detected by immunohistochemistry staining for CD79a (Figure 4.2). B cell representation was minimal suggesting a lack of involvement of B cells in ALCL lymphomagenesis in this model system.

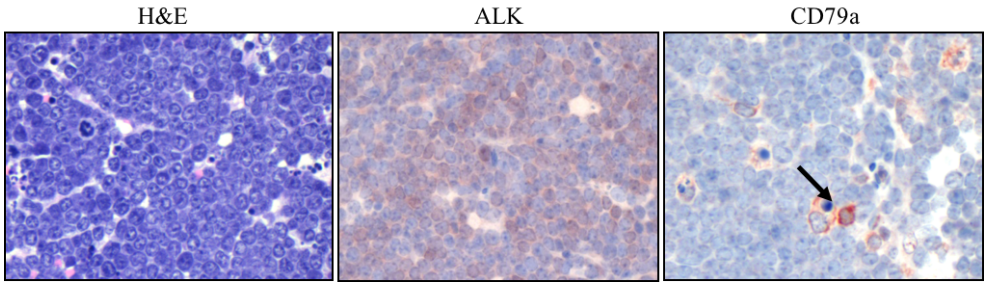


Figure 4.2 B cells are not prevalent in tumours arising in the CD4/NPM-ALK/OTI mouse: *Representative tumour from a CD4/NPM-ALK/OTI mouse was stained with H and E as well as NPM-ALK and CD79a, the latter indicative of B cells.*

4.2.3 CD4/NPM-ALK/OTI mice express RAG in peripheral tumours

RAG expression is repressed by tonic TCR signalling following successful positive selection in the thymus (Roose et al., PLOS Biology 2003). Therefore, RAG is not generally expressed in peripheral T cells unless in the context of receptor revision (Serra , et al., 2002). Tumours isolated from CD4/NPM-ALK/OTI mice were analysed for the presence of RAG transcripts by RT-PCR (figure 4.3). In all cases assessed, RAG transcripts were present suggesting that ongoing RAG activity perhaps through recognition of cryptic RSS sites or ongoing endogenous TCR gene rearrangement might be involved in lymphomagenesis.

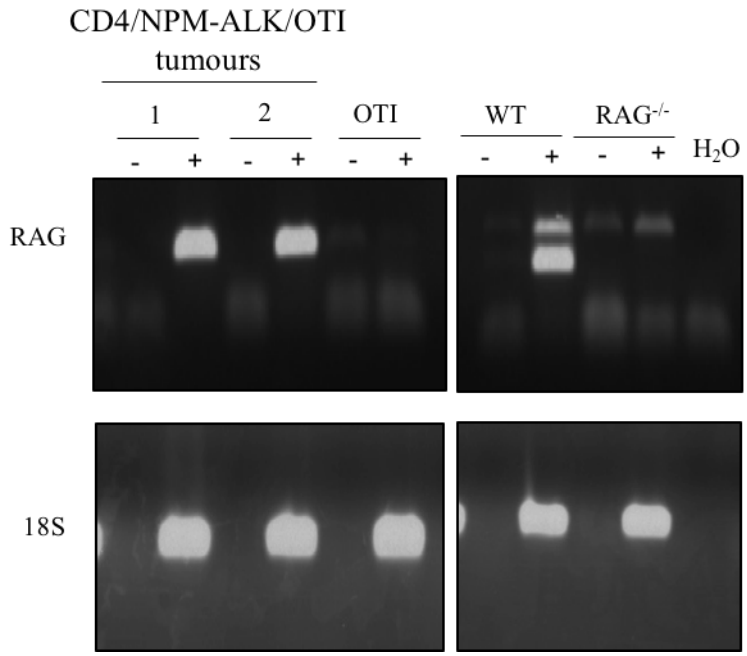


Figure 4.3: RAG transcripts are detectable in tumours derived from CD4/NPM-ALK/OTI mice
RT-PCR was performed for RAG2 with cDNA prepared from tumours isolated from CD4/NPM-ALK/OTI mice (2 representative examples are shown of 4 tumours analysed), OTI = cDNA isolated from peripheral lymph nodes, WT =cDNA isolated from thymi, RAG$^{-/-}$= cDNA isolated from thymi. The top panel shows transcripts for RAG2 and the lower panel for the 18s control PCR.

4.2.4 CD4/NPM-ALK/OTII/RAG2$^{-/-}$ mice do not generate ALCL like disease

In order to assess the effects of RAG deletion in CD4/NPM-ALK/OTII transgenic mice, the CD4/NPM-ALK/OTII line was back-crossed to a RAG$^{-/-}$ background. Mice were aged for tumour development and on showing clinical signs were culled for post mortem analysis. Only 18% of the mice developed lymphoma (2/11) with the majority displaying signs of inflammation on post mortem (e.g. myocarditis, lung congestion) but in the absence of tumour development (table 4.2). Overall survival varied in comparison to the RAG competent CD4/NPM-ALK/OTII mouse depending on the disease phenotype observed (figure 4.4). The two thymic lymphomas analysed by histology and FACS were ALK+ with a CD4SP phenotype still expressing components of the OTII receptor (Vα2 20.9%) although

this was less than the receptor expression levels observed in the NPM-ALK negative litter mate (Vα2 63%) (figure 4.5c).

Mouse	Age	Location	Disease
1	143	Peritoneal	Acites
2	105	Thymus	Lymphoma (ALK+ CD3+)
3	83	Heart and lung	Multifocal granulomatous inflammation with heart myocarditis acute and lung congestion
4	91	Liver	Multifocal coagulative necrosis with infiltration of neutrophils. No Lymphoma
5	NA	Fight wounds	Unknown
6	235	NA	No disease
7	198	NA	No disease
8	39	Peritoneal	Inflammation
9	191	Liver	Hepatitis
10	66	NA	No tumours
11	76	Thymus	Lymphoma (ALK+)

Table 4.2 CD4/NPM-ALK/OTII/RAG$^{-/-}$ mice rarely generate lymphoma: *Table shows summary of disease type, location and age of onset.*

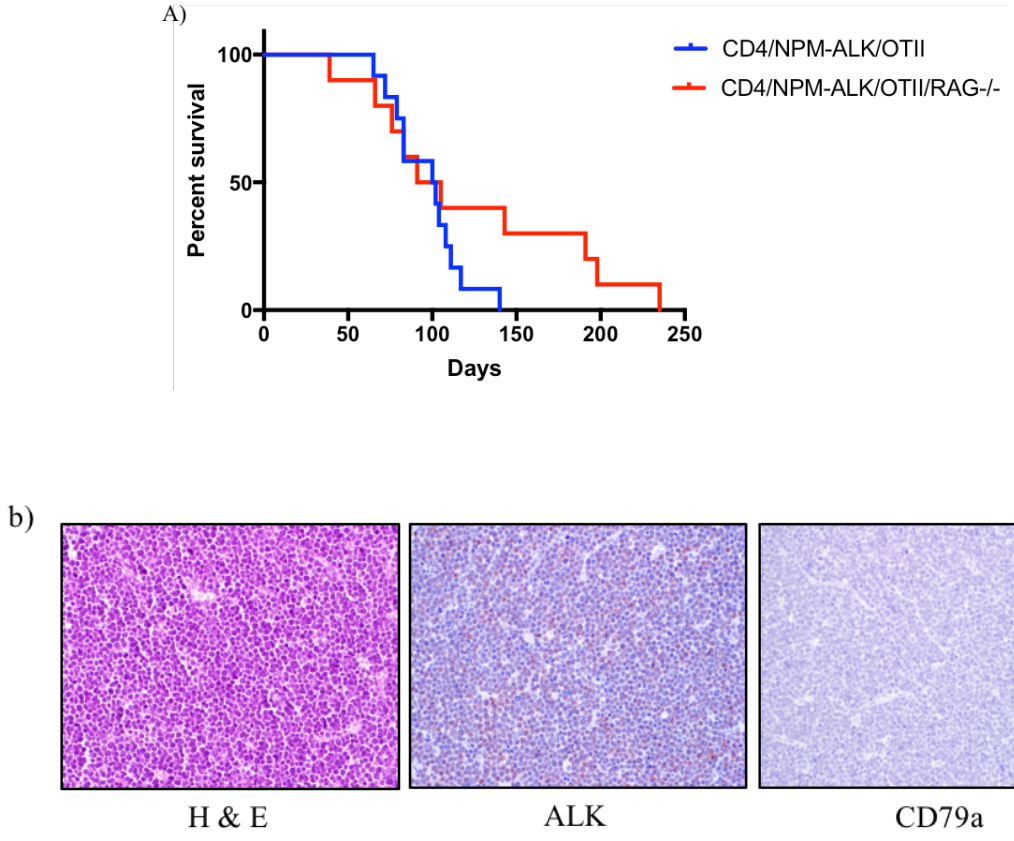


Figure 4.4 CD4/NPM-ALK/OTII/RAG$^{-/-}$ do not generate ALCL like disease:
(a)KAPLAN-MEIR survival curve CD4/NPM-ALK/OTII n=16 median survival=101 days, CD4/NPM-ALK/OTII/RAG2-/- n=11, median survival= 98 days, p=0.1658 (log rank mantel cortex) *(b) Histology representative of a thymic lymphoma showing H&E stain along with ALK and a B cell marker (CD79a).*

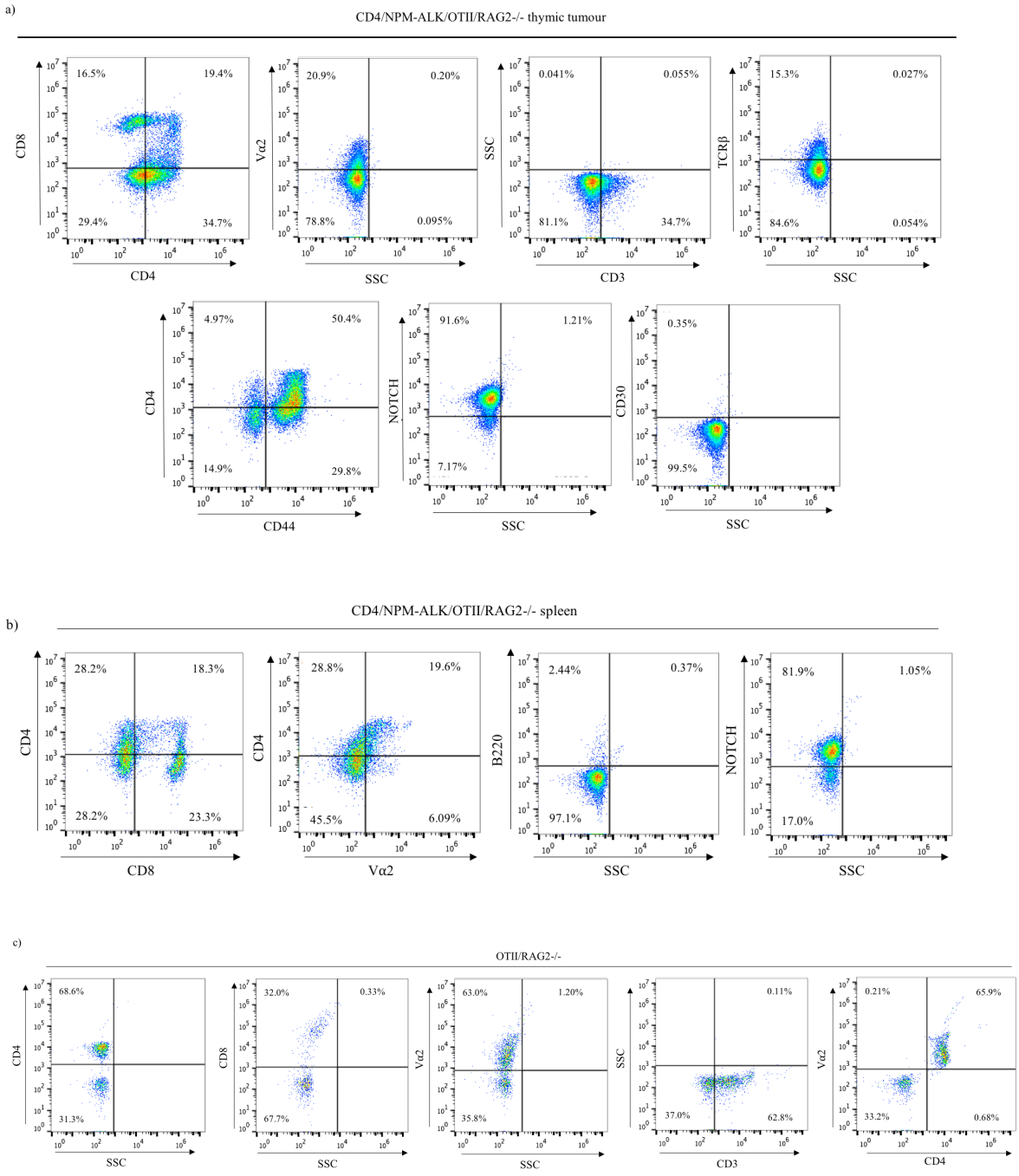


Figure 4.5 CD4/NPM-ALK/OTII/RAG$^{-/-}$ mice occasionally develop thymic lymphomas: *(a) Shows flow cytometry representation of a thymic lymphoma. (b) phenotype of peripheral lymphocytes of tumour-presenting mice. (c) Analysis of thymocytes from an NPM-ALK negative age-matched littermate.*

4.3 Discussion

Driven by RAG, the process of V(D)J recombination is considered itself be a risk factor for oncogenic mutagenesis due to its ability to introduce DSBs (Jackson & Jeggo, 1995). Indeed, it has been shown to be a contributor to lymphoma and leukaemia generation (Hubner , et al., 2004) (Miljuskovic, et al., 2015). Low levels of RAG transcript expression have been detected in human ALCL cases, potentially indicating a role for RAG in the development of ALCL (Knecht, et al., 1993) and for the CD4/NPM-ALK transgenic mouse model, deletion of RAG results in a significant, although subtle increase in survival (Malcolm , et al., 2016). However, the CD4/NPM-ALK transgenic model is not as good a mimic of human ALCL unlike the previously described CD4/NPM-ALK/OTI and therefore the effects of RAG activity were investigated in this latter mouse strain. Furthermore, back-crossing to a RAG knockout background served to ensure that only the transgenic TCR was expressed in the mice, without endogenous rearrangements occurring. For this same reason, the CD4/NPM-ALK/OTII transgenic mouse was back-crossed onto a RAG knockout background.

Genotype	Features of model	Disease phenotype	Conclusions	Reference
CD4/NPM-ALK	NPM-ALK expression from the CD4 promoter (expression throughout thymic development from DN1)	Thymic lymphoma mainly mature CD4SP and CD4/CD8 DP	NPM-ALK is oncogenic in thymic T cells	Malcolm et al., 2016
CD4/NPM-ALK/RAG$^{-/-}$	TCR gene rearrangement not possible	Thymic lymphoma containing T cells (CD4CD8 DP) but with an absence of surface TCR expression	Thymic tumour development is independent of RAG activity	Malcolm et al., 2016
CD4/NPM-ALK/OTI (no stimulation)	Express a TCR specific for ovalbumin on CD8 T cells, capable of endogenous TCR rearrangements	Peripheral ALCL: Hallmark cells, CD30+, TCR- (molecular TCR rearrangements present for the OTI receptor)	OTI transgene inhibits thymic tumour development/promotes peripheral disease	Malcolm et al., 2016
CD4/NPM-ALK/OTI/RAG$^{-/-}$	Mice only express TCR specific for ovalbumin (no endogenous TCR rearrangements)	Hepatocellular carcinoma, fibro sarcoma, gastrointestinal stromal tumours	RAG is required for peripheral T cell lymphoma development	Chapter 4
CD4/NPM-ALK/OTII	Express a TCR specific for ovalbumin on CD4 T cells, cells capable of endogenous TCR rearrangements	Thymic lymphoma containing mature T cells of mainly CD4SP and occasional CD8+CD4+ DP but with an absence of surface TCR expression	CD4 helper T cells are not able to transform in the periphery	Chapter 3
CD4/NPM-ALK/OTII/RAG$^{-/-}$	Express a TCR specific for ovalbumin on CD4 T cells (no endogenous TCR rearrangements)	No disease reported apart from 2 ALK+ lymphomas	RAG (clonal TCR) is required for thymic lymphoma development	Chapter 4
CD4/NPM-ALK/Marilyn(f)	Express a TCR specific for the H-Y antigen on CD4 T cells	Thymic lymphoma of mature DP and mature CD4 SP phenotypes with loss of surface TCR expression	CD4 T cells are not able to transform in the periphery	Chapter 3
CD4/NPM-ALK/Marilyn(m)	Express a TCR specific for the H-Y antigen on CD4 T cells. The H-Y antigen is expressed in the male mice.	ALK negative disease suspected myeloid and haematopoietic malignancies	NPM-ALK cannot bypass negative selection	Chapter 3

Table 4.3 Summary of disease phenotypes observed in the transgenic mice of the indicated genetic backgrounds

4.3.1 TCR transgenic mouse mimics of ALCL require RAG for lymphoma development

Murine models of NPM-ALK to date, including those discussed so far within this thesis, have

shown a variety of pathologies as shown in (table 4.3). Whilst the parental CD4/NPM-ALK transgenic mouse model continues to develop thymic lymphomas in the absence of RAG, this is not the case for the TCR transgenic lines whereby neither CD4NPM/ALK/OTII nor CD4NPM-ALK/OTI develop thymic nor peripheral lymphoma respectively, on a RAG knockout background. These data suggest that RAG is essential for tumorigenesis in these scenarios. The fact that CD4/NPM-ALK/RAG$^{-/-}$ mice develop thymic lymphomas but with a majority DP phenotype shows that NPM-ALK allows T cells to bypass the β selection point but is not able to support successful clonal selection although this may be because it stalls thymic T cell development at the DP stage. Yet, CD4/NPM-ALK transgenic mice produce tumours of an SP phenotype arguing against this, as do female CD4/NPM-ALK/Marilyn transgenic mice (RAG competent background). However, the male counterpart showed that NPM-ALK is not capable of bypassing negative selection evident by the lack of ALK positive tumours observed.

RAG is expressed in waves during T cell development, firstly before the point of TCR β chain rearrangement, where once productive rearrangement occurs signalling via the pre TCR results in loss of RAG expression. RAG is then consequently re-expressed at the DP stage to allow for TCR α chain rearrangement, to replace the pTα, producing a mature αβTCR. Again,TCR signalling results in the silencing of RAG expression upon successful interaction between the TCR and MHC expressing antigen. RAG expression is controlled by a basal level of TCR signalling which is ligand independent. This low-level signalling is mediated by proximal signalling molecules LAT and SLP-76, calcineurin, NFAT, ERK and Abl (Kuo & Schlissel, 2009) (Roose , et al., 2003) (Patra , et al., 2006). All four mouse strains (CD4/NPM-ALK, CD4/NPM-ALK/OTII, CD4/NPM-ALK/Marilyn(f), CD4/NPM-ALK/RAG-/-) developed tumours restricted to the thymus and prevented the production of peripheral lymphoma, though only three were capable of successfully undergoing positive

selection, producing a mature T cell phenotype. Therefore NPM-ALK cannot mimic steps required for successful positive selection nor can it bypass negative selection. Interestingly the CD4/NPM-ALK/OTI mimic is the only strain so far that has produced peripheral lymphomas with hallmark cells for ALCL. $RAG^{-/-}$ mice are incapable of producing mature T or B cells yet established CD4/NPM-ALK/OTI tumours showed no evidence of B cell involvement or contribution (figure 4.2). Tumours also derived from this model showed evidence of RAG transcripts suggesting RAG expression in mature T cells, (figure 4.3) as the cell phenotype in this model was a null activated phenotype (Malcolm , et al., 2016). CD4/NPM-ALK/OTII tumours were not assessed for the presence of RAG production as the presence of β chain rearrangements in established tumours provided evidence of RAG expression and function, in conjunction the location of the tumour would also show RAG expression in uninvolved T cells. This suggests that in the presence of a Tg TCR, RAG plays a role in lymphomagenesis, perhaps in the removal of the TCR. Or that detection of RAG expression could be representative of immature T cells present within the tumour or deregulation of T cell maturation leading to the persistence of RAG expression (Loza & Perussia, 2002). These results could indicate towards a potential role of RAG in ALCL development though it is uncertain whether the role is extrinsic or intrinsic.

4.3.2 Intrinsic roles of RAG

RAG expression in the periphery is rare but possible in the context of TCR editing of TCRs responding to self-antigen. This has been shown in a TCR tg mouse where the Vβ5 Tg CD4 cells re-express RAG1 and RAG2 genes when exposed to a weak super antigen (Macmahan & Fink , 1998). It has been documented that some populations of CD8+ T cells are also capable of re-expressing V(D)J recombination machinery within the periphery, producing new TCRs in response to antigenic stimulation (Serra , et al., 2002). Perhaps NPM-ALK

permits the re-expression of RAG, in peripheral T cells, in tg mice even though it is not required for lymphomagenesis in the CD4/NPM-ALK/RAG2$^{-/-}$ model.

This process of re-expression of RAG is thought to be down to loss of the TCR and TCR associated signalling, which supresses RAG expression. During thymocyte development TCR dependent and independent signalling results in ERK and ABL activity which supresses RAG expression (Fink & Hale , 2010). However, NPM-ALK upregulates ERK suggesting that this constant signalling via ERK should consequently suppress RAG expression. Though this does not rule out an interference with RAG expression by other mechanisms affected by NPM-ALK. The activation of Akt inhibits a negative regulator of RAG, NFATc1 (Patra , et al., 2006) and NPM-ALK has been shown to activate the PI3K – Akt pathway suggesting that this may be the mechanism by which NPM-ALK bypasses RAG suppression, via ERK related mechanisms (Slupianek , et al., 2001).

RAG could also be expressed as an indirect effect of NPM-ALK. NPM-ALK has been shown to epigenetically silence proximal signalling components of the TCR signalling cascade for which activation of LAT and SLP76 are considered to be suppressers of RAG expression. Perhaps silencing leads to re-expression of RAG which interferes with cell surface TCR expression. In addition to this, the presence of RAG or absence for that matter plays a role in T cell fitness in terms of their ability to respond to DNA damage. OTI T cells derived from RAG$^{-/-}$ mice show an inability to repair double strand breaks in comparison to RAG competent cells. Stimulation of OTI Rag$^{-/-}$ cells with OVA displayed with reduced survival cells indicating that the presence of RAG during ontogeny is important for T cell fitness and survival. RAG activity during T cell development has been shown to provide protection and or fitness for T cells when exposed to stresses and proliferation later in their life span (Karo , et al., 2014). Therefore, the re-expression of RAG could contribute to cell survival maybe in response to two levels of T cell stimulation one being induced by the TCR and the secondary

the effects of NPM-ALK upregulating ERK activity downstream. A way to address whether the role of RAG is intrinsic or extrinsic would be to adoptively transfer CD4/NPM-ALK/OTI cells into a $RAG^{-/-}$ mouse or OTI $RAG^{-/-}$ cells into a CD4/NPM-ALK/OTI irradiated mouse and assess disease progression.

Interestingly the two thymic tumours recorded in the CD4/NPM-ALK/OTII/$RAG^{-/-}$ were positive for Notch following the same trend as the CD4/NPM-ALK$RAG^{-/-}$ model previously where NPM-ALK increased Notch expression to bypass the β selection point (Malcolm , et al., 2016). In this instance, it may be concluded that the two thymic tumours observed in the CD4/NPM-ALK/OTII/$RAG^{-/-}$ model were driven by Notch.

4.3.3 Treg or memory T cells may contribute to ALCL development

The influence of RAG in OTI transgenic mice has been documented. It is thought that half the peripheral T cell population are memory cells, by which $RAG1^{-/-}$ knockout OTI mice have about half the peripheral T cell population of RAG competent mice (Clarke , et al., 2000). This, suggests that in the absence of RAG, OTI mice lose their memory T cell population and potential Treg populations as well. OTI positive cells with rearranged α chain can be selected for by MHC class II, which with the presence of RAG allows for this as endogenous α chains can still be rearranged and expressed (Clarke , et al., 2000). This can also give rise to the production of Treg cells (DiPaolo & Shevach , 2009). CD4SP tumours were observed in thymic lymphomas derived from CD4/NPM-ALK, CD4/NPM-ALK/Marilyn and CD4/NPM-ALK/OTII mice (Chapter 3) (Malcolm , et al., 2016). Though in the absence of RAG CD4/NPM-ALK/$RAG^{-/-}$ mice only displayed with DP tumours suggesting that productive α chain rearrangements and expression is essential to the formation of SP tumours.

Isolated Treg cells containing rearranged α chains from OTII mice have been shown to respond to an unknown peptide expressed within the thymus of C57BL/6 mice, present late in thymocyte development, in the thymic medulla (DiPaolo & Shevach , 2009). Perhaps recognition of this peptide, in the thymus of CD4/NM-ALK/OTII mice, may play a role in lymphomagenesis and if these Treg cells are absent in $RAG^{-/-}$ this may account for a lack of tumours observed in the CD4/NPM-ALK/OTII/$RAG2^{-/-}$ model (table 4.1). Perhaps removing the presence of RAG form either the CD4/NPM-ALK/OTII or CD4/NPM-ALK/OTI depletes mice of the "cell of origin" hinting that ALCL may be derived from either Treg or memory T cells only present in RAG competent mice. This also indicates an importance for α chain rearrangement and expression. Previous work has shown 16/17 ALCL demonstrated evidence of α chain rearrangement by array CGH and α chain transcripts were detectable in 10/15 of these positive cases whereas array CGH of α chain rearrangements were only present in 3/17 cases (Malcolm , et al., 2016). This again points to an importance for the α chain, though NPM-ALK can bypass the β selection point NPM-ALK cannot bypass positive selection leading towards a reliance on the α chain. Therefore, with this in mind, the role of Treg or memory T cells should be addressed. This could be achieved by sorting Treg and memory T cells from mice and adoptively transferring them into recipient C57BL/6 mice to address if these cellular populations establish disease.

4.3.4 RAG and cellular fitness

Mice studied within this thesis were housed in a barrier facility, presumed to be pathogen-free but know to be positive for *Helicobacter hepaticus*, particularly the rack on which the mice described herein were housed. In immunocompetent mice, this infection tends not to cause an issue although in immunocompromised mice there may be an inflammatory response in the gut and liver. Enterohepatic *helicobacter* infection in mice results in a disease

resembling inflammatory bowel disease and *H. hepaticus* has also been linked to hepatocellular carcinoma in A/J mice (Chichlowski & Hale , 2009). The presence of RAG is thought to play a role in the fitness of innate lymphoid cells (ILC), these protect against pathogens at mucosal surfaces such as the gut and lungs. 30-50% of these cells in an OTI/RAG mouse showed history of RAG expression during ontogeny and as seen in the OTI/RAG$^{-/-}$ cells ILCs from RAG$^{-/-}$ mice had higher gH2AX levels indicating a role for RAG in development of fitness of lymphocytes (Karo , et al., 2014). This could be the case for the OTII mouse as well and could be a plausible explanation for infection related illnesses observed in these mice, which did not seem to affect other colonies in the housing facility (table 4.2). 2/11 (18%) of mice presented with thymic lymphomas yet the remainder showed no signs of tumour development and those that did generate illness this was due to infection like symptoms but no sign of malignancy.

Hence, it is conceivable that the presence of these organisms in the RAG$^{-/-}$ models could be responsible for HCC and GIST observed in CD4/NPM-ALK/OTI/RAG2$^{-/-}$ mice (table 4.1). However, NPM-ALK was expressed in these tumours suggesting that it too may have played a role, perhaps due to leaky expression from the promoter (figure 4.1). Or that due to the expression of NPM-ALK being driven by the CD4 promoter, there could be a chance that NPM-ALK may be expressed in additional cells outside of the T cell repertoire. By which monocytes, macrophages and dendritic cells all express CD4 (Fillon , et al., 1990) (Vremec , et al., 2000). Though analysis of T cells showed that NPM-ALK was not solely restricted to the CD4 lineage as protein could be detected in CD8 cells and during T cell development as early as the DN1 stage (Malcolm , et al., 2016). Given that these tumours arose at a relatively late stage in life for these mice, it is most likely that these tumours are age and environment-related, perhaps combined with low-level expression of NPM-ALK. This then begs the question as to why these mice do not develop lymphoid malignancies like the parental

CD4/NPM-ALK/OTI strain, indeed CD4/NPM-ALK/RAG$^{-/-}$ mice still present with mediastinal lymphoid malignancies. Therefore, combined, these data are suggestive of a role for RAG in peripheral T cell lymphoma generation but not thymic disease.

4.4 Summary

This chapter sought to investigate the contribution of RAG to lymphomagenesis in the context of NPM-ALK-induced T cell lymphoma. Whilst patient tumour tissues express RAG, its contribution to disease pathogenesis remains to be elucidated. This data shows that in the absence of RAG, CD4/NPM-ALK/TCR tg mice do not develop lymphomas and due to the detection of RAG transcripts there may be an intrinsic or extrinsic role of RAG in ALCL lymphomagenesis.

5 Examining the role of the TCR towards lymphomagenesis

Main aims:

- Address the effects of TCR signalling in the presence of NPM-ALK
- Determine whether there is a role for infection
- Is the presence of NPM-ALK and a functional TCR not compatible with lymphomagensis?

5.1 Introduction

The contribution of infection towards tumorigenesis and the particular organisms involved have become increasingly more evident (Chang & Parsonnet , 2010)((Mager , 2006) (Martin & Gutkind , 2009). The mechanisms by which infections contribute to tumorigenesis can occur either by direct effects of the microorganism upon the cell (e.g. HPV and cervical carcinoma) (Munger , et al., 2004) or via a sustained chronic inflammatory response (e.g. Helicobacter and gastric cancer) (Wotherspoon, et al., 1991), giving rise to mutagenesis. Direct stimulation of immune cells is another mechanism in which infectious microorganisms can contribute towards or be considered as a driver of lymphomagenesis. In MALT lymphoma, for example, BCR signalling contributes to lymphomagenesis by driving growth and survival, via activation of the NF-kB pathway (Gascoyne , 2003)(Niemann & Wiestner, 2013). Though not fully established, T cell lymphomas are also capable of being driven by antigen receptor activity and transformation of the cell can be dependent upon the presence of a TCR. For example, a PTCL mouse model has been shown to be dependent upon intact TCR signalling for cellular transformation whereby proximal signalling is retained in order to drive the disease (Wang & et al, 2011).

ALK positive ALCL has rarely been linked to an infectious aetiology, except for the cutaneous form of the disease (Lamant , et al., 2010) and (Kang , et al., 2015). Although association of infectious organisms or foreign antigens as a contributing factor tends to be more predominant in ALK –ve ALCL cases, the most recent being breast implants (Ye, et al., 2014). Whilst an infectious aetiology in ALK+ ALCL has not been identified, this does not discount a 'hit and run' mechanism providing the secondary impetus (Skinner , 1976). NPM-ALK can be detected within cord blood samples (1.95%) and in peripheral blood from healthy individuals, although it is very unlikely that this proportion of individuals would generate ALCL, due to ALCL only representing 10-15% of paediatric non-Hodgkin lymphomas (Laurent , et al., 2012) (Maes & et , 2001) (Trumper , et al., 1998) (Ferreri, et al., 2012). Therefore, the reason as to why some people develop ALCL and others do not is unknown.

ALCL is defined by a "null" cell with an activated T cell phenotype, expression of granzyme B and perforin. Yet patient tumours, cells lines and tumours derived from the CD4/NPM-ALK mouse mimics, to date and presented within this thesis, all lack the expression of the cell surface TCR complex, even in models forcibly expressing TCRs via transgenesis (Malcolm , et al., 2016) (Chiarle , et al., 2003) (Alexander & Turner , 2005) (Chapter 3). Cell surface expression of the TCR is lacking in human ALCL cases, although cells from established tumours do show rearrangements of their TCR α and β chains, whereby 58% of cases have fully rearranged $\alpha\beta$ chains (Malcolm , et al., 2016). Transcripts and intracellular protein are also detectable in tumours derived from CD4/NPM-ALK/OTII mice (Chapter 3). This indicates that cells are producing the TCR and should therefore be capable of expressing the receptor. In addition, cell lines and patient samples lose expression of proximal signalling molecules, although components of the TCR signalling cascade, i.e. MAP Kinase, is

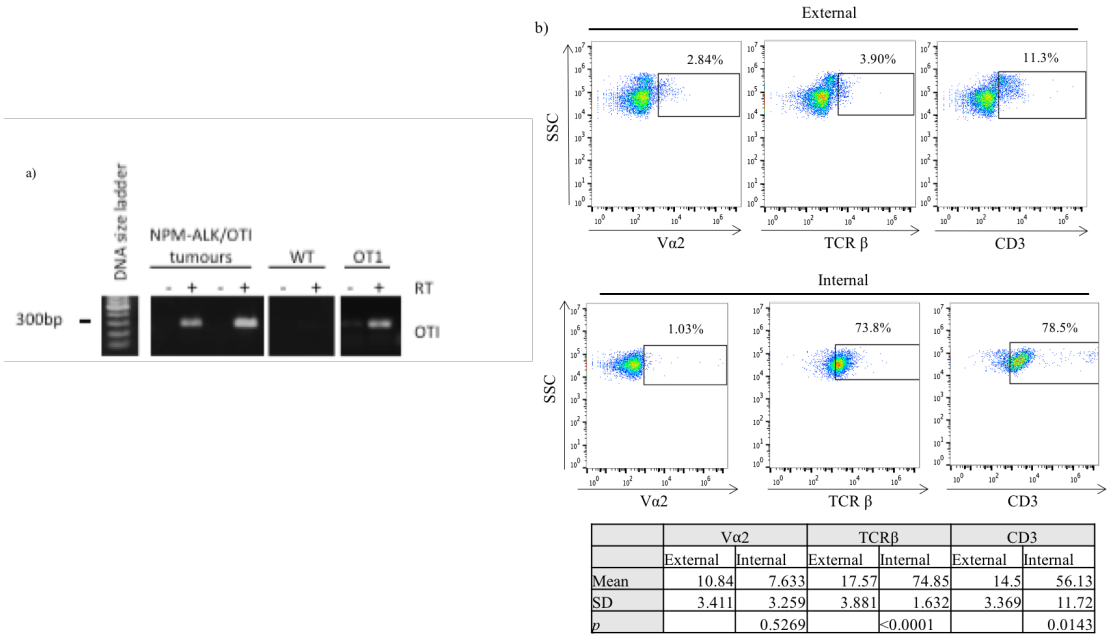
upregulated further downstream by NPM-ALK, mimicking TCR signalling (Bonzheim , et al., 2004) (Turner , et al., 2007). Signalling via the TCR may account for the activated phenotype observed and the loss of the receptor. Upon receptor antigen engagement leading to consequent T cell activation results in the internalisation of the T cell receptor (Jose, et al., 2000). Stimulation via the TCR, therefore, could explain this activated phenotype and the absence of cell surface TCR expression (Finetti, et al., 2009). These observations further indicate interference and perhaps incompatibility between NPM-ALK and TCR expression. Hinting that even though signalling via the TCR may not necessarily be a driver of ALCL it could be required in the events that contribute towards cellular transformation.

The CD4/NPM-ALK/OTI model displayed evidence for a functional TCR to be required for thymic egress and development of peripheral disease. However, the presence of a functional TCR is not a requirement in order for NPM-ALK to simply transform cells as illustrated in the CD4/NPM-ALK/RAG$^{-/-}$ mouse (Malcolm , et al., 2016). Nor is it the case that a functional TCR alone was required to generate peripheral disease as demonstrated in chapter 3 wherein MHC class II restricted mice produce lymphomas in the mediastinum. However, the CD4/NPM-ALK/OTI mouse did produce the closest ALCL mimic to date indicating some importance for the TCR in the context of MHC class restriction. The survival differences between the two MHC class II restricted models hinted towards a role for signalling strength via the TCR. This chapter aims to address the role of infection in ALK+ ALCL and the interplay between NPM-ALK and the TCR using the CD4/NPM-ALK/OTI mouse mimic.

5.2 Results

5.2.1 Established tumours from CD4/NPM-ALK/OTI mice lack cell surface expression of the TCR but still produce OTI protein

The CD4/NPM-ALK/OTI model develops peripheral tumours with hallmark cells but tumour cells lack cell surface expression of the OTI TCR (Malcolm , et al., 2016). Indeed, an absence of cell surface expression, of the TCR, is consistently observed in all NPM-ALK/TCR transgenic mice assessed in chapter 3. To elucidate further the fate of the TCR, cells from tumours isolated from CD4/NPM-ALK/OTI mice were assessed for OTI TCR transcripts and intracellular protein. OTI transcripts were detected in tumour cells (figure 5.1a) suggesting post-transcriptional regulation of TCR expression. Indeed, intracellular proteins of the TCR complex (TCRβ chain and CD3) were detected (figure 5.1b) suggesting that NPM-ALK actively internalises the TCR and/or prevents its expression on the cell surface. Internal expression of Vα2 was not present, which due to presence of transcripts for the OTI TCR could suggest that perhaps the protein was being degraded.



	Vα2		TCRβ		CD3	
	External	Internal	External	Internal	External	Internal
Mean	10.84	7.633	17.57	74.85	14.5	56.13
SD	3.411	3.259	3.881	1.632	3.369	11.72
p		0.5269		<0.0001		0.0143

Figure 5.1: CD4/NPM-ALK/OTI tumour cells produce OTI transcripts and express intracellular TCRβ and CD3:
(a) RNA was extracted from tumour cells of the indicated genotypes and RT-PCR conducted to detect the presence of the OTI transgene. Data are representative of 4 tumours analysed. WT = wild-type C57BL/6, OTI = RNA isolated from lymph nodes, NPM-ALK/OTI = RNA from CD4/NPM-ALK/OTI tumours, RT = reverse transcriptase (b) Tumour cells were isolated from CD4/NPM-ALK/OTI transgenic mice and analysed for expression of the TCRβ and TCRα chains as well as CD3ε both on the cell surface (top panel) and intracellularly (bottom panel) by flow cytometry. Data were firstly normalised for the isotype control antibody and are representative of 3 tumours analysed.

5.2.2 Strong stimulation, via the TCR, of NPM-ALK positive cells affects cell viability

As tumours arising in mice expressing NPM-ALK do not produce cell-surface TCR, the effects of NPM-ALK on TCR expression and stimulation were assessed *in vitro*. T cells were isolated from the spleens of 5-week-old pre-tumour CD4/NPM-ALK mice (in which established tumours lack cell surface TCR expression (Malcolm , et al., 2016) (Chiarle , et al., 2003) mice and C57BL/6 mice and stimulated for 24 hours with anti-CD3 (10µg/ml) alone or in combination with anti-CD28 (2µg/ml) (figure 5.2a). Cell viability was assessed using an MTT assay showing that NPM-ALK positive cells had suppressed proliferation in comparison to NPM-ALK negative cells (anti-CD3; p=0.0019, anti-CD3anti-CD28;

p=0.0003). Likewise, T cells isolated from CD4/NPM-ALK/OTI mice demonstrated a reduced proliferative capacity in comparison to OTI T cells upon stimulation (anti-CD3 and anti-CD28; *p*=0.0154). A similar result was also observed when cells were exposed to the OTI specific OVA peptide (SINFEKL) (*p*=0.0224) (figure 5.2b). Prior to stimulation OTI cell surface receptor expression was determined showing that in both OTI and CD4/NPM-ALK/OTI mice cell surface receptor expression was similar (figure 5.2c).

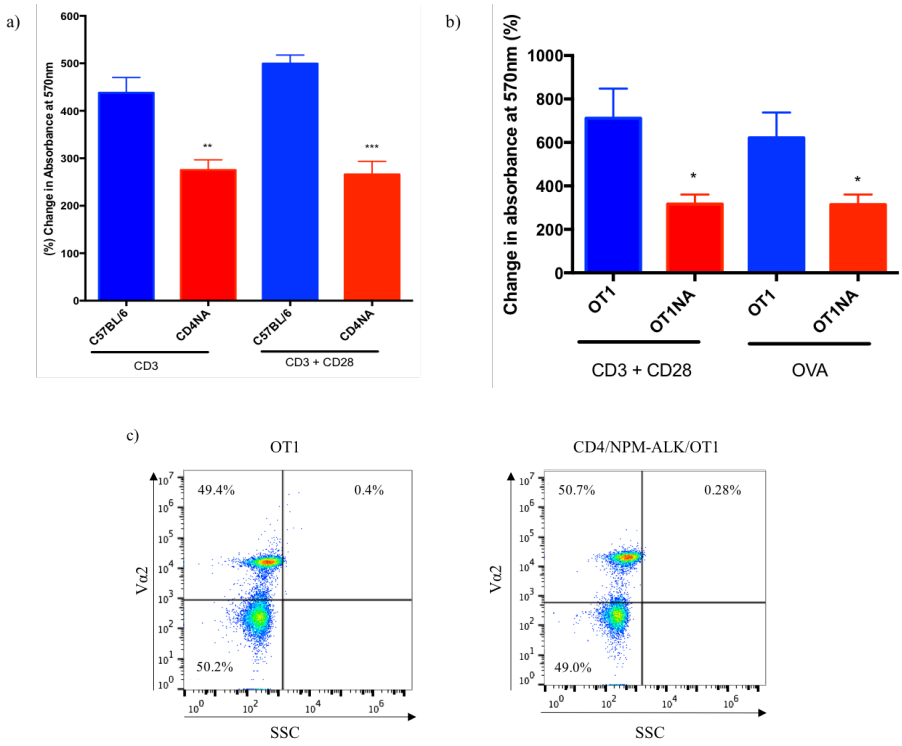


Figure 5.2: NPM-ALK-expressing OTI T cells show reduced proliferation in response to TCR engagement *(a) T cells derived from spleens of 5 week old C57BL/6 and CD4/NPM-ALK mice were stimulated with anti-CD3 alone or in combination with anti-CD28 antibodies and viability assessed by MTT assay 24 hours later of 3 biological repeats ** p= 0.0019, ***p=0.0003 Data represent means and standard deviations of biological and technical triplicates (b) Stimulation of T cells derived from spleens and lymph nodes of 8 week old CD4/NPM-ALK/OTI or OTI mice stimulated with anti-CD28 and anti-CD3 antibodies or OVA 257-264 peptide and viability assessed 24 hours later by MTT assay. Data presents means and standard deviations of biological and technical triplicates. *p=0.0154 and *p=0.0224 respectively (c) Representative of 4 flow cytometry plots of OT1 (Vα2) cell surface expression on T cells, prior to stimulation.*

5.2.3 NPM-ALK is permissive towards weak signalling via the TCR

Strong stimulation upon engagement of the TCR seems to be prohibitive towards cell proliferation in the presence of NPM-ALK. It has previously been shown that NPM-ALK is able to substitute for signals normally produced downstream of an engaged TCR (Turner , et al., 2007) suggesting that this, together with stimulation through the TCR may result in suppression of cellular proliferation. In essence, 'over-stimulation' of NPM-ALK expressing T cells has a pejorative effect. Hence, in order to address whether variation of the cumulative effects of stimulation through the TCR and NPM-ALK expression affects proliferation, peptides of differing avidity for the OTI TCR were applied to T cells *in vitro* (fig 5.3a). Proliferation was determined via CFSE dilution over a period of 72 hours and as previously observed, stimulation with anti-CD3 and anti-CD28 or OVA peptide resulted in reduced proliferation of NPM-ALK expressing T cells. Interestingly, peptides with weaker avidity for the OTI TCR, specifically the G4 peptide, was permissive of cell proliferation in the presence of NPM-ALK with a significant increase over wild-type OTI T cells (p=0.0072), an effect that was not observed with the E1 peptide, the weakest agonist of the OTI TCR (figure 5.3b). These data were mirrored with the expression of CD25, a marker of T cell activation, whereby following stimulation of NPM-ALK expressing T cells with the G4 peptide, a significant increase (p=0.0009) in cell surface expression was noted (figure 5.3c). A reduction in CD25 expression in the presence of NPM-ALK was seen on T cells expressing NPM-ALK and stimulated with OVA, anti-CD3 plus anti-CD28 although this was not significant. These results suggest that NPM-ALK is permissive of weaker signalling via the TCR and therefore may favour antigens with lower avidity for a given TCR.

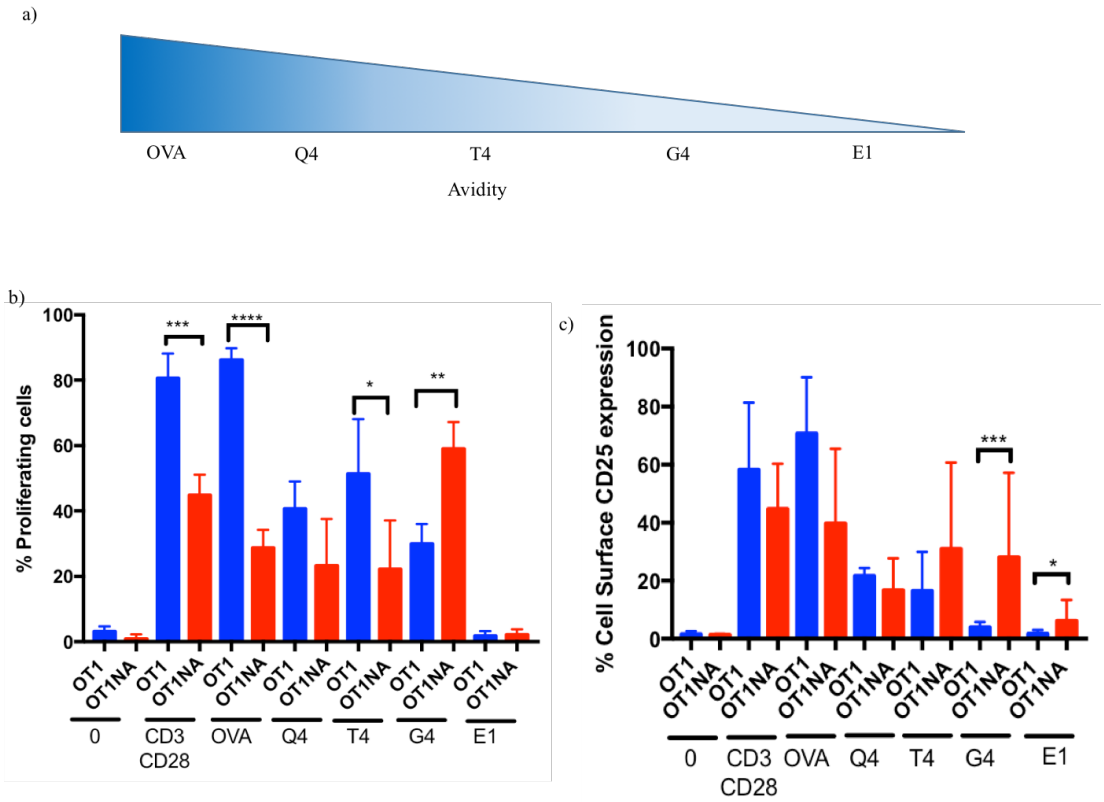


Figure 5.3: Weak signalling via the TCR in CD4/NPM-ALK/OTI pre-tumour T cells shows an increase in proliferation and activation

*(a) Peptides of differing avidity for the OTI TCR were applied in vitro to T cells as indicated. (b) Pre-tumour T cells derived from lymph nodes and spleens of 10-week-old CD4/NPM-ALK/OTI and OTI mice were stimulated in vitro with peptides or anti-CD3 and anti-CD28 as indicated. Over 72hours, proliferation was determined via CFSE dilution. Data represents means and standard deviations of 4 biological repeats gated on the CD8 population, ***p= 0.0003, ****p=<0.0001, *p=0.0412, **p=0.0072. (c) T cell activation was determined by cell surface expression of CD25. Data represents means and standard deviations of 4 biological repeats. ***p=0.0009, *p=0.0202.*

5.2.4 Weak stimulation via the TCR, in CD4/NPM-ALK/OTI mouse T cells, leads to an increase in ERK activation:

Activation of T cells via the TCR triggers proximal signalling, recruitment of proteins to the intracellular TCR and ultimately production of IL-2 and other proteins through activation of transcription factors including AP-1, NFkB and NFAT amongst others. The downstream

effects of TCR ligation are dependent on a number of variables including the avidity of the antigen. Hence, to address (a) the effects of stimulation via the TCR on proximal signalling in the presence of NPM-ALK and (b) to determine whether proximal signalling is still intact, pre-tumour T cells were stimulated and expression and activation of proximal signalling determined via Western blot. Pre-tumour T cells derived from CD4/NPM-ALK/OTI and OTI littermate mice were stimulated *ex vivo* for 30 minutes with either anti-CD3 and anti-CD28, OVA, G4 or E1 peptides. Western blot analysis shows that proximal signalling is still intact as phosphorylated proteins can still be detected (figure 5.4a). Whilst not exhaustive, these data show that ERK phosphorylation, following TCR ligation, is not detectably different to wild-type OTI cells except in the case of stimulation with the G4 and E1 peptides whereby NPM-ALK expression results in increased levels of pERK possibly accounting for the increased cellular proliferation observed (figure 5.3b). Interestingly, in the presence of NPM-ALK regardless of stimulation, the mobility of ZAP 70 is higher suggestive of a smaller protein perhaps lacking a post-translational modification which does not alter phosphorylation at tyrosine 319.

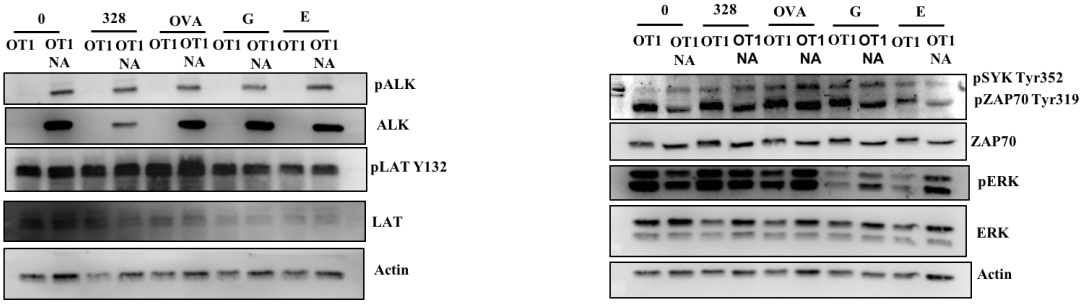


Figure 5.4: CD4/NPM-ALK/OTI cells show intact proximal signalling and increased ERK activity in response to weak agonists:
Cells were stimulated for 30 minutes with the indicated antibodies/peptides prior to cell lysis and Western blot analysis for the indicated proteins. These data are representative of two independent experiments.

5.2.5 *In vivo* stimulation of pre-tumour CD4/NPM-ALK/OTI mice:

Prior data gleaned from *in vitro* analyses, suggests that NPM-ALK expression affects cellular response to antigenic stimulation and that strong stimuli may have a pejorative effect on cell proliferation. Therefore, to determine whether this response affects tumour growth *in vivo*, CD4/NPM-ALK/OTI mice were exposed to a murine herpes virus expressing the OVA (SINFEKL) peptide (MHV-OVA) (Smith , et al., 2007). The CD4/NPM-ALK/OTI mice develop an ALCL-like disease in the absence of exogenous T cell stimulation with a penetrance of 100% (Malcolm , et al., 2016). Pregnant CD4/NPM-ALK/OTI mice were administered MHV-OVA intranasally 1 week after plugging (gestation day 1 starts one day after plugging). Then offspring were exposed to the virus intraperitoneally, to ensure that infection was systemic and not maintained within the lungs. Virus was then administered monthly, for three months, post weaning and monitored for tumour development (figure 5.5). Overall survival in comparison to uninfected mice was much greater (245.5 days compared to 167) and interestingly, the majority of mice did not generate lymphoma, but instead hepatocellular carcinoma and sarcomas which were positive for the expression of NPM-ALK (table 5.1, figure 5.6a). Mice that did develop lymphoma (2/12) did not display with hallmark cells, typical of human ALCL, as did control mice. It could be suspected that these were B cell which is common in aged mice of the OTI strain, though remains unknown as these tumours were not analysed. Even though the occurrence of lymphoma is less common in these mice, OTI T cells are still detectable in uninvolved lymph nodes (figure 5.6b) suggesting that lack of lymphoma was not due to clearance of all OTI T Cells as may occur in acute stimulation. Conversely, the phenotype of mice infected only after weaning (1 month of age) and monthly thereafter, were not affected by virus administration in terms of disease presentation, phenotype and survival producing half (4/8) developing atypical peripheral T cell lymphoma with hallmark cells as previously seen in this strain (Table 5.2, Figure 5.7a,b).

Histology performed on these tumours showed them to be driven by NPM-ALK as expected. However, 2/8 mice presented with thymic lymphomas for which a representative image is shown in figure 5.7c (and peripheral cells of that mouse figure 5.7d). Flow cytometry, gating on the tumour population, showed this tumour to be DN, lacking expression of the OTI receptor but showing expression of TCRβ. Indicating that this tumour may have arisen from an early stage of development which may not have expressed the OTI receptor due to the presence of RAG. In contrast, the majority of peripheral T cells in this mouse, expressed the OTI receptor and CD8 (figure 5.2.7d). The remaining 2 mice of this cohort did not develop lymphoma within a 200-day time frame at which point they were culled.

Mice that were only subjected to virus *in utero* (table 5.3, figure 5.8a,b) again developed atypical lymphomas of this strain with hallmark cells. Flow cytometry and histology representation of a splenic lymphoma showed tumours to be driven by NPM-ALK with infiltrating activated T cells, indicated by the expression of CD28 and B220 when gating on tumour cells (figure 5.8b). Whilst these data are suggestive of a discordance between NPM-ALK expression and antigenic stimulation in the development of lymphoma, they suggest that the time of exposure to infection, during development, may be critical and this event may be protective against ALK+ALCL development.

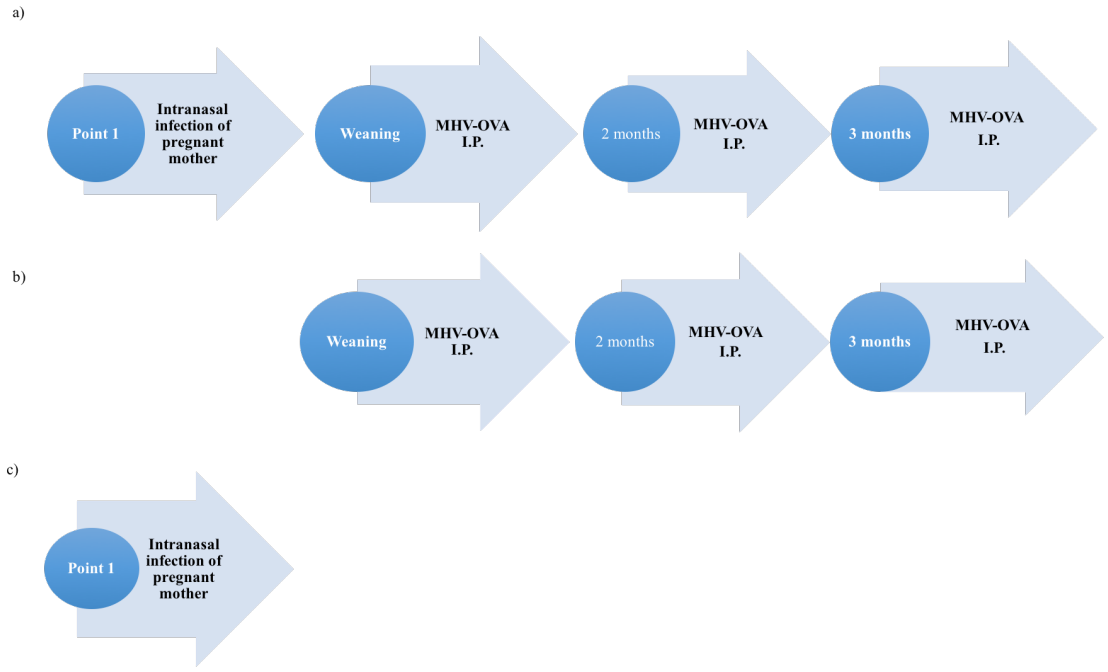


Figure 5.5 Summary of virus administration

Mouse number	Survival (days)	Histopathology diagnosis
1	123	Histiocytic sarcoma
2	208	Lymphoma
3	230	Hepatocellular carcinoma (HCC)
4	261	HCC
5	268	HCC
6	157	HCC
7	183	Lymphoma
8	313	HCC / sarcoma
9	313	Beginnings of HCC
10	313	Beginnings of sarcoma

Table 5.1: In utero and ex utero exposure to MHV-OVA prevents lymphomagenesis: *Summary of disease phenotype and overall survival of CD4/NPM-ALK/OTI mice exposed to MHV-OVA in utero and ex utero. HCC=Hepatocellular carcinoma.*

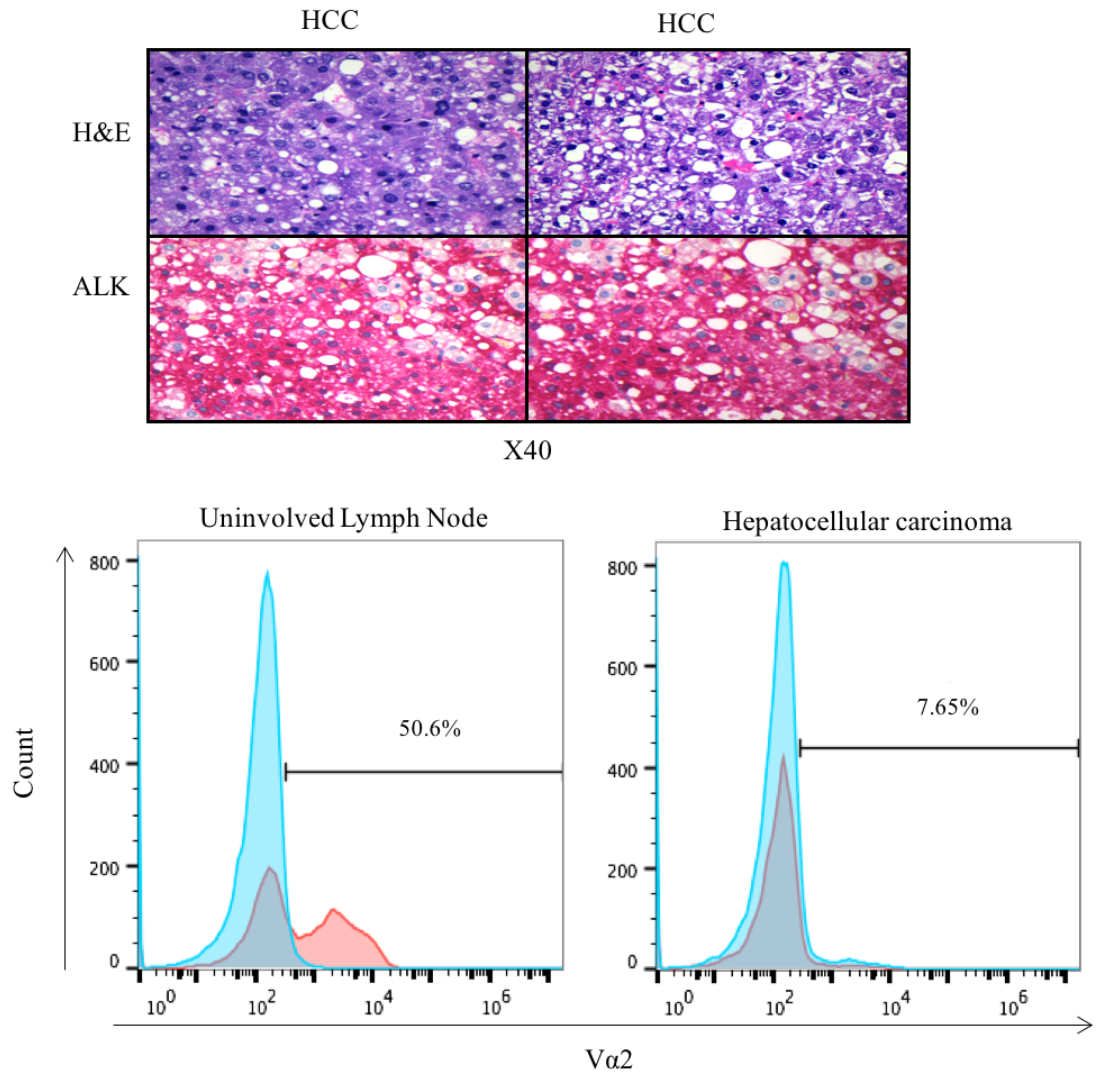
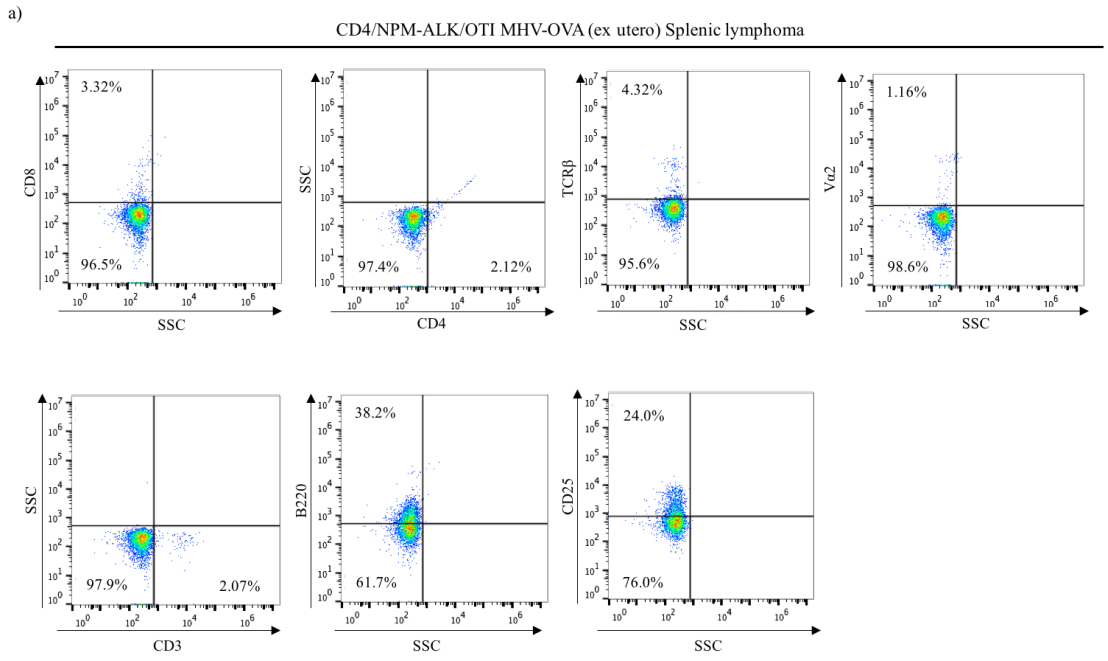


Figure 5.6 Exposing CD4/NPM-ALK/OTI mice to MHV-OVA during gestation and after weaning prevents lymphomagenesis:
(a) Atypical hepatocellular carcinoma presenting in CD4/NPM-ALK/OTI mice following i.n. infection of the mother with MHV-OVA followed by i.p. infection at weaning and monthly infections for a further 3 months. The top panel shows staining of tissue sections with Haematoxylin and Eosin and the lower panel immunohistochemistry with an anti-ALK antibody. (b) Flow cytometry assessment of OT1 expression on T cells derived from an uninvolved lymph node of a mouse presenting with HCC (orange histogram) compared to the isotype control antibody (blue histogram) (left panel) and of HCC cells (right panel).

Mouse	Survival (days)	Histopathology diagnosis
1	144	Peripheral lymphoma
2	176	Lymphoma with hallmark cells
3	159	Thymic tumour
4	267	Lymphoma with hallmark cells
5	158	Thymic tumour
6	136	Peripheral lymphoma
7	152	No tumours
8	400	No tumours

Table 5.2: MHV-OVA infection ex utero only does not affect disease presentation nor survival

Summary of survival and disease phenotype in CD4/NPM-ALK/OT1 mice exposed to MHV-OVA via intraparinetal injection post gestation.



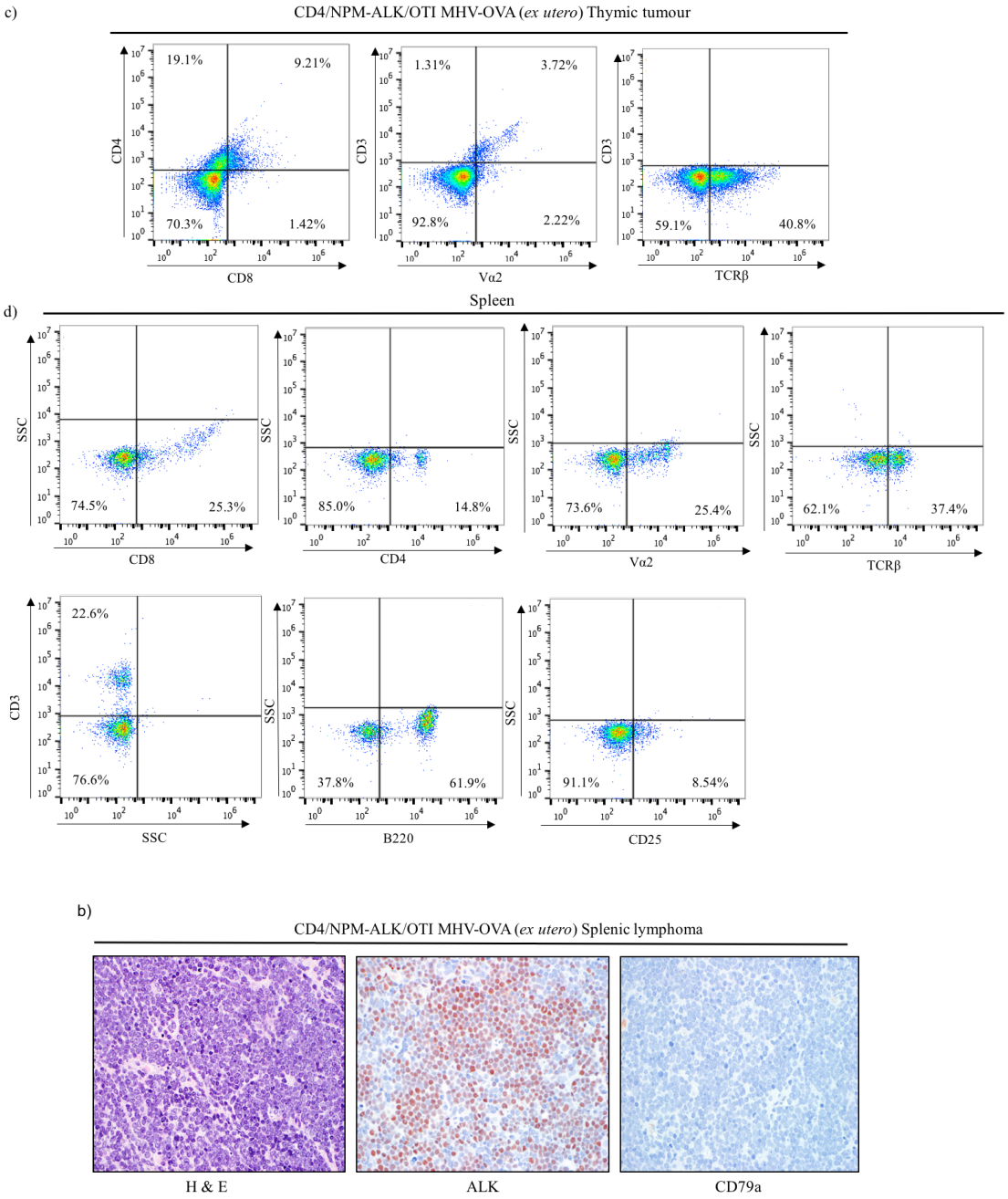


Figure 5.6: Effects of virus administration post weaning:
Flow cytometry and histology representations of tumours derived from CD4/NPM-ALK/OTI MHV-OVA (ex utero). (a) Representation of splenic lymphoma, (b) Histology performed on splenic tumour showing H&E, immunohistochemistry shows ALK staining and staining for B cells (CD79a). (c) FACS representation of a thymic lymphoma, (d) representation of peripheral lymphocytes derived from that mouse.

Mouse	Survival (days)	Histopathology diagnosis
1	200	Lymphoma
2	142	Lymphoma
3	280	Developing lymphoma
4	280	Developing lymphoma

Table 5.3: Exposure to MHV-OVA in utero only does not affect tumour phenotype:
Summary of overall survival and disease in CD4/NPM-ALK/OT1 mice exposed to MHV-OVA in utero only.

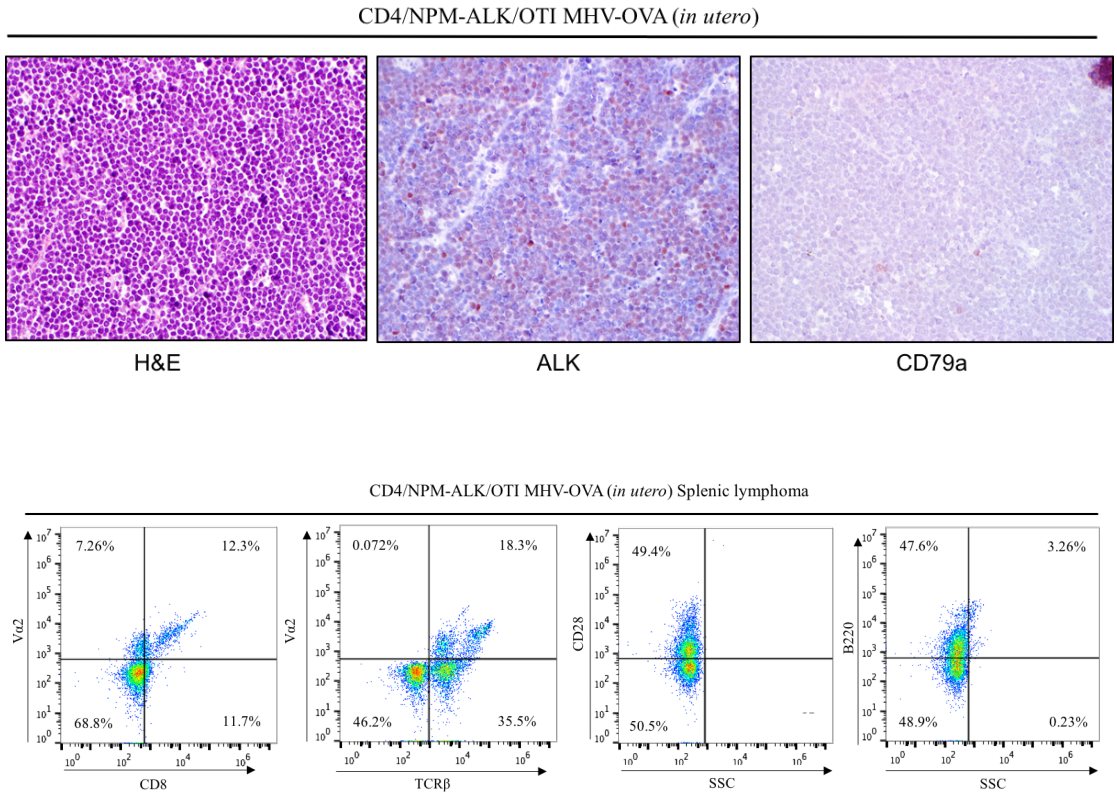


Figure 5.7: CD4/NPM-ALK/OT1 mice exposed to MHV-ova in utero only develop ALCL-like lymphoma:
Flow cytometry example showing cell surface staining on a tumour derived from this model. B220 is indicative of activated T cells. (Renno , et al., 1998)
Flow cytometry example of cell surface markers from a tumour derived from CD4/NPM-ALK/OT1 mouse only exposed to MHV-ova in utero.

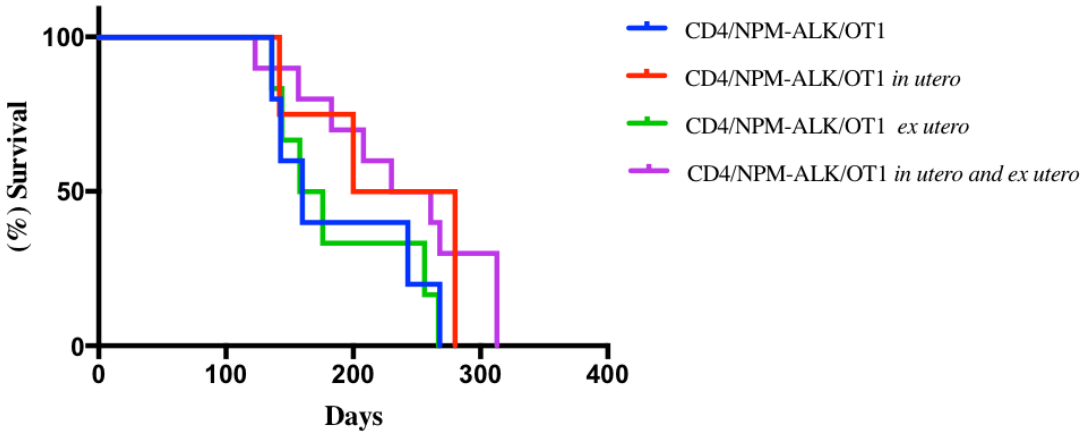


Figure 5.8: Overall survival of CD4/NPM-ALK/OT1 mice exposed to MHV-OVA: *Kaplan Meir survival curve, median survival of CD4/NPM-ALK/OT1 mice = 160 days, CD4NPM-ALK/OTI in utero and ex utero MHV-OVA exposure = 245 days, CD4/NPM-ALK/OT1 ex utero exposure alone = 167 days, CD4/NPM-ALK/OTI in utero exposure alone = 240 days. All points are not significant as determined by Log-rank (Mantel-Cox) test.*

Mouse strain	Description of line	Median survival age	Disease pathology	Histology
CD4/NPM-ALK/OTI (MHV-OVA *in* and *ex utero*)	CD4/NPM-ALK mouse with the MHC-Class I restricted OTI receptor, recognizing OVA, exposed to OVA expressing murine herpes virus pre partum and offspring exposed monthly for 3 months.	245 (12 mice)	HCC, GIST	HCC and Sarcoma
CD4/NPM-ALK/OTI (MHV-OVA *ex utero*)	CD4/NPM-ALK/OT1 mice exposed to MHV-OVA post weaning, monthly, for 3 months.	167 (8 mice)	Lymphoma	Lymphoma with Hallmark cells
CD4/NPM-ALK/OTI (MHV-OVA *in utero*)	Pregnant mothers exposed to MHV-OVA at around day 5 of gestation.	240 (4 mice)	Lymphoma	Lymphoma with Hallmark cells

Table: 5.4 Summary of disease phenotypes in CD4/NPM-ALK/OT1 mice exposed to MHV-OVA

5.3 Discussion

Human ALCL tumours do not express the TCR complex (TCRβ and CD3), CD8 and rarely express CD4 despite having an activated T cell phenotype (Foss , et al., 1997). Mouse models developed to date and those discussed within this thesis also display with loss of the TCR especially those with receptors expressed via transgenesis. The first question arising from this observation was whether NPM-ALK positive cells were still capable of producing the Tg TCR or whether it was post-transcriptionally or post-translationally affected. Transcripts and protein were detectable in established tumour cells from CD4/NPM-ALK/OTII (showing transcripts for α and β chains) (chapter 3) and CD4/NPM-ALK/OTI mice (figure 5.1) hinting towards interference mediated by NPM-ALK for TCR cell surface expression. As the OTI receptor is forcibly expressed under the control of the H-2Kb promoter, a 700-bp fragment Ig H chain enhancer located to the promoter (by transgenesis) in the T cells of this model, these data suggest that tumour cells actively lose the TCR and associated proximal signalling in order to transform. Zhang et al transduced primary CD4+ T cells with NPM-ALK, which transformed cells, expression of a cell surface TCR was lost (Zhang, et al., 2013). Hence, NPM-ALK (or another mechanism) down-regulates and interferes with expression of the OTI/OTII TCR and other TCR components and hence that a functional TCR might be inhibitive towards lymphoma development.

This phenomenon has also been noted with the stimulation of T-ALL cells with anti-CD3 (Trinquand & et al, 2016). This is an interesting and novel concept as other PTCLs express a TCR and in some cases, utilise TCR signalling as a driver of disease (Warner , et al., 2013) (Wang & et al, 2011), which does not seem to be the case for ALK positive ALCL. This begs to address the question as to what mechanism may be responsible for the loss of the TCR.

Upon T cell activation, the TCR is internalised via an LCK, PKC and clathrin mediated mechanisms (Crotzer, et al., 2004) (Martinez-Martin, et al., 2011). This internalisation upon stimulation could be the trigger for initial loss of the receptor in NPM-ALK expressing tumours. So perhaps antigenic engagement, by the TCR, is an initial "trigger". Human herpes virus 6 (HHV-6) evades the immune system via its U24 protein which inhibits CD3 recycling to the cell surface. This internalisation is independent of ZAP-70 and LCK which are sequestered within the cell, preventing receptor recycling (Sullivan & Coscoy , 2007). Therefore, interference with receptor recycling could also be a potential mechanism for NPM-ALK especially as it has been shown to epigenetically silence ZAP-70 and LCK (Ambrogio , et al., 2009). It could be assumed that perhaps stimulation/antigenic engagement via the TCR may lead to receptor internalisation and then interferes, via NPM-ALK, with receptor recycling. Future studies should look into TCR recycling in the presence of NPM-ALK post-stimulation.

5.3.1 High avidity antigenic stimuli fail to activate T cells expressing NPM-ALK:

The effect of stimulation, via the TCR, upon NPM-ALK positive cells derived from mouse models was assessed to see if this was incompatible with NPM-ALK activity. Strong stimulation (CD3+CD28 or OVA) of pre-tumour cells derived from CD4/NPM-ALK and CD4/NPM-ALK/OTI mice resulted in reduced proliferation and cellular activation as determined by CD25 expression (figures 5.2 and 5.3). The differences observed were not due to the expression level of the TCR as prior to stimulation the levels of receptor expression were assessed (figure 5.2c) and deemed to be similar between NPM-ALK positive and negative mice. Western blot analysis (figure 5.4) also provided evidence that proximal signalling was not significantly impaired, as previously reported in ALCL (Bonzheim , et al., 2004) (Ambrogio , et al., 2009). This is in contrast to data published by Ambrogio et al who

expressed NPM-ALK in the ALCL, ALK- cell line MAC-1 and showed that proximal signalling was diminished after 72 hours due to loss of expression of proximal signalling molecules via western. However, in this study, proximal signalling proteins were only analysed at 30 minutes following stimulation and therefore a longer incubation period may yield different results. In addition, MAC-1 cells have an activated helper T cell phenotype CD2+ CD4+ CD25+ CD30+ HLA-DR+ and do not express a cell surface TCR (Kadin , et al., 1994) (Ambrogio , et al., 2009).

Though NPM-ALK has been shown to impair TCR proximal signalling, it does activate distal pathways and components of TCR signalling for example NPM-ALK upregulates the MAPK-ERK1/2 pathway, inducing transcription from the IL2 promoter evidentially mimicking TCR signalling (Turner , et al., 2007). Likewise, ITK-SYK, a translocation breakpoint product expressed in some cases of peripheral T cell lymphoma, also mimics TCR signalling, although in this instance proximal signalling activity is increased and therefore tolerated by the cells (Pechloff & et, 2010). Therefore, as ITK-SYK does not require removal of the TCR why does NPM-ALK? Excessive NPM-ALK expression causes cells to undergo high levels of apoptosis via activation of DNA damage response pathways (Ceccon & et al , 2016) therefore the combination of TCR-induced signalling and NPM-ALK could be expected to have the same effect as excessive NPM-ALK expression and activity. As such, whilst stimulation of the TCR has been shown to contribute towards lymphomagenesis, in the case of ALCL it may have the opposite effect. This phenomenon has also been observed in T-ALL where Trinquand et al showed that strong stimulation via the TCR in T-ALL cells lead to leukemic cell death (Trinquand & et al, 2016). When ALCL cell lines are stimulated via CD30 this leads to apoptosis of ALCL via upregulation of pro-apoptotic molecules (Nishikori , et al., 2005). CD30 activates ERK1/2, MAPK, JNK and p38 MAPK pathways and the

canonical and alternative NFkB pathways, via the reduction of TRAF2 (Harlin , et al., 2002) (Watanabe , et al., 2005). If high avidity binding and strong stimulation via the TCR in the presence of NPM-ALK is not compatible with cell proliferation, perhaps weaker avidity antigens, possibly even self-antigens are implicated in the pathogenesis of ALCL.

5.3.2 NPM-ALK is permissive towards weak avidity antigenic stimulation *in vitro*

During T cell development signal intensity and the degree of intensity to which the T cell responds varies. For instance, DP thymocytes are more sensitive to TCR ligands than the SP cells, due to changes in expression of genes associated with TCR proximal signalling events. This can be via *Themis*, *Tespa1* and *Scn4b* which are expressed at higher levels in DP cells compared to more mature thymocytes (Paster , et al., 2015). There is also an alteration in the sensitisation of T cells to peptides in the periphery in comparison to the thymus whereby peripheral cells are less sensitive (Hogquist & Jameson , 2014). Results derived from Tg TCR NPM-ALK mouse models discussed in chapter 3 hinted towards a role for TCR signalling strength and positive selection. CD4/NPM-ALK mouse mimics showed differences not only between MHC class restriction but also within the same MHC class II restricted category in terms of disease, overall survival and phenotype. For instance, both OTII and Marilyn female mice developed thymic lymphomas yet the Marilyn females had a greater onset of disease generation indicative of a role for signalling strength (chapter 3).

Stimulation of T cells derived from pre-tumour CD4/NPM-ALK/OT1 mice with peptides of varying signalling strengths (OVA>Q4>T4>G4>E1) addressed the impact of peptides with varying signalling avidity, via the TCR, in the presence of NPM-ALK. The weak agonist, G4,

permitted a greater degree of proliferation in NPM-ALK positive cells over negative and this was supported by increased levels of CD25 (figure 5.3). Interestingly, despite the E1 peptide not giving rise to cellular proliferation, the degree of activation, as determined by cell surface CD25 expression, was higher in NPM-ALK positive cells. Increased CD25 expression upon activation without proliferation could be due to a partial activation of the T cell (Croft , et al., 1997) or the effects of NPM-ALK upon the cell. It could be suggested that a weak stimulus promotes the transformation of the cell via NPM-ALK. NPM-ALK promotes high levels of CD25 expression when transduced into CD4+ primary T cells (Zhang et al) by which this is mediated by STAT5 and STAT3 activation of CD25 genes (Marzec, et al., 2013). It was shown in these two studies that NPM-ALK utilises the IL-2 signalling pathways for transformation. Therefore, this increase in CD25 expression may be indicative of the initiation of NPM-ALK mediated cellular transformation.

The presence of increased expression of pERK (figure 5.4) in response to G4 and E1 peptides in comparison to NPM-ALK negative cells potentially provides a mechanism for the increase in proliferation observed in response to the G4 peptide. The reasons why no proliferation was recorded in response to E1 despite CD25 expression and pERK are not immediately apparent but could be due to a lack of activation of other TCR-induced pathways. As TCR mediated cellular proliferation can be independent of cytokine production and requires interaction of Vav1 with phosphorylated CD3 ITAMs mediating the recruitment of Notch 1 and proliferation induction by c-Myc (Guy , et al., 2013). Or the activity of NPM-ALK itself is promoting ERK signalling and expression of CD25, as previously mentioned.

It is known that stimulation via the TCR can elicit negative feedback mechanisms to allow for ligand discrimination. This is via intracellular components which regulate how the TCR perceives the signal to ensure that a weak agonist does not lead to strong signalling. (Edwards & Evavold , 2011). This activity can be achieved via proximal negative feedback mechanisms regulated by PTPN22, SHP1, DOK1, DoK2, HPK1, STS1/STS2 and PGM/AcP (Acuto , et al., 2008). For example, a weak agonist activates negative regulation of signalling via the interaction between the agonist and CD3ς chain phosphorylation, interfering with ZAP70 localisation to the TCR. SHP-1 and PTPN22 also play key roles in the negative regulation of the TCR in response to weak ligands (Jenkins , et al., 2009) (Edwards & Evavold , 2011) (Salmond , et al., 2014). ALCL cells have also been shown to produce FOXO3 (Gu, et al., 2004) which is known to negatively regulate PTPN22 (Brownlie & Zamoyska, 2013). In conjunction with epigenetically silencing proximal signalling molecules, NPM-ALK interferes with the negative feedback control of ERK signalling. This is via downregulation of *DUSP22* which inhibits TCR signalling via inactivation of MAPK and ERK2 (Zeng & Feldman , 2016) (Ambrogio , et al., 2009). In the presence of NPM-ALK loss of SHP-1 occurs in ALCL cells, thus signalling in response to weak ligands may be stronger due to interference with negative regulation (Han , et al., 2006). This provides evidence hinting towards NPM-ALK negatively effecting proximal signalling whilst removing negative feedback mechanisms to regulate stimulation levels. Therefore, could NPM-ALK mimic the effects of proximal TCR signalling regulatory molecules?

Further investigation between the interplay of NPM-ALK and negative regulation of TCR signalling would be interesting especially looking at THEMIS, SHP-1, PTPN22 which all play a role in monitoring signalling strength and discrimination of weak self-peptide from strong agonists. The strongest stimulation, via anti-CD3 and anti-CD28, resulted in reduced ALK expression (figure 5.4) which could indicate that strong signalling via the TCR may in

fact interfere with ALK expression which would also requires further investigation. If weak signalling is permissive towards lymphoma development what could be the source of this in ALCL development?

Once in the periphery naïve T cells are maintained via normal T cell homeostasis. This is achieved via TCR interactions with weak peptides in the periphery providing a tonic signal promoting T cell survival whilst avoiding activation, to an extent where one would see an active response (Sprent & Surh, 2011). Maintenance of this level of homeostasis is normally achieved by contact through self pMHC complexes and IL-7 (Tan , et al., 2001). The self-peptides to which the TCR is exposed are those presented during the process of (or to elicit) positive selection in the thymus and are also responsible for maintaining this tonic signalling in peripheral naïve T cells (Kieper , et al., 2004). Positive selection requires sustained ERK activation, which along with maturation of thymocytes is dependent upon continuous ERK activation and sustained TCR/peptide-MHC interaction (McNeil , et al., 2005). This sustained response could be the requirement for NPM-ALK to actively downregulate the TCR and silence proximal signalling. Could the response of NPM-ALK positive cells to weak signalling hint towards a role for T cell homeostasis/ tonic signalling within the periphery or encountered during the positive selection process?

5.3.3 The importance of ZAP70: is there a specific role for proximal signalling in ALCL lymphomagenesis

Western blot analysis showed that proximal signalling was still intact in NPM-ALK positive cells in the context of ZAP70 and LAT phosphorylation and expression (figure 3.4). Interestingly the mobility of pZAP70 and ZAP70 was altered in NPM-ALK positive cells. ZAP70 is essential for TCR mediated signalling, once activated, via LCK phosphorylation,

ZAP70 becomes active and consequently activates LAT and SLP76 leading to activation of signalling cascades further downstream of the engaged TCR (Wang , et al., 2010). ALCL cases have been shown to lack expression of ZAP70, due to epigenetic silencing (Bonzheim , et al., 2004) (Ambrogio , et al., 2009). As discussed T cell responses to stimulation are regulated to limit intensity signals. The TCR signalosome can be targeted for degradation and/or internalisation by ubiquitin ligases after activation. Therefore, stimulation via the TCR results in ubiquitination of Zap-70 and disruption of this process results in increased ZAP-70 signalling (Ivanova & Carpino , 2016). The slower migration observed may hint towards ubiquitination as studies addressing the effects of stimulation of the TCR complex upon ZAP70 in this study showed that a greater migration of ZAP-70 was due to ubiquitination (Ivanova & Carpino , 2016). Is this mobility of ZAP-70 an indication towards NPM-ALK targeting ZAP70 for degradation and as a first port of call to silence proximal signalling? This mobility difference was not observed in additional proximal signalling molecules though the state of LCK and SLP76 were not determined here. However, Ambrogio et al showed that after 72 hours of NPM-ALK transduction into MAC-1 cells expression of ZAP-70 was lost, though it should be noted that MAC-1 cells lack a cell surface TCR complex (Foss , et al., 1997) (Bonzheim , et al., 2004). As 10-week-old pre-tumour mice still express proximal signalling molecules and show activation, this further hints towards a secondary or even tertiary hit for transformation and alteration to the TCR and proximal signalling. Though loss of the TCR and associated proximal signalling renders ALCL cell lines unable to be stimulated through the normal T cell activation route, studies have looked at the effects of stimulants such as IL-2 and chemicals capable of activating the T cell by bypassing TCR signalling (Ito, et al., 2011). Western blot analysis also pointed towards potential degradation of ZAP70 in pre-tumorigenic CD4/NPM-ALK/OTI mice and so perhaps the expression of cell surface TCR is dependent upon ZAP70 expression and loss of ZAP70 is the primary

target for NPM-ALK to downregulate and silence proximal signalling (figure 5.4). As the TCR signalosome is internalised and ubiquitinated upon stimulation via the TCR, NPM-ALK may induce and maintain negative regulation of proximal signalling. Studies addressing the state of ZAP70 after TCR activation in healthy T cells suggest that negative regulation could be exerted via a complex between Crk, PI3K, the E3 ubiquitin ligase Cbl and p85 (Gelkop et al 2001). Indeed, NPM-ALK upregulates the PI3K/Akt pathway and epigenetically silences proximal signalling, so promotion of a negative feedback mechanism for NPM-ALK which regulates proximal signalling, in response to activation, is a possibility. Silencing of ZAP70 and LCK could be responsible for interference of receptor recycling (Sullivan & Coscoy , 2007) a mechanism by which NPM-ALK may interfere with TCR expression. Therefore, the role of NPM-ALK and specific proximal signalling molecules of TCR signalling needs to be addressed.

5.3.4 *In vivo* stimulation of NPM-ALK T cells

In vitro TCR engagement with high avidity ligand hinted towards strong stimulation, via the TCR, in the presence of NPM-ALK to be detrimental towards cell proliferation and activation (figures 5.2 and 5.3). In contrast, NPM-ALK is permissive towards weak avidity antigen-induced signalling via the TCR (figure 5.3). The role of infections in tumorigenesis is well acknowledged (Chang & Parsonnet , 2010) (Mager , 2006) (Martin & Gutkind , 2009). In the context of ALCL ALK-ve and ALK +ve, cutaneous ALCL cases have been linked to insect bites and breast implants (Lamant , et al., 2010) (Ye, et al., 2014), indicating a role for infection.

There are various studies associating maternally acquired infections during gestation to the generation or increased risk of paediatric cancers, mainly haematological, for example, Herpes viruses such as the Epstein Barr virus, leading to an increased risk of paediatric ALL (Lehitinen et al.,, 2003) (Mckinney, et al., 1999) (Roman , et al., 1997). In this study, the role of infection was addressed by administration of a murine herpes virus expressing Ovalbumin to CD4/NPM-ALK/OTI mice in three different patterns detailed in (figure 5.5). The presence of OVA elicits a strong stimulation via the TCR with infection starting in the lungs and spreading to the lymphatic system. MHVγ 68 is capable of crossing the placenta, and can be transferred via breast milk and vertical transmission, making it a suitable virus for this study (Francoise , et al., 2013). Previous studies have shown in the HTVL mouse model that OVA mediated T cell activation promoted tumourigenesis (Rauch , et al., 2009).

The MHV-OVA virus was administered to pregnant CD4/NPM-ALK/OTI mothers and their offspring to assess a potential role for infection in ALK+ lymphomagenesis during gestation and post gestation. CD4/NPM-ALK/OTI mice exposed to virus *in utero* and *ex utero* did not develop lymphomas instead hepatocellular carcinomas, sarcomas and gastrointestinal stromal tumours (figure 5.6). Mice had a greater survival over uninfected mice with a median survival of 245 as opposed to 160 and OTI expression was still detectable in uninvolved lymph nodes (figure 5.6b). As Infection of OTI mice with MHV-OVA shows acute replication of the virus in the lung, liver and spleen (Braaten , et al., 2005). This could explain the presence of HCC's and pneumonia-like symptoms in these mice due to prolonged inflammation. Or this could be due to age of mice, leaky expression of NPM-ALK, or the presence of *Helicobacter* infection within the housing facility, as similar pathologies were observed in the CD4/NPM-ALK/OTI/RAG$^{-/-}$ mice (chapter 4). In contrast, mice only exposed to MHV-OVA post-weaning (n=8) developed a mixture of pathologies half of which developed lymphoma with hallmark cells (figure 5.7). However, 2/8 developed thymic

lymphomas and 2/8 had no disease pathology. More mice need to be studied under these conditions to determine a full trend. Survival was not prolonged in these mice in comparison to those uninfected (median survival 167). As half of the population developed peripheral lymphoma with hallmark cells a preliminary conclusion could be that post weaning administration of MHV-OVA has no effect upon disease generation in the CD4/NPM-ALK/OTI model. Mice only exposed to virus during the gestation period (n=4) still generated null cell lymphoma with hallmark cells (figure 5.7) but time taken for disease development was much greater, 240 days, than those not exposed or those just exposed to virus post-gestation. Preliminary data suggests that there is some form of protective effect against lymphomagenesis when embryos are exposed to stimulation elicited by MHV-OVA or via the mother's milk. Could variation between virus administration cohorts indicate a potential role for breast feeding and perhaps an alteration in the microbiome? Alterations to the microbiome in laboratory mice have shown to either increase the rate of lymphoma disease onset or protect against disease onset (Yamamoto M L, 2013).

OVA should elicit a strong T cell response and it was hypothesised that either observations *in vitro* (figure 5.2) of negative responses to high avidity stimuli would be translated *in vivo,* or alternatively that infection would increase the rate of lymphomagenesis. Instead the effects observed *in vitro*, i.e high avidity antigen being detrimental to cellular proliferation, were not seen *in vivo* unless mice were exposed to stimuli *in utero* and *ex utero.* A potential explanation for this phenomenon could be that the initial exposure, during gestation/weaning, may prime T cells to the peptide which when re-exposed, may result in a much stronger T cell response emulating the same effect as observed *in vitro*. In the case of *ex utero* exposure only, continuous exposure to the virus may direct T cells to become tolerant to the peptide thus mice become anergic to infection. As ALCL is rare, maybe those that are not exposed to a common microorganism or antigen early in life are the ones that generate the disease? Is

there a role for acute infections to be considered as protective towards ALCL development? The Hygiene hypothesis postulates that "the lack of exposure to common microorganisms during the early stages of life results in abnormal or aberrant immune responses leading to increased incidences of autoimmune disease, allergies and cancers" (Greaves , 2009). It could be the case that this may be translated to *in utero* protection as well. Exposure to microorganisms within the first years of life is essential in humans, according to this hypothesis. In the context of mice perhaps exposure within the first month is essential, potentially linking a role to breast feeding as MHV-OVA is shed in breast milk. NPM-ALK+ ALCL cases have shown aberrant TCR production which could also lead to an aberrant immune response, which could be another factor (Malcolm , et al., 2016). Maternal infections are linked to increased risk of haematological malignancies when acquired pre-partum though if exposure to common microorganisms helps to provide a protective effect, patients may have non-serious symptoms or be asymptomatic and therefore the infection will not be documented making the epidemiology of this harder to document and thus study. Either way these results link a protective effect of exposure to infection during foetal development or the early stages of life although further studies and repeat experiments would be required to confirm this observation.

An additional progression from this would be to address the *in vivo* effects of OVA epitopes of different affinities to see if weak agonists promoted tumourigenesis by engineering the MHV68 virus to express these peptides. Or to complement the observations with the CD4/NPM-ALK/Marilyn(m) mouse in chapter 3 the CD4/NPM-ALK/OTI mouse model could be backcrossed onto the RIPmOVA model where OVA is expressed under the rat insulin promotor expressing OVA in pancreatic islet beta cells, kidney proximal tubular cells and the thymus (Kurts , et al., 1996). This mouse line has been generated CD4/NPM-ALK/OTI/RIPmOVA, however preliminary results from two mice (disease presentation

started during the final drafting of this thesis) present with B cell lymphomas in NPM-ALK negative mice and those positive for NPM-ALK so far do not seem to differ from disease observed in the NPM-ALK negative OTI mice.

This chapter aimed to address whether stimulation via the TCR influenced disease generation, progression and phenotype. Whether lymphomagenesis would follow the pattern of that previously described for antigen receptor driven lymphomas, where TCR or BCR signalling contributed to disease generation? Instead the opposite was proven, strong stimulation via the TCR was inhibitive towards disease generation indicating a role for weak TCR signalling as a potential instigator of transformation in the development of ALCL. This chapter links a protective role of early exposure to microorganisms or antigens to be protective towards lymphomagenesis in ALCL.

5.4 Main conclusions

- Incompatibility between NPM-ALK and strong avidity stimulation via the TCR
- *In utero and ex utero* stimulation of CD8 T cell skewed NPM-ALK mice prevents ALCL like disease.
- Interference with clonal selection and central tolerance leads to a variation of tumour phenotype and location.
- Signalling strength via the TCR may play a role in lymphomagenesis
- NPM-ALK may interfere with regulation of proximal signalling in response to a weak stimulus.

6 Discussion

6.1 Overview of thesis aims

Lymphoma covers a diverse range of diseases characterised by tumour location and cellular phenotype, presumed cell of origin and disease course. This cancer subgroup consequently has a broad range of aetiologies, which can be subtype specific, although many key instigators remain unknown. Peripheral T cell lymphomas (PTCL), a group to which ALCL belongs, are considered aggressive proliferating cancers consisting of mature T cells. In ALK+ and ALK- ALCL, the cell of origin has been hard to define, as it does not follow the normal pattern of a PTCL due to loss of the majority of pan T cell markers (CD3, TCR, CD8, CD4, CD28) and an inability to define a particular genetic signature (CD4 and CD8) (Eckerle & et al.,, 2009). Occasional expression of CD4, CD3, CD5 and the production of IL-17 has hinted towards the potential for a Th17 or Th2 cell of origin. However, the presence of granzyme B and perforin is more indicative of an activated cytotoxic T cell (Bonzheim & et al .,, 2008) (Bonzheim , et al., 2004) (Matsuyama, et al., 2011). Loss of the TCR and associated proximal signalling molecules (Ambrogio , et al., 2008) also sets ALCL aside from other PTCLs which have been shown to utilise the TCR and proximal signalling for transformation and to drive disease development (Warner , et al., 2013). Thus, in conjunction with NPM-ALK's ability to upregulate signalling pathways, normally distal to the TCR, this poses the question as to why ALCL cells do not express a TCR and associated proximal signalling.

It has proven difficult to mimic ALCL in mouse models through targeted expression of NPM-ALK until the development of the CD4/NPM-ALK model (Chiarle , et al., 2003) in which T lymphomas were observed (Turner & Alexander, 2005). Tumours developing in the CD4/NPM-ALK model retain co-receptor expression (CD8 and CD4) but do not display the TCR complex, yet disease is also restricted to the mediastinum limiting its likeness to human

ALCL. When this model is backcrossed onto the OTI TCR transgenic mouse line to generate CD4/NPM-ALK/OTI mice (Malcolm , et al., 2016), with peripheral tumours lacking all TCR surface markers (CD3, TCR, CD8, CD4) giving a "null cell" phenotype and hallmark histopathology present, better mimicking the respective human disease. This suggests that a functional, clonal TCR is required during ontogeny, for thymic egress, to establish peripheral disease. Interestingly, established tumours lose the forcibly expressed OTI receptor suggesting it is either surplus to requirement or is pejorative to disease development (Malcolm , et al., 2016).

Given these findings, this thesis aims to discover the mechanisms required for NPM-ALK-driven lymphomagenesis and identification of the potential cell of origin. This was addressed using the CD4/NPM-ALK ALCL model as a base to establish novel double and triple transgenic mice to study the influence of (a) a T cell skew, (b) functional TCR expression, (c) clonal selection, (d) the role of RAG, (e) the role of a ligand as a surrogate for infection (f) the role of direct stimulation via the TCR. The findings from all mouse models are mentioned in (Table 6.1).

6.2 Summary of ALCL mouse mimics:

All ALCL mouse mimics discussed and presented are summarised in table 6.1. Briefly, only one previous mimic produces peripheral ALCL-like disease, the CD4/NPM-ALK/OTI, representing the best described model of ALCL to date (Malcolm , et al., 2016). All models restricted to MHC class II, via transgenic TCRs (CD4/NPM-ALK/OTII and CD4/NPM-ALK/Marilyn(f)), develop thymic lymphomas still expressing both CD4 and CD8 (DP) or just CD4 (CD4SP) but do lose cell surface expression of the TCR tg receptor (chapter 3). TCR tg mice on $RAG^{-/-}$ backgrounds (CD4/NPM-ALK/OTII/$RAG^{-/-}$ and CD4/NPM-ALK/OTI/$RAG^{-/-}$) rarely generate lymphoma as opposed to the CD4/NPM-ALK/$RAG^{-/-}$ line, which produced thymic lymphomas (chapter 4) (Malcolm , et al., 2016). This was also the

case for infected CD4/NPM-ALK/OTI mice infected with MHV-OVA (chapter 5). Male CD4/NPM-ALK/Marilyn(m) mice expressing the H-Y antigen, for which the Marilyn TCR is specific, generated ALK-ve haematopoietic malignancies and ALK+ thymic lymphomas although ALK+ tumours appeared to derive from T cells expressing endogenous TCRs as opposed to the transgenic receptor (chapter 3). All-together the mouse strains described herein hint towards a role for RAG, clonal TCR presence and antigenic stimulation in disease pathogenesis. However, the CD4/NPM-ALK/OTI is still the closet mimic of ALCL developed to date.

Genotype	Features of model	Disease phenotype	Conclusions	Reference
CD4/NPM-ALK	NPM-ALK expression from the CD4 promoter (expression throughout thymic development from DN1)	Thymic lymphoma mainly mature CD4SP and CD4/CD8 DP	NPM-ALK is oncogenic in thymic T cells	Malcolm et al., 2016
CD4/NPM-ALK/RAG$^{-/-}$	TCR gene rearrangement not possible	Thymic lymphoma containing T cells (CD4CD8 DP) but with an absence of surface TCR expression	Thymic tumour development is independent of RAG activity	Malcolm et al., 2016
CD4/NPM-ALK/OTI (no stimulation)	Express a TCR specific for ovalbumin on CD8 T cells, capable of endogenous TCR rearrangements	Peripheral ALCL: Hallmark cells, CD30+, TCR- (molecular TCR rearrangements present for the OTI receptor)	OTI transgene inhibits thymic tumour development/promotes peripheral disease	Malcolm et al., 2016
CD4/NPM-ALK/OTI/RAG$^{-/-}$	Mice only express TCR specific for ovalbumin (no endogenous TCR rearrangements)	Hepatocellular carcinoma, fibro sarcoma, gastrointestinal stromal tumours	RAG is required for peripheral T cell lymphoma development	Chapter 4
CD4/NPM-ALK/OTII	Express a TCR specific for ovalbumin on CD4 T cells, cells capable of endogenous TCR rearrangements	Thymic lymphoma containing mature T cells of mainly CD4SP and occasional CD8+CD4+ DP but with an absence of surface TCR expression	CD4 helper T cells are not able to transform in the periphery	Chapter 3
CD4/NPM-ALK/OTII/RAG$^{-/-}$	Express a TCR specific for ovalbumin on CD4 T cells (no endogenous TCR rearrangements)	No disease reported apart from 2 ALK+ lymphomas	RAG (clonal TCR) is required for thymic lymphoma development	Chapter 4
CD4/NPM-ALK/Marilyn(f)	Express a TCR specific for the H-Y antigen on CD4 T cells	Thymic lymphoma of mature DP and mature CD4 SP phenotypes with loss of surface TCR expression	CD4 T cells are not able to transform in the periphery	Chapter 3
CD4/NPM-ALK/Marilyn(m)	Express a TCR specific for the H-Y antigen on CD4 T cells. The H-Y antigen is expressed in the male mice.	ALK negative disease suspected myeloid and haematopoietic malignancies	NPM-ALK cannot bypass negative selection	Chapter 3
CD4/NPM-ALK/OTI (MHV-OVA *in* and *ex utero*)	Expresses a TCR specific for Ovalbumin (OVA) on CD8 T cells. Mice are exposed to OVA via a MHV-OVA. Pregnant mice are infected with MHV-OVA and offspring, monthly, for 3 months thereafter.	Hepatocellular carcinoma and GIST	*In utero* and *ex utero* exposure to MHV-OVA prevents lymphoma generation	Chapter 5
CD4/NPM-ALK/OTI (MHV-OVA *ex utero*)	Expresses a TCR specific for Ovalbumin (OVA) on CD8 T cells. Mice are exposed to OVA via a MHV-OVA, *ex utero*.	Lymphoma with hallmark cells, null cell phenotype and loss of the TCR	*Ex utero* exposure to MHV-OVA does not alter disease phenotype but decreases disease latency	Chapter 5
CD4/NPM-ALK/OTI (MHV-OVA *in utero*)	Expresses a TCR specific for Ovalbumin (OVA) on CD8 T cells. Mice are exposed to OVA via a MHV-OVA. Pregnant mice are infected with MHV-OVA.	Lymphoma with hallmark cells, null cell phenotype and loss of the TCR	*In utero* exposure to MHV-OVA does not alter disease phenotype but increases disease latency	Chapter 5

Table 6.1: Summary of all CD4 promoter driven NPM-ALK mice

6.3 The cellular origin of ALK+ ALCL

The cellular origin of ALCL is still unknown, widely debated and thought to arise within the thymus indicated by ALCL cell lines carrying a genetic signature of an ETP (Moti , et al., 2014). Speculations of the T cell phenotype postulate T helper, Treg and a Th17 phenotype due to the production of IL-17, FOXP3, IL-10, TGFβ and the occasional expression of CD4 (Bonzheim & et al .,, 2008) (Matsuyama, et al., 2011) or an activated cytotoxic T cell due to expression of granzyme B, perforin and TIA-1 (Krenacs , et al., 1997) (Foss , et al., 1996) (Bonzheim , et al., 2004). However, studies have not been able to align the genetic signature of ALCL to a specific T cell subset (Eckerle & et al.,, 2009), raising the possibility that the cellular immunophenotype of ALCL may be a consequence of NPM-ALK expression and activity rather than the cellular origin. For instance, sustained upregulation of the MAPK/ERK pathway by NPM-ALK and NPM-ALK's ability to transform via IL-2 induced cell signalling may explain the activated phenotype (Marzec , et al., 2007) (Marzec, et al., 2013).

CD4/NPM-ALK mouse mimics, on tg TCR backgrounds, gave the greatest insight into a potential cellular preference for NPM-ALK (chapter 3). Those with a transgenic receptor restricted to MHC class I (CD4/NPM-ALK/OTI), developed peripheral lymphomas with hallmark cells and still remains the closet ALCL mimic to date. Restriction of CD4/NPM-ALK to MHC class II and hence a CD4 skew (CD4/NPM-ALK/OTII, CD4/NPM-ALK/Marilyn(f)) resulted in the development of thymic lymphomas with loss of the Tg TCR (chapter 3). These results indicat that a) a functional TCR, on its own, is not a requirement for peripheral disease generation and b) there was a preference for CD4 or CD8 cells and associated signalling. Perhaps NPM-ALK has a preference for CD4 leading to earlier transformation of the cell, although Marilyn mice did have a greater overall survival, closer

to that of the CD4/NPM-ALK/OTI model (161 vs 181). Alternatively, a CD8 skew (CD4/NPM-ALK/OTI) due to the presence of peripheral lymphomas with a null cell phenotype and hallmark cells closely mimicking ALCL (Malcolm , et al., 2016). Unlike the OTII mouse, CD4/NPM-ALK/Marilyn(f)'s produced more DP tumours with a greater disease latency, suggesting that tumours were arising from an earlier developmental stage. This could also indicate that the differences in signalling strengths between the receptors may play a role as the speed at which Marilyn cells moved through the thymus seemed to be faster when T cell development was analysed (chapter 3, figure 3.3, 3.9).

The inability of the OTII or Marilyn receptor to affect the disease presentation, overall survival and cellular phenotype of the CD4/NPM-ALK was unexpected considering the disease and phenotype observed in the CD4/NPM-ALK/OTI mimic. The addition of a transgenic receptor, not altering disease progression and phenotype, whilst on a RAG competent background, has been noted in the PTEN knockout mice. The CD4-cre-PTEN δ (PTEN knockout mouse) develops peripheral lymphomas with a semi mature CD4+CD69+ phenotype driven by the (14;15) translocation. When this model was backcrossed onto the OTII RAG competent background tumour phenotype and location of disease was not altered (Liu , et al., 2010). The lack of alteration in disease phenotype of the parental CD4/NPM-ALK may be due to the characteristics of the OTII and Marilyn receptors rather than T cell skew. Perhaps OTII is not capable of supressing endogenous TCR expression on a RAG competent background to the same extent as OTI and thus tumours arise from T cells expressing endogenous TCRs. Chapter 3 concluded that whilst NPM-ALK caused downregulation of the cell surface OTII receptor during thymocyte development, downregulation of the OTI receptor was not observed. Thus, the different characteristics of the receptors may lie within their signalling strength and the speed in which cells migrate through the thymus.

When the CD4/NPM-ALK/OTI and CD4/NPM-ALK/OTII mimics were both placed onto a $RAG^{-/-}$ background, lymphoma generation was reduced (chapter 4). It was considered that the absence of specific cell types, such as B cells, may contribute towards lymphomagenesis. Immunohistochemistry, however, showed absence of B cells within established tumours, indicating a lack of contribution to disease generation (chapter 4). Studies have shown that OTI $RAG^{-/-}$ mice lose memory and T reg cells, due to the lack of endogenous α chain rearrangements, allowing for cells to be selected on MHC class II (Clarke , et al., 2000) (DiPaolo & Shevach , 2009). As the absence of RAG resulted in a lack of lymphoma in CD4/NPM-ALK/OTI/$RAG^{-/-}$ and CD4/NPM-ALK/OTII/$RAG^{-/-}$ mice, it could be suggested that tumours may arise from either a T reg or memory T cell.

The differences between the transgenic mice within this thesis, in conjunction with previous models, has helped to shed some light into the origin of ALCL, though the cell of origin still cannot be concluded without future work. Therefore, though the origin is still debateable, results from this thesis indicate specific cellular subsets to be addressed and an importance towards α chain rearrangements and expression in ALCL lymphomagenesis. Or that the capacity to delete the TCR is lost in the absence of RAG.

6.4 A Role for RAG in ALK+ ALCL lymphomagenesis

As mentioned previously, the absence of RAG leads to a loss of T cell subsets in TCR transgenic mice. This may be a cause of the different phenotypes observed between the $RAG^{+/+}$ and the $RAG^{-/-}$ CD4/NPM-ALK TCR tg models (chapter 3 and 4). However, the detection of RAG protein in the CD4/NPM-ALK/OTI tumours hints towards a direct effect of RAG upon the cell (chapter 4).

The ability to express endogenous TCR α or β chains via V(D)J recombination may explain some of the disease phenotypes observed. It was established, in the CD4/NPM-ALK/OTI mimic, that tumours were derived from T cells positive for the OTI receptor, and the same observation was also noted in CD4/NPM-ALK/Marilyn(f) tumours (chapter 3) (Malcolm , et al., 2016). However, CD4/NPM-ALK and CD4/NPM-ALK/OTII mice displayed with clonal and oligoclonal D-J rearrangements of the TCRβ chain, indicating that the OTII receptor did not have the ability to fully suppress endogenous TCR production. However, NPM-ALK driven lymphomagenesis is not dependent upon TCR β rearrangements at the β selection point, but whether it is dependent upon the α chain is unknown (Malcolm , et al., 2016).

α chain rearrangements and the consequent presence of the α chain, along with its importance in NPM-ALK driven lymphomagenesis, was suggested from results obtained with the MHC class II restricted, TCR tg CD4/NPM-ALK mouse variants in chapter 3. The tumour phenotypes observed in the CD4/NPM/ALK, CD4/NPM-ALK/OTII and CD4/NPM-ALK/Marilyn(f) predominantly produced CD4SP tumours as opposed to only DP tumours, as seen in the CD4/NPM-ALK/RAG$^{-/-}$ line (chapter 3) (Malcolm , et al., 2016) suggesting successful positive selection had taken place, which requires an αβ TCR. When a PTEN$^{-/-}$ model, which develops semi-mature CD4+ CD69+ peripheral lymphomas driven by the t(14;15) translocation, was placed onto an OTII RAG$^{-/-}$ background, tumours became thymic and 50% developed DP tumours, with a less mature phenotype and low-level expression of TCRβ. Interestingly none of the RAG$^{-/-}$ tumours had the t(14;15) clonal translocation (Liu , et al., 2010). This hinted towards the importance of RAG during ontogeny to form the translocation driving tumours, in this particular mouse model. The occasional thymic tumours, in the CD4/NPM-ALK/OTII/RAG$^{-/-}$ model, and tumours from CD4/NPM-ALK/RAG$^{-/-}$ all expressed ALK (chapter 3) (Malcolm , et al., 2016), suggesting that RAG does not directly affect NPM-ALK expression.

The detection of RAG transcripts within the peripheral null cell tumours, derived from the CD4/NPM-ALK/OTI model, was unusual (chapter 4). Mature peripheral T cells usually lack the expression of RAG, suppressed by TCR mediated tonic signalling (Kuo & Schlissel, 2009) (Roose , et al., 2003) (Patra , et al., 2006). However, peripheral expression of RAG has been shown in mature T cells, for example in the peyers patch, in T cells with a double positive, mature activated T cell phenotype (Kondo , et al., 2003). RAG can also be re-expressed to revise T cell receptors in self-reactive peripheral T cells (Cooper , et al., 2003). The loss of cell surface tg TCRs in established tumours, derived from the NPM-ALK TCR tg mice, may indicate a role for RAG in TCR receptor editing. RAG helps to promote survival and fitness of the cell, which may also contribute to the disease phenotypes observed (chapter 4) (Karo , et al., 2014) especially those associated with infection like pathologies in the CD4/NPM-ALK/OTII/RAG$^{-/-}$ mouse.

Other than a potential role in receptor editing and cellular fitness, RAG may mediate its effects via other means. The process of V(D)J recombination, driven by RAG, has been implicated in increasing the expression of oncogenes and interfering with tumour suppressor genes. This has been shown with the MTCP1/TCL1 oncogenes, where increased expression in the T cell lineage has been increased via juxtapositions of these oncogenes to the TCRα and/or TCRβ loci (Woiciechowsky, et al., 2001) (Hagihara , et al., 2001). The ATM$^{-/-}$ mouse model, which develops thymic lymphomas, showed that RAG was not essential for disease generation but did increase disease latency (Petinoit , et al., 2002). It had been shown in this model that RAG was essential towards tumorigenesis developing due to translocations with the TCRα/δ locus. Absence of RAG, however, showed that the lack of ATM was still able to promote lymphomagenesis, although with a later onset (Petinoit , et al., 2002). Delayed onset of thymic lymphomas were also observed in RAG deficient p53/eb mice (Liao , et al., 1998), but a lack of tumor development has not been noted for thymic lymphomas, this is in line

with observations by Malcolm *et al*. Therefore, the absence of lymphoma development, only seen in the TCR tg NPM-ALK mice, is unknown (chapter 4). Are the differences observed, in the absence of RAG, due to the loss of particular cell populations or the absence of RAG function upon the cell itself?

Newrzela *et al.* developed NPM-ALK positive tg TCR cells, by transducing cells derived from RAG component MHC class I restricted mice, OTI and P14 (Newrzela , et al., 2012). RAG competent TCR tg, NPM-ALK positive cells still developed lymphoma when adoptively transferred into $RAG^{-/-}$ hosts, suggesting that the role of RAG in NPM-ALK mediated lymphomagenesis is an intrinsic one, though this cannot elucidate the specific T cell populations in which NPM-ALK was transduced into (Newrzela , et al., 2012). Human cases of ALCL and RAG expression are inconclusive where RAG expression varies between ALCL cases analysed (Malcolm , et al., 2016). Though the role of RAG in ALCL is unclear, results from these mouse mimics show the presence of RAG and T cell subsets lost upon its absence to be essential in ALCL lymphomagenesis in these tg TCR mimics.

6.5 NPM-ALK and clonal selection

The CD4/NPM-ALK/$RAG^{-/-}$ line illustrated that NPM-ALK was capable of bypassing the β selection process, driven by Notch 1 activity (Malcolm , et al., 2016). However, tumours derived from this model only displayed a DP phenotype suggesting that these cells may not have undergone positive selection, as the maturity of the cell was not analysed (Malcolm , et al., 2016). Tumours developed in the CD4/NPM-ALK/Marilyn(f) mimic illustrated that tumours were derived from a mature cellular phenotype, CD4SP cells, and an intermediate between the DP mature and SP immature cells (chapter 3) suggesting successful positive selection had taken place. Despite this, tumours were restricted to the mediastinum hinting towards an inability for thymic egress, perhaps due to loss of the TCR or another mechanism.

This suggested that NPM-ALK positive cells were successfully undergoing positive selection, indicating that the process of positive selection plays a role in lymphomagenesis.

The CD4/NPM-ALK/Marilyn(m) mimic enabled an investigation into the role of negative selection for which the Marilyn receptor recognises the H-Y antigen, only expressed in male mice, and therefore T cells within this model undergo deletion via excessive negative selection. This mimic produced a variety of diseases, mainly ALK-ve, strongly indicating that NPM-ALK is unable to bypass negative selection (chapter 3). However, ALK+ve thymic lymphomas were observed within this model and so it was concluded that these had arisen from T cells expressing endogenous TCRs other than the Marilyn receptor (chapter 3). Due to this observation, this model on a RAG knockout background would help to further confirm the inability of NPM-ALK to bypass negative selection. However, RAG seemed to be essential for lymphomagenesis in the other TCR transgenic NPM-ALK mimics (chapter 4), therefore it could be likely that any ALK related disease observed within this model would not be present on a $RAG^{-/-}$ background. Therefore, NPM-ALK can bypass β selection, but not positive selection in the absence of RAG and also cannot bypass negative selection and therefore cellular egress is not possible in the absence of the OTI receptor.

NPM-ALK may cause dysregulation of TCR signalling which may impact developmental stages within the thymus leading to restriction to the thymus and early transformation. The role of β selection and positive selection may contribute to ALCL lymphomagenesis via TCR-mediated stimulation. However, NPM-ALK cannot bypass negative selection nor can it bypass tolerance in terms of thymic egress without the presence of a specific TCR.

6.6 The role of the TCR in ALCL lymphomagenesis

ALCL cell lines and tumours all lack cell surface expression of a TCR, also observed in all tumours derived from CD4/NPM-ALK transgenic mouse models previously discussed within

this thesis (chapters 3 and 5) (Bonzheim, et al., 2004) (Chiarle , et al., 2003) (Malcolm , et al., 2016). Human ALCL lose proximal signalling proteins but have an activated phenotype, indicated by CD30+, granzyme B, and perforin expression. This would suggest that signalling/activation via the TCR must occur at least during lymphomagenesis or activation during ontogeny contributing towards lymphomagenesis. The CD4/NPM-ALK/OTI model showed that a functional TCR was required for the development of peripheral disease giving the closest ALCL mimic to date (Malcolm , et al., 2016). The loss of the forcibly expressed tg TCRs (chapter 3 and 5), were not due to a lack of production but instead interfered with cell surface receptor expression, likely mediated by NPM-ALK. Cell surface expression of the OTII receptor in CD4/NPM-ALK/OTII transgenic mice was even absent during thymocyte development.

Other PTCL mouse mimics have shown that disease generation and progression is reliant upon the presence of the TCR and associated signalling. For example, the LN3 mouse requires a rearranged TCR (Serwold , et al., 2010). Wang *et al* showed in a snf5 knockout mouse model that T cell development was blocked at DN3, and that lymphoma generation did not occur unless the OTI transgene was present, producing mature T cells in the periphery, developing CD3+CD8+ T cell lymphomas (Wang, et al., 2011). This is interesting as it suggests that the presence of a functional TCR is a requirement for peripheral ALCL like disease in CD4/NPM-ALK/OTI mice, although the TCR is not expressed on ensuing tumours. Only in the context of MHC class I restriction was a TCR shown to establish peripheral disease as the CD4/NPM-ALK TCR tg MHC class II restricted mice developed thymic lymphomas lacking cell surface TCR expression (chapter 3). In fact, loss of the TCR was noted during thymocyte development in the CD4/NPM-ALK/OTII mimic, in contrast pre-tumour CD4/NPM-ALK/OTI mice did not show a reduction in cell surface TCR

expression (chapter 3). This loss of receptor in pre-tumour CD4/NPM-ALK/OTII mice could be an explanation for the lack of egress and peripheral disease.

Differences in signalling strength between the OTI and OTII, and Marilyn and OTII may be a reason for the different phenotypes observed: the receptors have different avidities for antigen, and thus different levels of tonic signalling, which has been noted to vary across TCR tg mice (Azzam , et al., 1998) (Azzam , et al., 2001). The P14 and OTI TCR transgenic lines, both MHC class I restricted, when engineered to express NPM-ALK, cells showed disparity in tumour formation and phenotype despite being of the same skew. NPM-ALK OTI cells displayed with a null cell phenotype whereas P14 displayed with a CD4 phenotype (Newrzela , et al., 2012). Though CD4/NPM-ALK/Marilyn females did not show overall loss of the receptor as indicated by the β chain (Vβ6), loss of the receptor, was observed at the DN3 stage of development alongside an increase in endogenous TCRβ expression (chapter 3). This raises the question as to whether events during thymocyte development lead to transformation at the DN3 stage, potentially via TCR activation as this stage would be the first point for pre TCR signalling in order to maintain cellular survival.

The processes of clonal selection do and can occur at earlier time points during development in the case of TCR transgenic mice (Hogquist & Jameson , 2014). Therefore, if the processes occur earlier, stimulation via the TCR will also, perhaps leading to earlier transformation. Various stages of thymocyte development are plausible for TCR mediated stimulation: (a) at the point of β selection (pre TCR stimulation in non TCR transgenic mice) (b) at positive selection, (c) TCR tolerance stage (central and peripheral), and (d) in the periphery. Therefore, due to alterations at the β selection stage, (seen in the CD4/NPM-ALK variants) along with the presence of mature and semi-mature T cell phenotypes (chapter 3), indicating successful positive selection, this is suggestive of activation or stimulation via the TCR contributing to lymphomagenesis in the thymus.

Newrzela *et al* developed NPM-ALK positive tg TCR+ cells, by transducing NPM-ALK into mature T cells derived from the RAG component MHC class I restricted mice, OTI and P14. Those transplanted with OTI NPM-ALK cells developed peripheral lymphoma within 30-80 days (phenotype CD30+, no TCR, no CD8/ CD4), while P14 NPM-ALK was less than 100 days (phenotype CD5, CD4, CD25, CD30 no TCR). From the WT cells transduced with NPM-ALK, only 2/6 developed lymphoma after 161 or 229 days, respectively. This suggests that tumour development was reliant on TCR specific cells (in this study). Presence of CD30 expression was not observed in the CD4/NPM-ALK/OTI NPM-ALK model (Malcolm , et al., 2016). This study followed the same pattern with regards to loss of the tg TCR, although present with CD30 expression, and the duration till disease onset was much less (Newrzela , et al., 2012). Therefore, literature and results from chapter 3 suggest that the specificity or clonality of the TCR matters in NPM-ALK induced lymphomagenesis, evident by the alterations in tumour phenotype and location observed between the TCR transgenic mice.

6.7 A role for direct TCR stimulation

The absence of a TCR and NPM-ALK's ability to prevent cell surface expression of the TCR, occur via unknown mechanisms. As NPM-ALK upregulates signalling components further downstream of the TCR and the presence of the type of transgenic TCR dictates the type of tumour that develop and its location (chapter 3), these suggest a potential role for stimulation via the TCR in lymphomagenesis. The cell of origin is thought to be thymic/ or earlier (Moti , et al., 2014) (Hassler , et al., 2016) yet mediastinal disease is rarely recorded in humans therefore cells must egress the thymus and do so via a functional TCR. This suggests that NPM-ALK positive cells must be able to go through development and undergo TCR mediated stimulation events within the thymus. CD4/NPM-ALK/OTI hinted towards a need for functional TCR expression to produce peripheral disease with hallmarks of ALCL

(Malcolm , et al., 2016) and CD4 skewed models (Marilyn and OTII) (chapter 3) hinted towards successful progression through positive selection. However, the TCR was not expressed in tumours, suggesting that perhaps signalling directly, and the level of signalling, may play a role in NPM-ALK mediated lymphomagenesis. The $Snf5^{-/-}$ mouse model has shown lymphomagenesis and proliferation to be dependent upon TCR signaling (Wang, et al., 2011).

Two studies have expressed NPM-ALK in the ALK-ve cell line MAC-1 and CD4+ primary T cells. The MAC-1 study showed that after 72 hours, the addition of NPM-ALK led to loss of expression of proximal signalling proteins and that this was via epigenetic silencing (Ambrogio , et al., 2008). The other showed that expressing NPM-ALK in primary CD4+ cells transformed them into ALCL cells expressing CD30+ and lost cell surface expression of the TCR complex, both indicative of an incompatibility between the TCR and NPM-ALK (Zhang, et al., 2013). However, neither of these studies took into account the role of antigenic stimulation of the TCR; MAC-1 cells already lack expression of a TCR and the CD4+ study expressed NPM-ALK into previously stimulated T cells in order to facilitate transduction. This thesis concludes that in lymphomagenesis there is a role for stimulation via the TCR in the presence of NPM-ALK.

Thymic negative selection assessed in the CD4/NPM-ALK/Marilyn(m) mice provided evidence of the inability of NPM-ALK to rescue cells after a strong antigenic stimulation in the thymus and that positive selection (weak activation) was a requirement for the generation of SP and mature DP tumours in the CD4/NPM-ALK/Marilyn(f) mice (chapter 3). The absence of proximal signalling, in conjunction with upregulation of TCR signalling further downstream, by NPM-ALK suggests that perhaps the TCR is lost due to an incompatibility, perhaps excessive signalling, as overexpression of NPM-ALK impacts cell viability (Ceccon

, et al., 2016). ITK-SYK, another hyperactive kinase expressed in PTCL, also upregulates TCR signalling but is more dependent upon proximal signalling and does not lose expression of the TCR (Pechloff & et, 2010). When pre-tumour peripheral T cells from the CD4/NPM-ALK/OTI mice were stimulated strongly *ex vivo* with anti-CD3 and anti-CD28 or OVA (chapter 5) this proved to be the case. Strong stimulation via the TCR in the presence of NPM-ALK resulted in a reduction in activation and proliferation. Incompatibility between an oncogene and TCR signalling has been noted recently in T-ALL where stimulation with anti-CD3 lead to cell death (Trinquand & et al, 2016). Due to the adverse effects of strong signalling via the TCR, providing a strong selective pressure in the presence of NPM-ALK, shows that strong stimulation via the TCR to be an antagonist towards ALCL development.

Results obtained from the CD4/NPM-ALK/Marilyn(f) and CD4/NPM-ALK/OTII mimics hinted towards signalling at the β selection checkpoint or positive selection playing a role in this. Additionally, the survival time differences between the two mice suggested that the level of signalling via the TCR contributed to lymphomagenesis. This was shown to be true when pre-tumour cells were exposed to epitopes of varying avidity (chapter 5). The weakest epitope (G4) resulted in a greater degree of proliferation and activation in comparison to NPM-ALK negative cells. Western blot analysis also showed an increase in expression of pERK. Therefore, it could be suggested that weak stimulation may play a role in lymphomagenesis whether it is weak stimulation within the thymus (positive selection) or periphery (tonic signalling) and dependent upon the T cell skew. These results also indicate interference by NPM-ALK with T cell homeostasis. The role of an oncogene upsetting homeostasis has been observed with the MTCP1 oncogene, implicated in T-cell prolymphocytic leukemia, which unsettles CD8 homeostasis via Akt signaling, producing CD8 tumours in mice although the human disease is CD4 (Joiner , et al., 2007).

Intact proximal signalling was observed along with TCR expression prior to stimulation thus showing the opposite to previous studies by Ambrogio *et al* and Zhang *et al* hinting towards a need for a stimulus to provide a selective pressure to silence proximal signalling and downregulation of the TCR. However, the role of signalling is signal strength dependent showing the inverse to what is normally observed in the presence of strong stimulation.

6.8 A role for infection

It has been proposed that ALCL may initiate within the thymus or earlier, whilst *in utero*. NPM-ALK has been detected within 1.95% of cord blood samples and the protein can be detected in the peripheral blood of healthy individuals (Laurent , et al., 2012) (Trumper , et al., 1998). In combination with this, ALCL has been linked to an ETP signature linking acquisition to the thymus or perhaps earlier (Moti , et al., 2014). The requirement for a specific functional TCR to establish peripheral disease indicates a role for a secondary "hit", which as a common theme could be via T cell activation through the TCR or as a consequence of the inflammatory response. However specific infectious aetiologies have not been linked to systemic ALK+ ALCL, but cutaneous ALK+ ALCL cases have been linked to tick, bug and mosquito bites (Lamant , et al., 2010) (Ye, et al., 2014) suggesting a potential for a role of infection.

The CD4/NPM-ALK/OTI model hinted that a functional TCR is important during ontogeny, producing the closest ALCL mimic to date. This model was therefore exposed to a Murine Herpes virus expressing ovalbumin (chapter 5). Stimulation, via OVA administration, of a mouse model expressing the HTLV-1 tax oncogene, under granzyme b promoter, along with the expression of the ovalbumin specific DO11.10 TCR (MHC class II restricted) promoted tumourigensis by exacerbating the tumour phenotype (Rauch , et al., 2009). In contrast to this study the increase in tumour development was dependent on the time point by which mice were exposed to the virus in the CD4/NPM-ALK/OTI mimic (chapter 5). Mice derived from

pre-exposed mothers (*in utero*) and consequent exposures post weaning did not generate lymphoma. Those that were exposed post-weaning still generated lymphoma with hallmark cells and a decrease in disease latency observed (167 days as opposed 181 days median survival). Those that were only exposed whilst *in utero* still developed disease associated with the CD4/NPM-ALK/OTI model, however, with a much greater latency (240 days) (chapter 5). This hinted towards a protective effect against 2 step exposures of the virus either *in utero* or during the weaning process and thereafter. Mice not pre-exposed either whilst *in utero* or during the weaning process displayed a decrease in disease latency. This initial exposure therefore seemed to apply a protective effect against lymphomagenesis.

Maternal infections during pregnancy have been linked to an increased risk of leukaemias and lymphomas (Roman , et al., 1997), however, results from this thesis suggest otherwise, in the case of ALCL. One explanation for this could be that early exposure is priming T cells to the virus and secondary exposure of that agent leads to a strong T cell response, producing the effect observed with strong stimulation as seen in the *in vitro* studies. Alternatively, this pattern is following that of the hygiene hypothesis where exposure to common and diverse microorganisms during the early stages of life protect against cancer development. This has been documented with the reduced incidence of ALL and day care attendance (Gilham , et al., 2005). Breast feeding or lack thereof has also been noted to play a role in paediatric cancer development increasing the risk of lymphomas and leukaemia's (Martin , et al., 2005). It could be that early exposure to microorganisms is preventative in ALK driven ALCL, and that due to the rarity of ALCL it may be due to a lack of exposure to common agents. Therefore, to elucidate this further the CDR3 regions of the TCR in ALCL cases could be sequenced to identify a common agent between cases, based on the amino acid sequence of the CDR3 region of the TCR (Epstein , et al., 2014).

6.9 Summary of key findings and concluding remarks

The key findings from this thesis are as follows:

1. NPM-ALK cannot bypass negative selection and requires positive selection. Hinting that positive selection may play a role in the disease.
2. NPM-ALK, via an unknown mechanism, interferes with cell surface TCR expression
3. RAG is essential for the development of lymphoma in TCR transgenic NPM-ALK expressing mice
4. The phenotypic skew and/or the characteristics of specific tg TCR influence the development of ALCL-like disease.
5. NPM-ALK may have a preference towards a Treg or memory T cell
6. NPM-ALK and a functional TCR are not compatible.
7. NPM-ALK is permissive towards weak stimuli hinting a role towards a weak stimulation either via positive selection within the thymus or tonic signalling in the periphery.
8. *In utero* and post weaning exposure to T cell stimulation prevents lymphoma generation hinting towards the hygiene hypothesis and a protective role from early exposure to infection.

Overall this thesis displays evidence linking an incompatibility between NPM-ALK and a functional TCR, a role for weak stimulation and an important role for RAG and clonal selection in shaping the ALK+ ALCL phenotype. A schematic, representing an updated version of the proposed events in ALK+ ALCL, based on the findings from this thesis, is shown in figure 6.1.

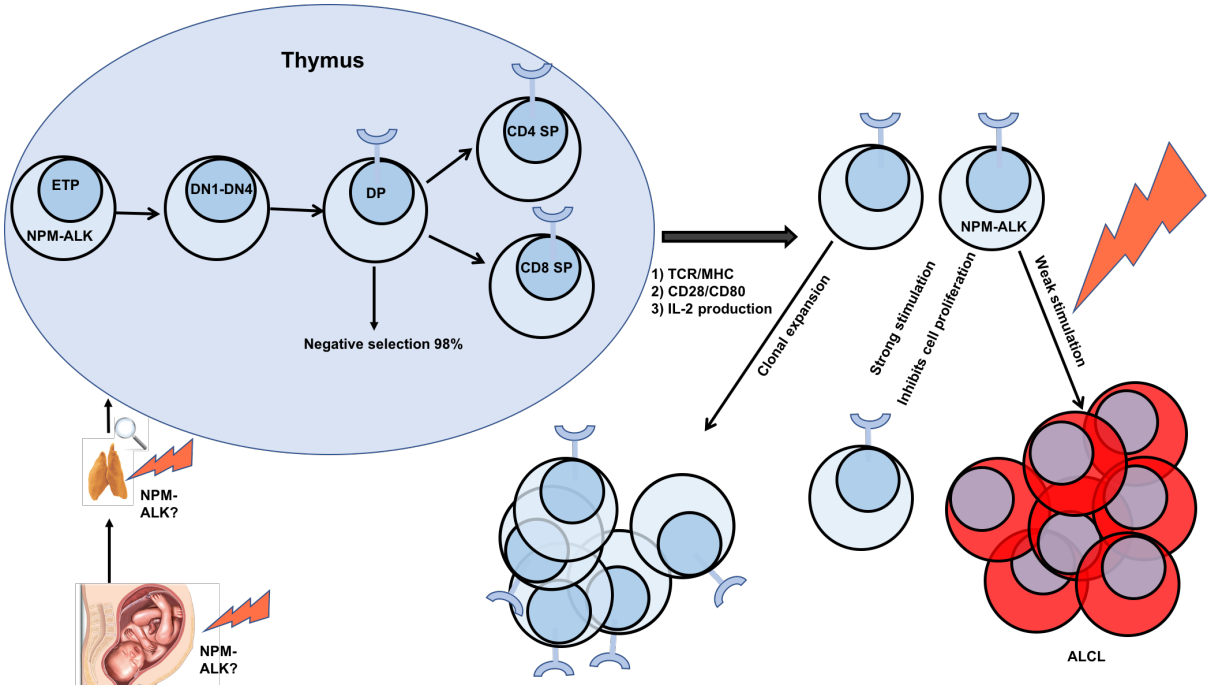


Figure 6.1: Updated theory of ALCL origins:
1)Acquisition of NPM-ALK occurs in a ETP or earlier. 2) Exposure to infectious agents pre- and post-partum have a protective effect against ALCL. 3)NPM-ALK bypasses the β selection point but is incapable of bypassing negative selection. 4) Positive selection may play a role in ALK+ lymphomagenesis. 5) A functional TCR, with a CD8 skew, is required for thymic egress. 6) Strong stimulation inhibits function and weak stimulation may promote transformation.

7 Future directions

This thesis has produced a greater insight into the potential mechanisms shaping ALK positive ALCL. Hinting towards areas for further investigation, uncovering the mechanisms of lymphomagenesis in ALCL.

7.1 Determining the cell of origin Treg or memory:

Chapters 3 and 4 indicate a preference of NPM-ALK for a CD8 phenotype shaping disease and a potential for a Treg or memory T cell to phenotype to be associated with ALK+ ALCL. To address the potential role for a Treg or a memory T cell of origin, these populations should be sorted from pre-tumorigenic cells of the CD4/NPM-ALK/OTI model and assessed to see if these populations establish disease when adoptively transferred into C57BL/6 mice. P14 and OTI studies by Newrzela *et al.,* show that the role of RAG could be intrinsic though this study did not address particular subsets (Newrzela , et al., 2012).

7.2 TCR proximal signalling and NPM-ALK

Chapters 3 and 5 indicate an incompatibility between signalling through the TCR and NPM-ALK expression, a putative role for NPM-ALK in preventing TCR surface expression and a potential preference for specific components of proximal signalling (Zap70). Therefore, to address an interference with TCR expression by NPM-ALK and a role for specific components of TCR proximal signalling, generation of cells expressing a functional TCR and NPM-ALK would help to address some of these questions. Transduction of NPM-ALK into human T cells and measurements of TCR cycling in the presence of NPM-ALK may shed more light into this.

To compliment the results observed with the pre-tumour mouse cells studies (chapter 5) Jurkat cell lines stably expressing a doxycycline inducible construct were established during this project. The NPM-ALK construct was transduced into Jurkat cell lines defective in components of TCR signalling and TCR cell surface expression and are as follows: α chain-/-

, β chain -/-, Zap70-/-, LAT-/-, LCK-/-, SLP76-/-. These cell lines should be assessed initially for the ability of NPM-ALK to upregulate characteristic markers of ALCL and interference with the TCR and proximal signalling molecules. To address the effect of stimulation via the TCR looking at ERK activation, IL-2 production and TCR recycling in the presence of NPM-ALK. Thus, attempting to identify whether NPM-ALK is reliant on the presence or absence of specific components of the TCR and associated proximal signalling and whether NPM-ALK interferes directly with TCR cycling.

Identification of infectious organisms in ALK+ ALCL

Chapter 5 indicates a role for infectious agents in ALK+ALCL but was deemed, dependent on point of exposure, to be protective. CDR3 region: infect mice with epitopes of different strength to see if this affects disease development in CD4/NPM-ALK/OTI mice

Next generation sequencing of the CDR3 region should be performed to look for common antigenic sequences.

In addition, to compliment the MHV-OVA study epitopes of the OVA peptides of weak avidity, such as G4, could address whether weak stimulation speeds up tumour development.

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Appendix 1

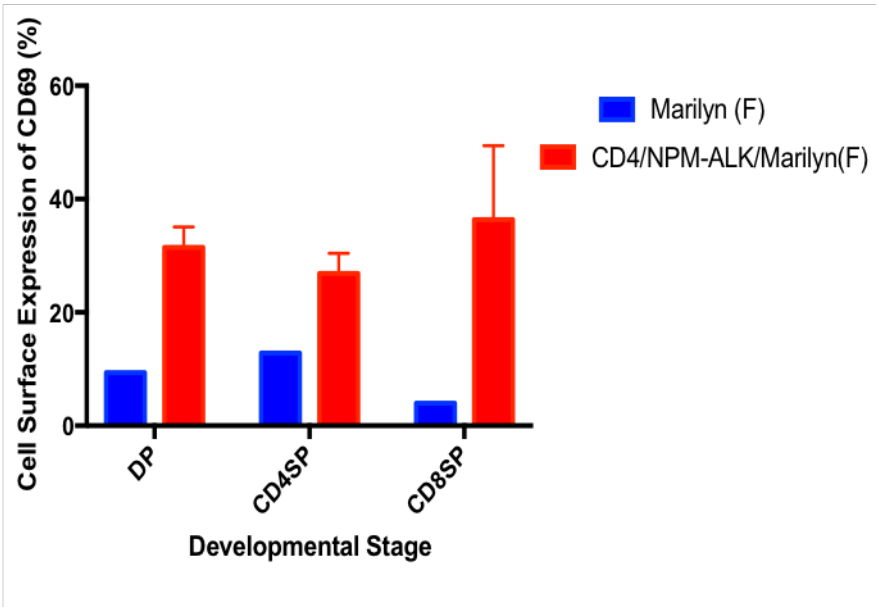

Figure A1: CD4/NPM-ALK/Marilyn(f) mice upregulate CD69 in the mature stages of thymocyte development *Samples were prepared for FACS analysis as previously described. (a-b) example of CD69 staining on a DP (a) and a CD4 SP (b) tumour derived from CD4/NPM-ALK/Marilyn(f). (c)Summary of cell surface CD69 expression on pre-tumour CD4/NPM-ALK/Marilyn(f)(n=2) and NPM-ALK(n=1) negative littermates.*