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Miguel Chaves Ferreira

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ACADEMIE DE PARIS
UNIVERSITÉ RENÉ DESCARTES – PARIS V

FACULTÉ DE MÉDECINE RENÉ DESCARTES – PARIS 5
- site Necker -

THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ DE PARIS V

Spécialité
Immunologie

Présentée par
Miguel Chaves Ferreira

Présentée et soutenue publiquement le 23 Novembre 2012

THE ROLE OF CYCLIN D1 IN LYMPHOPOIESIS

Professeur Marc Delpech	Président
Professeur Olivier Coqueret	Rapporteur
Docteur Ana Cumano	Rapporteur
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"One had to be a Newton to notice that the moon is falling,

when everyone sees that it doesn't fall."

Paul Valéry (1871-1945) French poet and philosopher

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I eagerly started by trading the social world of "bourgeois Paris" for a round the clock dedication to my work both to prove myself I could find my way in science and make a difference and also to repay for the great chance that Benedita had provided me by inviting me without a scholarship of my own.

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Abbreviations

5FU 5-fluorouracil

ACE accessibility control element

AGM aorta-gonad-mesonephros

APC antigen presenting cell

AR androgen receptor

ATM ataxia-telangiectasia mutated

ATR ataxia-telangiectasia and Rad3-related protein

Bcl B-cell lymphoma/leukaemia

BCR B-cell receptor

bHLH basic helix–loop–helix

BM bone marrow

Bp base pair

BrdU bromodeoxyuridine

BRE butyrate response element

bZip basic region-leucine zipper

Cbfβ core-binding factor β

CBP CREB-binding protein

CCL chemokine (C-C- motif) ligand

CCND1 cyclin D1 mRNA

CCND2 cyclin D2 mRNA

CCND3 cyclin D3 mRNA

CCR chemokine receptor

CD62L L-selectin

Cdc cell division cycle

Cdk cyclin dependent kinase

Cdki cyclin dependent kinase inhibitors

CDR3 third complementarity-determining region

ChiP chromatin immunoprecipitation

Chip-chip chromatin immunoprecipitation (ChIP) technique combined with microarray technology (chip)

CIA collagen-induced arthritis

CIN chromosomal instability

CLP common lymphocyte progenitor

CMP common myeloid progenitor

CRE tyrosine recombinase enzyme

CREB cAMP response element-binding protein

CSF1R colony-stimulating factor 1 receptor gene

CSF2 colony-stimulating factor 2

Ctr control

CYL cyclin-like proteins

D1$^{-/-}$ cyclin D1 cyclin box deficient mice

DC dendritic cell

D-MEM dulbecco's modified Eagle's medium

DMP1 dentin matrix acidic phosphoprotein 1

DN double negative cells ($CD4^{-}$ $CD8^{-}$)

DNA deoxyribonucleic acid

DP double positive cells ($CD4^{+}$ $CD8^{+}$)

EAE experimental autoimmune encephalomyelitis

EGFP enhanced green fluorescent protein

ER estrogen receptor

ETP early thymic progenitor

EYFP enhanced yellow fluorescent protein

FBXO31 F-box motif of F-box only protein 31

FCS foetal calf serum

Flk2 fms-like tyrosine kinase receptor 3

Flt3 fms-like tyrosine kinase receptor 3

FoxP3 forkhead box P3

G gap phase

GFP green fluorescence protein

GMP granulocyte-monocyte progenitors

HAT histone acetyl transferase

HDAC histone deacetylase

HC heavy chain

HEV high endothelial cell

HGF/SF hepatocyte growth factor/scatter factor

HRG heregulin beta 1

HSC haematopoietic stem cell

IP intra peritoneal

IV intra venous

IC intracellular domain

ICAM intercellular adhesion molecule

ICD intra-cellular domain

Id3 inhibitor of DNA binding 3

IFNγ interferon γ

Ig immunoglobulin

IgH immunoglobulin heavy chain

IgL immunoglobulin light chain

IL interleukin

Inr initiation sequence

IP intraperitoneal

IPEX polyendocrinopathy, enteropathy, X linked

ITAM immunoreceptor tyrosine-based activation motif

IVM intravital microscopy

LC light chain

LCMV lymphocytic choriomeningitis virus

LFA-1 leukocyte function-associated antigen-1

Lin lineage markers

LMPP lymphoid-primed multipotent progenitor cells

LN lymph node

Lox loxP site

LSK early hematopoietic progenitor

LT-HSC long term reconstituting haematopoietic stem cell

Lti lymphoid tissue inducer

M mitosis phase

Mad CAM mucosal addresin cell adhesion molecule

MEC medullary epithelial cells

MEP megakaryocyte-erythrocyte progenitor

MHC major histocompatibility complex

MPEC memory precursor effector cells

MPF maturation promoting factor

MPP multipotent progenitor

mRNA messenger ribonucleic acid

mTOR mammalian target of rampamycin

Myb myeloblastosis viral oncogene homolog

MyoD myogenic differentiation factor D

NGF nerve growth factor

NK natural killer cell

NKT natural killer T-cell

NLS nuclear localisation signal

p107 retinoblastoma-like protein 1

p130 retinoblastoma-like protein 2

P21 Cdk2 inhibitor p21Cip1

P27 Cdk2 inhibitor p27Kip1

P50 nuclear factor-κβ

PCR polymerase chain reaction

PEST peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T)

PNAd peripheral node addressin

PPARγ peroxisome proliferator-activated receptor γ

qPCR quantitative PCR

R point restriction point

RAG recombination-activated gene

Rb retinoblastoma

RbPL large loop within the pocket domain of retinoblastoma

Reg regenerating gene product

RNA ribonucleic acid

RSS recombination signal sequences

Runx RUNT-related transcription factor

S synthesis phase

S1P sphingosine 1-phosphate receptor

SCS subcapsular sinus

shRNA short haipin RNA

SLEC short-lived effector cells

SPL spleen

SRC1 steroid receptor co-activator 1

SRE serum response element

SRR site specific recombination

STAT signal transducer and activator of transcription 3

ST-HSC short term reconstituting haematopoietic stem cell

TAFII 250 transcription factor II B

T-ALL T-cell acute lymphoblastic leukaemia

T-bet T box transcription factor

TCF T-cell factor

TCM central memory cell

TCR T-cell receptor

tD1 truncated version of *CCND1* composed of exons 4 and 5

TEF effector memory cell

TF transcription factor

Tfh follicular helper T-cells

Th T helper cell

THRβ1 thyroid hormone receptor β

TLR toll-like receptor

TN triple negative cell ($CD3^{-}$ $CD4^{-}$ $CD8^{-}$)

TN1 $CD44^{+}$ $CD25^{-}$ cell

TN1a $CD117^{+}$ $CD24^{-}$ cell

TN1b $CD117^{+}$ $CD24^{+}$ cell

TN1c $CD117^{int}$ $CD24^{hi}$ cell

TN1d $CD117^{-}$ $CD24^{+}$ cell

TN1e $CD117^{-}$ $CD24^{-}$ cell

TN2 $CD44^{+}$ $CD25^{+}$ cell

TN3 $CD44^{-}$ $CD25^{+}$ cell

TN4 CD44$^-$ CD25$^-$ cell

TNR trinucleotide repeat

TPA 12-O-tetradecanoylphorbol-13-acetate

TRE TPA response element

Treg regulatory T-cell

UTR untranslated region

UV ultraviolet

V(D)J variable, diversity and joining gene segments

VCAM vascular cell adhesion molecule

Introduction

Haematopoiesis

All blood cells are generated from multipotent haematopoietic stem cells (HSCs).

The term "stem" is commonly referred to cells with self-renewal capacity and with the ability to generate all the different lineages of a specific system.

HSCs were first recognised by rescuing lethally irradiated mice who suffered from bone marrow (BM) failure with an injection of unirradiated BM cells (Ford et al, 1956).

These cells were not only capable of self-renewal during the life span of the organism but also of differentiating into all blood cell precursors. These HSC are found in the liver during foetal life and in the BM in adults.

Blood formation is split in two different stages, the primitive and definitive haematopoiesis. Primitive haematopoiesis in mice initiates at day E7,5 in the blood islands of the extra-embryonic yolk sac (Cumano et al, 1996; Dzierzak & Medvinsky, 1995). The definitive haematopoiesis characterised by the HSC with long term reconstitution potential appear on day E10,5 in the aorta-gonad-mesonephros (AGM) region which colonise the foetal liver starting at day E11 (reviewed in (Ginhoux & Merad, 2011)).

Although haematopoiesis poly-functionality was starting to be unravelled, it was only in 1961, with the findings of Till and McCulloch that the term "stem cell" was introduced. These two scientists discovered that the poly-functionality of haematopoiesis did not arise from a population of progenitor cells. Instead, a single undifferentiated pluripotent-cell clone originated at least 4 blood cell types: monocytes, granulocytes, erythrocytes and megakaryocytes (Till & McCulloch, 2011).

During differentiation, the vast array of options that a progenitor cell can undergo is regulated by a few signalling pathways that orchestrate a network of biochemical interactions that, while cross-talking with each other, translate precise instructions to the cell machinery at the nuclear level. These interactions were first highlighted by Schofield, in 1978. He illustrated the importance of a niche to prevent maturation of a stem cell and determined its behaviour (Schofield, 1978). Since then the concept of niche has evolved to include specific cell types, anatomical locations, soluble molecules, signalling cascades and gradients, as well as physical factors, such as shear stress, oxygen tension and temperature (Adamo et al, 2009; Eliasson & Jonsson, 2010; Eliasson et al, 2010; Kulkeaw et al, 2010)

The isolation of HSCs and their characterisation was first performed by Irving Weissman's group at Stanford University in 1988 (Spangrude et al, 1988). Since this work, the field of haematopoietic stem cell research has grown exponentially. The main development pathways leading to HSC differentiation, including the main cell surface protein markers that delineate the differentiated populations is depicted in Figure 1.

Though not all secrets have been found some diagrams were drawn in an attempt to explain the development pathway through steps defined by the expression or no expression of certain cell surface proteins as it can be seen in figure 1.

The uncommitted HSCs capable of self-renewal can be separated in two groups: (i) the long-term reconstituting HSCs (LT-HSCs), which are capable of self-renewal and multi-lineage differentiation potential throughout life; (ii) the short term reconstituting HSCs (ST-HSCs), which result from the differentiation of the LT-HSCs and have a more limited self-renewal potential. Further differentiation of these ST-HSCs gives rise to the multipotent progenitors (MPPs), which no longer have the ability of self-renewal and are the precursors of the oligopotent progenitors. From this step onwards, the multipotency is lost and specific lineage restricted progenitors are originated. The population of MPPs has been further divided on 3 subpopulations which will not be discussed here. Lineage committed oligopotent progenitors populations derived from MPPs are the common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), megakaryocyte-erythrocyte progenitor (MEP) and granulocyte-monocyte progenitors (GMP) (Akashi et al, 2000; Kondo et al, 1997).

The discovery of both CLPs and CMPs illustrates that the first lineage commitment step of adult HSCs results in an immediate and complete separation of myelopoiesis and lymphopoiesis. Differentiation of the oligopotent progenitors populations gives rise to all mature blood cell populations (figure 1).

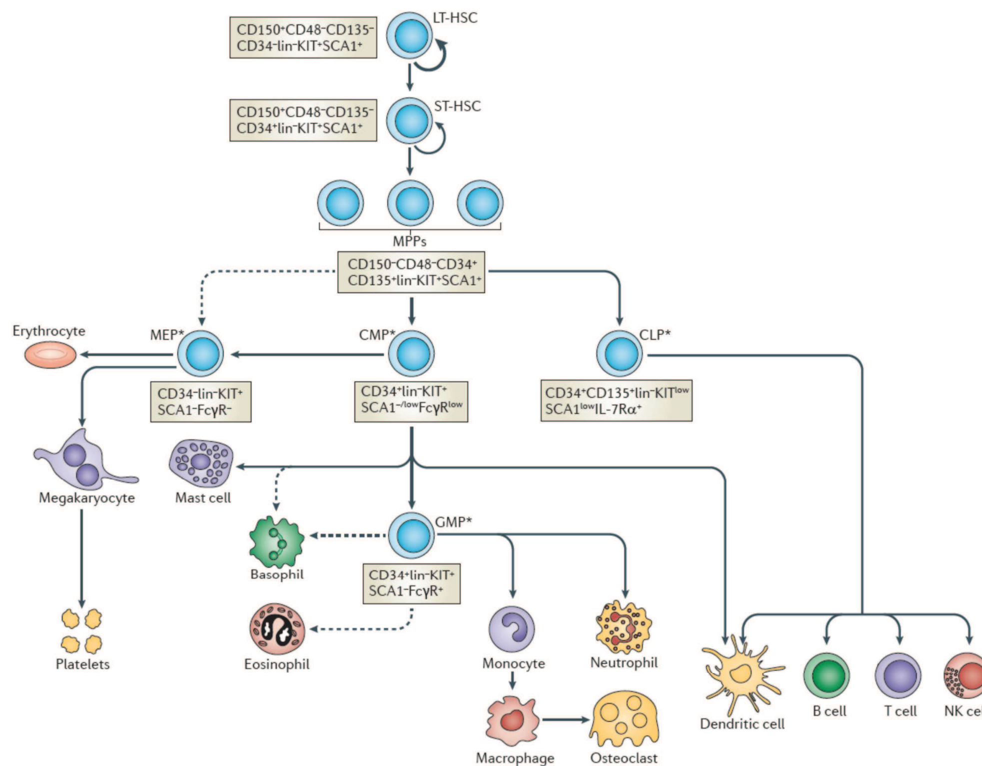


Figure 1: Haematopoiesis pathway in adult bone marrow

From top to bottom is a summarised model for the haematopoiesis pathway. The straight arrows represent the descendants. Curved arrows represent the ability to self-renewal. Grey boxes indicate the surface antibodies used in flow cytometry to determine the populations. Asterisks mark the oligopotent progenitors from which mature cells are originated. Figure from (Wang & Wagers, 2011).

T lymphocyte lineage commitment

The haematopoietic progenitors require a specific niche to undergo the sequential development processes, which will ultimately give rise to T-cell subsets with diverse antigen specificity. This niche exists within the thymus. Although all lymphocyte progenitors are originated in the BM and the development of other leukocytes continues mainly in the BM, the thymus is the sole organ that supports development of T lymphocytes. In the thymus the haematopoietic precursors undergo a series of cell-fate decisions processes to prepare them to become T-cell subsets.

Foetal mouse thymus is colonised by waves of bone marrow-derived precursors between days 10 and 13 of foetal life (Jotereau et al, 1987). All T-cells produced by the thymus descend from these colonising cells up to the first week after birth. The cells seeding the thymus after day 13 of foetal life will produce a second generation of T-cells, resulting in the displacement or dilution of the first generation starting at day 7 after birth. Further seeding of the thymus

occurs throughout adult life (Donskoy & Goldschneider, 1992; Scollay et al, 1986). Recent data shows, however, that thymocyte precursors in the thymus are capable of self-renewal, demonstrating that thymocyte survival is determined by competition between incoming progenitors and resident-cells (Peaudecerf et al, 2012).

All progenitor T-cells share an identical genome sequence and they acquire antigen specificity by recombining certain gene sequences in a locus called T-cell antigen receptor (TCR). These sequences are modified by a recombinase called RAG (recombination-activated gene) that mediates site specific recombination on the TCR locus. As the name indicates, this locus encodes a surface protein that recognises specific antigens in T-cells. The generation of different T-cell subsets with distinct functional properties in the thymus is regulated by spatiotemporal expression of a selected set of genes whose expression is dependent on transcription factors (TF). TF are nuclear proteins that bind specific genome sequences, alone or in a protein complex, activating or repressing the transcription of DNA.

Thymocyte differentiation

T-cell precursors within the thymus are characterised by the lack of expression of cell surface glycoproteins CD3, CD4 and CD8 and are therefore called Triple Negative (TN). Originally these cells were designated as double negative (DN) cells. Although this denomination is still used on many papers, the CD3 negative marker was added to eliminate a small subset of CD3 positive cells expressing either TCRγδ or TCRαβ NK1.1: the γδ T-cells and natural killer T-cells (NKT), respectively (Godfrey et al, 1992).

In adult mice, TN T-cell precursors in thymus can be further divided according to the expression of CD25 (the α chain of the IL-2 receptor) (Pearse et al, 1989; Shimonkevitz et al, 1987) and CD44 (Godfrey et al, 1993; Lesley et al, 1990). This results in the division on 4 stages that constitutes the classical thymocyte differentiation process. Starting from the least differentiated TN1 ($CD44^+$ $CD25^-$), followed by TN2 ($CD44^+$ $CD25^+$), TN3 ($CD44^-$ $CD25^+$) and finally TN4 ($CD44^-$ $CD25^-$) (figure 2).

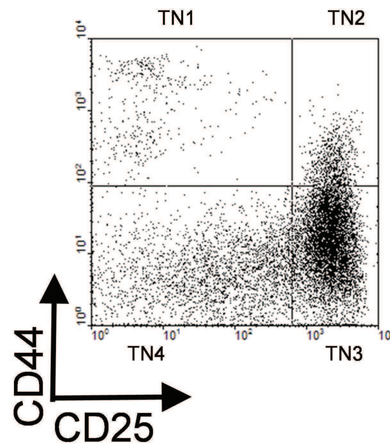


Figure 2: Thymus TN profile

Lineage negative cells of an adult mouse are ploted against CD44 and CD25 to determine the TN 1 to TN 4 profile.

The TN1 population is the most heterogeneous and can be further subdivided into 5 groups TN1a to TN1e (Porritt et al, 2004) (figure 3). Although all five populations are capable of generating T-cells *in vitro*, only TN1a and TN1b do so efficiently *in vivo*.

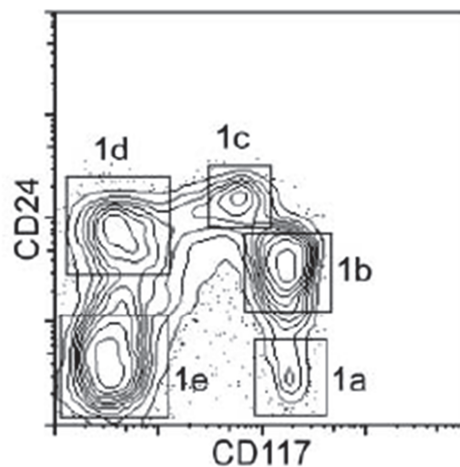


Figure 3: TN1 subsets characterisation

TN1 cells were ploted against cKit and CD24 to discriminate 5 subpopulations TN1a to TN1e. Image from (Porritt et al, 2004).

Another term widely used to define early T-cell precursors is the early thymic precursors (ETP), which encompass both TN1a and TN1b populations. The ETPs are characterised by a lack of

expression of lineage markers (Lin^-) for lymphoid and myeloid lineages and by the expression of c-kit, Sca-1 and IL-7R$\alpha^{neg/lo}$ (Allman et al, 2003).

Lymphoid-primed multipotent progenitor cells (LMPP) are haematopoietic progenitors found in the BM that are committed to develop into lympho-myeloid cells. The HSC become LMPP when their ability to follow erythroid and megakaryocyte lineages fates is greatly reduced. This event coincides with the expression of the fms-like tyrosine kinase receptor 3 (Flt3; also known as Flk2). LMPP cells have the potential to become T-cells, B-cells, Natural killer cells (NKs), dendritic cells (DCs) or macrophages (Adolfsson et al, 2005) and are thought to be the first progenitors to colonize the neonatal thymus (Luc et al, 2012).

Transcription factors and emergence of T-cells

Many transcription factors are critical for the differentiation of T-cells, including Notch, E2A, Heb and Runx family members, Myb, Id3, Gata 3, TCF 1, Bcl11b and Id3.

The Notch signalling pathway was first identified in Drosophila mutants (Morgan, 1917) and is a conserved pathway in many other organisms. Notch signalling is implicated on the differentiation of LMPPs into ETPs upon activation of Notch receptors by Notch ligands, delivered early after thymic entry (Sambandam et al, 2005; Ueno et al, 2002). The Notch family is constituted by four highly conserved Notch transmembrane receptors, Notch1, 2, 3 and 4 and five functional ligands: three orthologs of the Drosophila Delta (Delta or Delta-like [Dll] 1, 3, and 4) and two of the Drosophila Serrate (Jagged1 and Jagged2). Association with its ligand marks a series of proteolytic processes in the Notch transmembrane domain which results in the release of the intra-cellular domain (ICD). The released ICD of all four Notch receptors migrates to the nucleus where it complexes with the DNA-binding protein RBP-J (also called CSL) and recruits the coactivators PCAF and GCN5 and triggers the transcription activation of Notch target genes. Notch signalling plays not only a crucial role for the thymocyte differentiation but also in peripheral T-cells as well as B-cells functions.

A schematic representation of Notch signalling throughout Lymphopoiesis can be seen on figure 4.

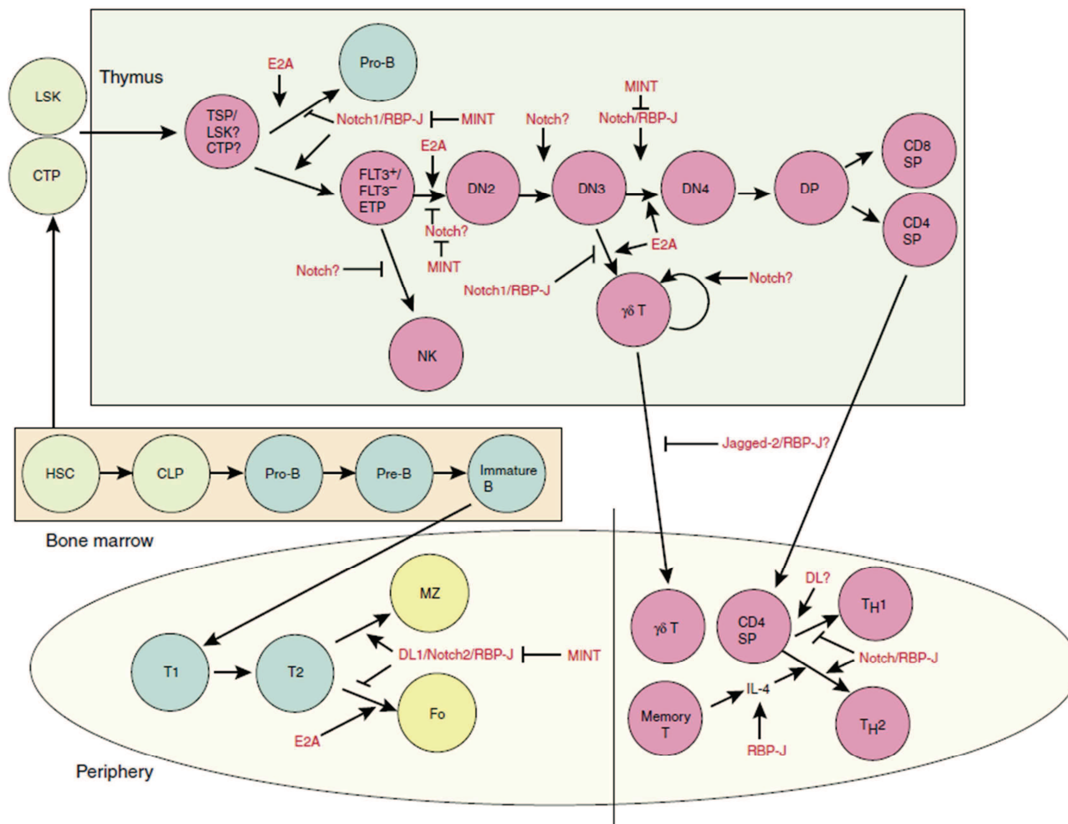


Figure 4: Notch role in Lymphopoiesis

Schematic representation of the steps in which Notch and other transcription factors play a key role in the development of lymphoid cells. A straight arrow represents the need of a particular TF to overcome a stage and a broken arrow represents the ability of a TF to block the development at a given stage. Question marks are present on those steps that are yet to be confirmed by molecular genetics. These data were obtained from loss of function mutants, γ-secretase inhibitor treatments and co-cultures with Notch ligand-expressing stromal cells. Of note, on the thymus compartment, as previously stated, the DN cells are equivalent to the TN cells. T1 and T2 are transitional B-cells; Fo, follicular cells and MZ, marginal zone cells. Image from (Tanigaki & Honjo, 2007).

The T-cell factor 1 (TCF-1 or T-cell specific transcription factor 7, TCF-7) is one of the critical downstream factors of Notch and is believed to imprint the T-cell fate by inducing the expression of T-cell essential transcription factors (Weber et al, 2011). These transcription factors include GATA-binding protein 3 (Gata 3) and B-cell lymphoma/leukaemia 11b (Bcl11b).

The Gata 3 transcription factor is implicated in the generation of ETPs and in the transition of ETP to the TN2 stage (Hosoya et al, 2009). Gata 3 is expressed at every stage of thymic development and has a fundamental role in maintaining Notch expression in ETPs (B. Rocha, personal communication). It is also involved in β selection (checkpoint to screen for successful TCRβ rearrangement and assembly of pre-TCR occurring on TN3 and TN4 stages deciding the

fate of the cell between αβ or γδ lineage) and is indispensable for CD4 thymocyte development. The expression of Gata 3 at each stage of thymic development is thought to be critical for the generation of mature T-cells (Pai et al, 2003).

The Bcl11b transcription factor is known to play an important role within the TN2 compartment. Although TN2 is not as heterogeneous as the TN1 group, it can be divided in two very important groups: (i) TN2a, which carry the potential to develop into at least three T-lineage alternatives, B-cells, DCs and NK cells; (ii) TN2b, which is the first population to be fully committed to T potential (Rothenberg et al, 2008). The TN2b subset is defined by a slightly reduced cKIT expression level when compared to the TN2a subset (Masuda et al, 2007). Bcl11b was reported to be essential for the transition of TN2a to TN2b (Rothenberg et al, 2010). Progenitor foetal liver cells deficient for Bcl11b (lethal on adult mice) were injected on irradiated mice and an arrest at TN2 stage with only a few cells making it to DP stage was reported (Ikawa et al, 2010)

The basic helix–loop–helix (bHLH) transcription factors E2A and HEB, also play important roles as critical gene regulators in early lymphocyte development. Both bHLH factors share a C-terminal bHLH (DNA binding and dimerisation) domain and conserved N-terminal activation domains.

E2A homodimers are essential for early B-cell development, whereas HEB/E2A heterodimers are dominant during T-cell development (Engel & Murre, 2001; Greenbaum & Zhuang, 2002).

The E2A acts at several time points (figure 4), the earliest being on LMPP stage in the BM. In the absence of E2A there is a decrease in LMPP, which translates to a decrease in ETP in the thymus (Dias et al, 2008). E2A was first described as having an important role in the development and commitment to the B-cell lineage by binding E2-box sites present in the immunoglobulin (Ig) enhancers but it has later been shown to be also essential for early T-cell development. In its absence, TN1 population increases reflecting a block in reaching TN2 stage, which is almost non-existing on $E2A^{-/-}$ mice. In addition, these mice are also prone to develop highly malignant T-cell lymphomas after 2 months of age. These lymphomas are associated with high levels of c-myc, which is explained by a non-random gain of chromosome 15 containing the c-myc gene (Bain et al, 1997).

The Heb transcription factor is essential for TN3 transition. In its absence a severe block at this stage is observed (Barndt et al, 2000). Together, these findings support an important role of both E2A and HEB in the TN3 stage which coincides with the β-selection. E2A and HEB

overexpression activates pTα promoter and enhancer (Petersson et al, 2002; Takeuchi et al, 2001).

E47, a splicing isoform of E2A promotes TCRβ gene rearrangements by allowing chromatin accessibility in a dose dependent regulation (Agata et al, 2007).

E2A and HEB were also described as being able to induce a diverse repertoire of TCRδ and γ on non-lymphoid cells (Ghosh et al, 2001). Moreover, E2A deficient mice show that E2A is necessary for the proper development of TCRγδ cells (Bain et al, 1999).

RUNT-related transcription factor (Runx) complexes are essential for early thymocyte development. They are composed of a DNA binding subunit encoded by the *Runx1, Runx2,* or *Runx3* genes and a common non-DNA-binding partner core-binding factor β (Cbfβ). The study of RUNT is complicated since a KO mouse for either Runx1 or Cbfβ results in a lethal phenotype at birth and is characterised by a complete lack of definitive haematopoiesis. The study of a hypomorphic Cbfβ allele showed a reduced foetal thymic cellularity and an increase in the percentage of $CD4^+$ cells. This was due to partial de-repression of the CD4 gene, which is normally silenced by Runx1 during the DN stages of thymocyte development. Most of these $CD4^+$ $CD8^-$ cells expressed low percentage of TCRβ. In fact, the differentiation block started at the ETP stage and affected its transition to DN2 and also from DN2 to DN3 (Talebian et al, 2007).

Runx1 (also known as AML-1, CBFA-2 or PEBP-2αβ) is required for maturation of megakaryocytes and differentiation of T and B-cells, but not for maintenance of HSCs in adult haematopoiesis (Ichikawa et al, 2004).

Runx1-Cbfβ regulates a broad spectrum of genes in the myeloid and lymphoid lineages, including *IL3* (interleukin 3) gene, *CSF2* (colony-stimulating factor 2 [granulocyte-macrophage] gene), *CSF1R* (colony-stimulating factor 1 receptor gene), *CD4* (CD4 antigen gene), and *Tcrδ* (T-cell receptor delta chain gene)(Growney et al, 2005).

Myeloblastosis viral oncogene homolog (Myb) plays an essential role at distinct stages of T-cell development in the thymus. The *Myb* proto-oncogene encodes a nuclear, DNA-binding protein (c-Myb). Complete ablation of *c-Myb* gene results in death at days 13-15 of embryogenesis due to severely disrupted patterns of erythroid and myeloid development. To study its function on lymphopoiesis the $Rag1^{-/-}$ blastocyst chimera system (Chen et al, 1993) was used. Results suggested however that c-Myb is not required for haematopoietic stem cell function itself. To

further assess its role on T-cell development the *Myb* locus was targeted for cell specific deletion using the CRE-loxP system. A functional TCR chain is generated by somatic recombination of numerous variable (v), diversity (d) and joining (j) gene segments (Brack et al, 1978; Weigert et al, 1978). Myb was found to be required for transition through TN3 stage for D_β to J_β and V_β to DJ_β recombinations. C-Myb was also found to be required for the survival of pre-selection DP thymocytes and efficient V_α to J_α recombination. Moreover, c-myb seemed to be more important for differentiation from the DP compartment to CD4 than CD8 SP thymocytes. Proliferative response of mature T-cells to mitogens was also shown to require c-Myb (Bender et al, 2004; Lieu et al, 2004).

The inhibitor of DNA binding 3 (Id3) is essential for overcoming TN3 stage. Id3 is a HLH protein that acts as a negative regulator of gene expression. Id3 exerts its function by heterodimerising with E-proteins and other bHLH proteins and therefore preventing their binding to DNA. bHLH proteins are likely to cooperate with Notch1 to prevent uncontrolled proliferation and thus promote TCR β-chain rearrangements. During the early stages of thymocyte development, the bHLH proteins regulate the expression of components involved in pre-TCR- and Notch-mediated signalling. Additionally, the E-proteins act to enforce the developmental and proliferation checkpoints prior to pre-TCR signalling. Upon pre-TCR signalling, during β-selection, E47 levels decrease as its inhibitor, Id3, is induced. The downregulation of E47 is hypothesised to constitute a feedback mechanism that ensures allelic exclusion. This allelic exclusion can be broken with the forced expression of E47 (Agata et al, 2007).

The further progression from the TN3 stage is promoted by the weakened bHLH activity and down-modulation of Notch1, releasing the developmental block allowing the cells to expand.

Below, figure 5 illustrates both the known surface markers and the known transcription factors through thymopoiesis.

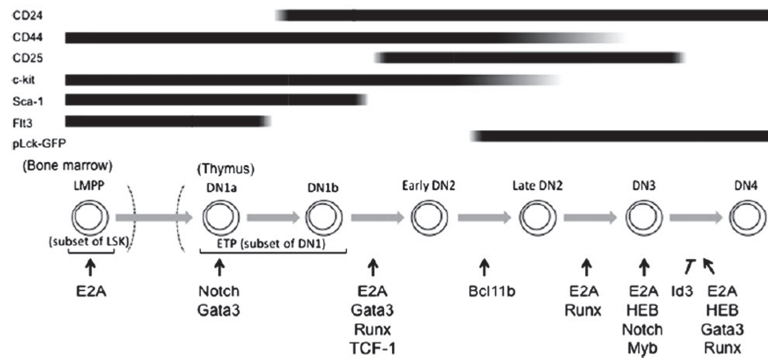


Figure 5: Surface markers and TF in thymopoiesis.

Several known TF are involved in the progression through the different stages of lymphopoiesis, from the LSK population in the BM to the TN4 population. Black arrows indicate at which stage a TF is required and the broken arrow indicates inhibition by a TF. On top the black bar represents when a cell marker, on the left, is expressed. Image from (Naito et al, 2011)

The T-cell Receptor

Progenitor cells committed to T-cell development become T-cells once they express a functional TCR. The TCR is a surface receptor composed by a complex of integral membrane proteins, expressed on all T-cells, capable of recognising antigens embedded in a major histocompatibility complex (MHC) and inducing a series of intracellular signalling cascades. This specific recognition of antigens is the hallmark of jawed vertebrate adaptive immune system. It was back in 1974 that T-cells were first found to be restricted to MHC for recognition of the lymphocytic choriomeningitis virus (LCMV) infected cells and trigger cell lysis (Zinkernagel & Doherty, 1974). TCR rearrangements results in the generation of a diverse antigen repertoire, which is capable of recognising virtually any foreign antigen. In humans, the diversity of antigen receptors is estimated to be over 10^8 (Wooldridge et al, 2010). As a result of this diversity-generating system, an efficient negative selection process is critical to prevent the generation of self-reactive receptors and ensuing auto-immunity.

Although TCR is constituted by several proteins, only a heterodimer constituted by a α-chain and a β-chain or by a δ-chain and a γ-chain bound together by a disulphide bound is responsible for antigen recognition. Most of TCR variability is focused on the bound antigenic peptide occupying the surface in contact with the receptor.

The three-dimensional structure of TCR is fairly similar to that of an antibody molecule. In the antibody, the centre of the antigen-binding site is composed by the third complementarity-

determining regions (CDR3) of the heavy and light chains. This structure is equivalent on the TCR, where the loops of TCRα and β chains composed of D and J gene segments act as the centre of the antigen-binding site. The periphery of TCR is also equivalent to that of an immunoglobulin. The CDR1 and CDR2 loops of immunoglobulins are encoded within the germline V gene segments of the TCRα and β chains.

V(D)J recombination is mediated by the RAG1 and RAG2 recombinase proteins (Oettinger et al, 1990; Schatz et al, 1989), whose expression is limited to precursor cells. Recombination by RAG complex targets conserved recombination signal sequences (RSSs) flanking all Ig and TCR gene segments (Sakano et al, 1979). V(D)J recombination is regulated by changes in chromatin. RAG complex recognition of RSSs will loop out segments of DNA and the recombinase activity splices the DNA. TCR loci reside in closed chromatin, inaccessible to both transcription and recombinase machinery. These loci only become accessible in an orderly fashion on lymphocyte development. The accessibility hypothesis is invoked to explain how a single recombinase complex could control the locus and allele specificity of V(D)J recombination. Stanhope-Baker, Schlissel and co-workers found that the specificity of RSS cleavage reflected the lineage and developmental stage of the cell population from which the nuclei were derived (Stanhope-Baker et al, 1996). Since then, it has also been shown that V(D)J recombinase is tightly regulated by promoters/enhancers, that serve as accessibility control elements (ACEs) that guide TCR assembly during lymphocyte development (Schlissel, 2003).

The order of rearrangement mediated by RAG1/RAG2 complex is D to J and then V to DJ (Khor & Sleckman, 2005) and starts in TCRβ locus. Recent studies have shown that, rather than a mechanism of complete allelic exclusion, TCRβ genes undergo intra-allelic ordering, which ensures that Vβ to DJβ is the final step of recombination and results in a feedback mechanism that prevents further rearrangements (Khor & Sleckman, 2005). The newly synthetized TCRβ protein associates with a pre-TCRα forming the pre-TCR complex expressed at the cell surface (Dillon & Fink, 1995). Pre-TCR signalling downregulates CD25 and generates a burst of proliferation, in a process termed β-selection (Buer et al, 1997). The RAG1/RAG2 complex becomes silenced and the cell can progress to the double positive stage ($CD4^+$ $CD8^+$) when RAG1/RAG2 complex is induced once again, and chromatin is made accessible by interleukine-7 (IL-7) signal transduction (Huang & Muegge, 2001). TCRα genes can now be recombined in a similar fashion to TCRβ locus although TCRα locus only contains V and J regions. In contrast to the β chain of the TCR, there is no allelic exclusion in the TCR α chain, so a T lymphocyte may

express two TCRα chains (Lacorazza & Nikolich-Zugich, 2004). The pairing of TCRβ and TCRα chains defines the antigen-binding site that ensures the specificity of the TCR.

The rearranged molecules associate with the γ, δ, ε and ζ chains of the CD3 molecule to form the complete TCR complex. CD3 is a membrane bound signal transduction complex which is able to mediate signal through immunoreceptor tyrosine-based activation motifs (ITAMs) (Dave, 2009).

TCR is capable of recognising specific antigens that are coupled to MHC. MHC molecules are polymorphic and a significant variation occurs among individuals. The newly synthetized TCR must be able to recognise the host's MHC complex. To ensure that TCR is capable of interacting with MHC/antigen complex, survival signals are transmitted through the TCR complex only upon antigen stimulation to the developing T-cells. This is called positive selection. The cells that fail to receive these signals undergo death by neglect (von Boehmer et al, 1989).

Besides positive selection, T-cells also undergo negative selection or central tolerance, where T-cells that recognise self-antigen are induced to undergo apoptosis. Positive selection is known to occur in the thymic cortex while negative selection occurs mostly in the medulla of the thymus (Kishimoto & Sprent, 2000; Sprent & Kishimoto, 2002). The reason why negative selection occurs in the medulla is that the repertoire of peptides in the cortex is very limited. Double positive thymocytes undergo a series of maturational changes once they successfully undergo positive selection. They now express high levels of TCR and subsequently cease to express one of the two co-receptor molecules, becoming either CD4 or CD8 single positive thymocytes. They also upregulate the chemokine receptor CCR7, inducing their migration from the cortex to the medulla. In the medulla, single positive thymocytes will be exposed to a complex network of self-antigens to ensure immunological tolerance to self-antigens. Transcripts encoding transcription factors, structural proteins, membrane proteins, hormones and secreted proteins are all expressed on thymic medullary epithelial cells (MECs). This expression is regulated by the AIRE transcription factor. In humans, AIRE deficiency causes autoimmune polyendocrinopathy syndrome type I (Anderson et al, 2002; Liston et al, 2003).

Recognising self-antigen poses the danger of auto-reactivity. To prevent this, high affinity TCR signals induce cell death (von Boehmer et al, 1989). T-cell development provides however ways to overcome this self-antigen recognition problem. Rather than becoming immediately

apoptotic, auto-reactive cells can undergo TCR editing (Mayerova & Hogquist, 2004), which requires the re-activation of the RAG1/RAG2 recombination complex.

TCR editing is not limited to the progenitor cells as originally thought (McMahan & Fink, 1998). The RAG1/RAG2 complex was reported to be induced in peripheral T-cells on germinal centres, which arise in spleen and lymph nodes after exposure to a superantigen (Collins et al, 1996). This TCR revision would be dependent on B-cells, suggesting direct interaction between revising T-cell and an antigen presenting cell (APC). Since mammals undergo thymic involution with age, this TCR revision would provide a means to produce new functional T-cells. Peripheral TCR revision also poses a risk of generating auto-reactive T-cells (Vaitaitis et al, 2003), but on the other hand, it could also be required to induce central tolerance, but it is not consensual if and when T cell revision actually occurs.

Only a small fraction of thymocytes manages to reach the final maturation stages. Nevertheless mouse spleen was reported to have approximately 2×10^6 unique TCRαβ clones, of about 10 cells each. This number falls short of the maximum theoretical diversity estimated to go up to 10^{15} but is large enough to maintain a high functional diversity since. TCR functional diversity is also increased by the fact that TCR antigen binding is degenerate, meaning that an antigen might be recognised by several different TCRs (Casrouge et al, 2000) and also because each TCR may recognise several different peptides with different affinities.

The organisation of the γ and δ locus is similar to that of the α and β. Of note, the δ chain locus is located between Vα and Jα gene segments of the TCRα chain, therefore any rearrangement for the TCRα chain genes results in deletion of the δ locus. TCRδ and TCRγ loci have much fewer V gene segments than TCRα or TCRβ loci. Despite the small number of possible variable regions, variability is ensured by junctional diversity. In peripheral lymphoid organs, only a small percentage (1-5%) of T-cells express the γδ receptors, but TCRγδ cells are very abundant elsewhere, mainly in the epithelia (Komori et al, 2006).

T-cell circulation

Once single positive thymocytes successfully undergo negative selection, after 1-2 weeks surveying self-antigens and undergoing final maturation changes, they are ready to leave the thymus and enter the peripheral blood stream, becoming mature T-cells.

As we have seen before, the thymus serves as a school to generate a pool of T-cells with functional TCRs capable of recognising virtually any foreign antigen. But in order to do so, it is crucial that T-cells circulate to establish an effective immune surveillance. Lymph nodes are organs of the immune system widespread thorough the body that recruit APCs, T-cells and other immune cells circulating in the bloodstream to mediate antigen recognition.

A multistep adhesion cascade is required for circulating naïve cells to enter into peripheral lymph nodes. This depends upon a very rapid increase in the adhesive activity of leukocyte integrins. L-selectin (also known as CD62L) is a cell adhesion molecule expressed on lymphocytes that binds to peripheral node addressin (PNAd), expressed on specialised post capillary venules, the high endothelial cells (HEV) of lymph nodes. L-selectin engagement slows down lymphocytes allowing them to roll along the surface of HEVs in the direction of the blood flow (Girard & Springer, 1995). While slowly rolling, lymphocytes that express chemokine receptor type 7 (CCR7) will bind their ligands, chemokine (C-C- motif) ligand 21 (CCL21) and ligand 19 (CCL19) (Baekkevold et al, 2001; Richmond, 2002; Sallusto et al, 1999), expressed on the luminal surface of peripheral lymph nodes HEV cells (Stein et al, 2000). This event triggers a signalling cascade resulting in the rapid activation of adhesion receptors of the integrin family such as leukocyte function-associated antigen-1 (LFA-1) (Warnock et al, 1998). Integrins bind to members of the Ig superfamily, which are expressed in the endothelium. Some of these members are the intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, vascular cell adhesion molecule-1 (VCAM-1) and mucosal addresin cell adhesion molecule Mad CAM-1 (Butcher & Picker, 1996; von Andrian & Mackay, 2000). The adhesion of intergrins will add the necessary strength to stop the rolling lymphocytes, resisting the sheer force of the blood flow. The homing process will end by flattening the arrested lymphocytes allowing them to transmigrate by diapedesis into the lymphoid tissue.

Figure 6 illustrates the homing of T-cells to lymph nodes.

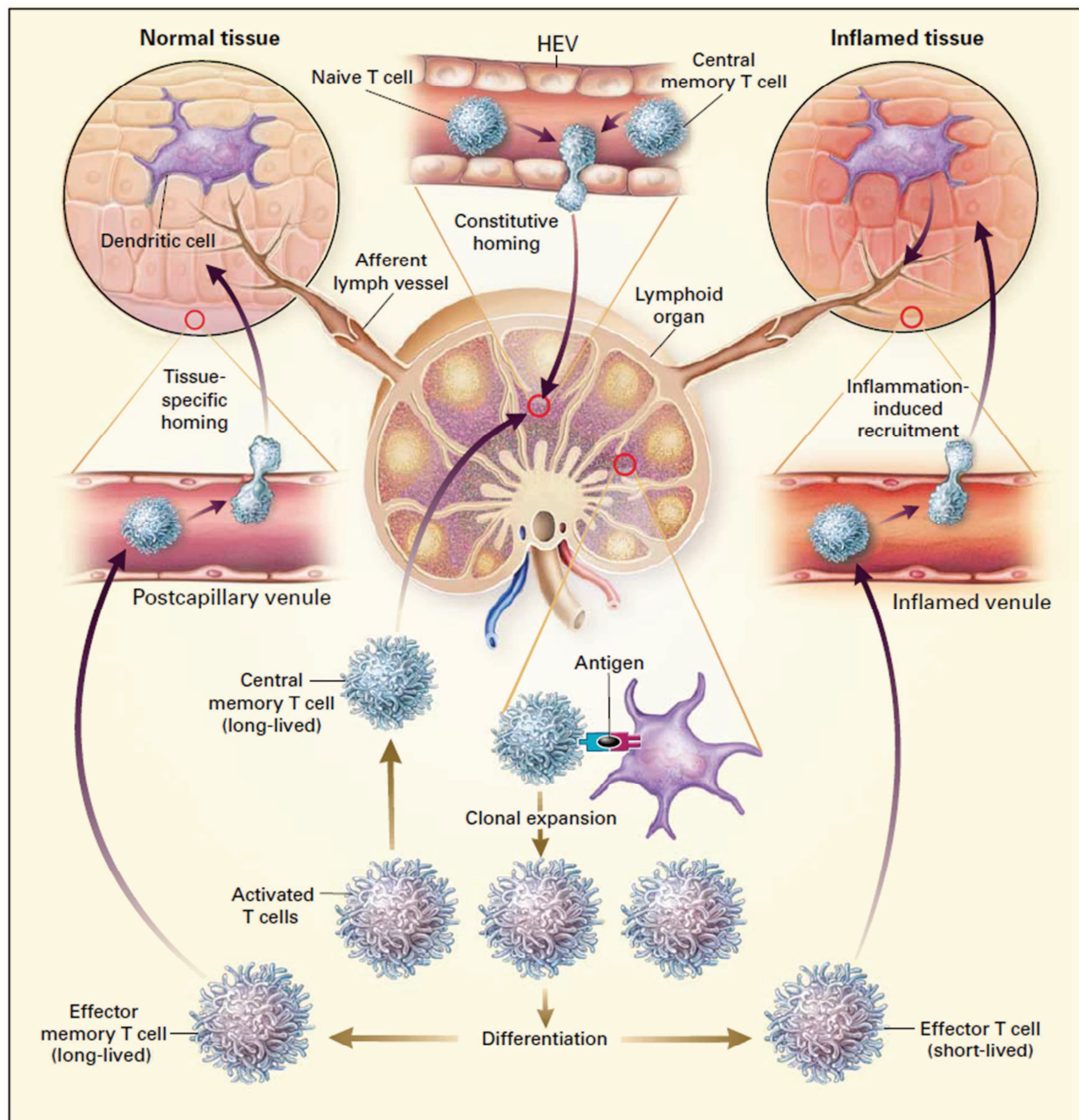


Figure 6: T-cell homing to the lymph nodes:

The image illustrates the process of diapedisis through the HEV cells, which allow the homing of T-cells to the lymph nodes, where they will contact with the antigen undergoing clonal expansion and differentiation into effector T-cells. Effector T-cells express receptors to exit the lymph node and return to the bloodstream. Among the effector T-cells, two populations can be discriminated; a population of short-lived effector T-cells that is recruited immediately to inflammation sites where it is most needed to combat infection and a population of long-lived memory cells. These long-lived memory cells can also be divided in two groups, one that has affinity to migrate to the lymph nodes expressing the same pattern of homing molecules as naïve T-cells; the central memory T-cells and another population of long lived cells that has a pattern of homing molecules which will allow them to migrate to peripheral tissue. These cells are called the effector memory T-cells. Both the long-lived effector memory T-cells and the short-lived effector T-cells have organ specific trafficking signals, which will determine to which organ they migrate. Image from (von Andrian & Mackay, 2000).

Naïve T-cells exit or “egress” lymphoid tissues into efferent lymph node by expressing the G protein-coupled sphingosine 1-phosphate receptor (S1P) (Mandala et al, 2002; Matloubian et al, 2004). From the efferent lymphatics they return to the blood stream through the thoracic duct. SP1 expression is also needed for thymic egress of naïve T-cells, along with the expression of CCR7 (Mandala et al, 2002; Morley et al, 2010; Yopp et al, 2004).

Naïve T-cells circulate through peripheral lymph nodes until they recognise a specific antigen. The homing from blood to tissue and back to blood again occurs as often as one to two times per day (Ford & Gowans, 1969).

Micro environmental compartmentalisation within the lymphoid tissue is thought to be mediated by CCR7 and other chemokine receptors. The close physical contact mediated in these compartments between T and B-cells allows for the extensive screening of MHC molecules and the initiation of specific immune response.

Activation, expansion and memory

Naïve mice have very small numbers of T-cells specific to a given foreign antigen. The frequency of such cells within the total CD4 and CD8 population has been estimated to be around 1 in 100,000 cells in both human and mice (Blattman et al, 2002; Obar et al, 2008).

After engagement with a peptide MHC ligand through the TCR (signal 1) and co-stimulatory molecules (signal 2) presented by an APC, naïve T-cells become activated, proliferate and differentiate into memory T-cells, thereby changing their chemokine receptor profile. This will enable them to circulate between lymphoid and peripheral tissues to deliver tissue specific immunity and surveillance of non-lymphoid sites. Depending on the strength and duration of the receptor signal, a naïve T-cell first differentiates into a central memory cell (T_{CM}) and then into an effector memory cell (T_{EF}) (Geginat et al, 2001). This pathway of differentiation, proposed by Lanzavecchia and co-workers, is directly contradicted by Ahmed and co-workers who propose that T_{EF} give rise to T_{CM} in a linear pathway (Wherry et al, 2003). A third model presented by Baron and co-workers proposes that T_{EF} and T_{CM} subsets have separate origins and are not inter-convertible (Baron et al, 2003).

Independently of the controversial origin of these subsets, T_{CM} cells were described as lymph-node homing cells without cytotoxic or inflammatory functions. These antigen-primed cells travel to secondary lymphoid organs, via the expression of L-selectin (CD62L) and CCR7. After

in vitro activation they express IL-2 but not IFNγ or IL-4. Upon secondary challenge T_{CM} are able to stimulate DC, help B-cells and generate a new wave of effector cells (Sallusto et al, 1999).

T_{EF} cells on the other hand do not express CD62L or CCR7, representing a readily available population of antigen-primed cells ready to migrate directly to the inflamed peripheral tissue. T_{EF} cells display immediate effector function, via expression of IFNγ and IL-4, mediating inflammatory signals or cytotoxicity (Zielinski et al, 2012).

Both T_{CM} and T_{EF} cells can be maintained *in vivo* for up to 10 years and their relative proportions do not change after antigen stimulation.

Activated T-cells enter an expansion phase, which lasts 7-8 days in mice (Butz & Bevan, 1998; Murali-Krishna et al, 1998) and approximately 14 days in human (Miller et al, 2008). At this phase, antigen specific T-cells undergo an important expansion. A single naïve T-cell may undergo more than 15 consecutive divisions generating over 50,000 descendants (Blattman et al, 2002).

Naïve T-cells must undergo changes in their metabolism to support their growth and differentiation. During TCR stimulation signals from growth factor cytokines like IL-2 and co-stimulatory CD28, lead to an increase in glycolysis by inducing the PI3K-dependent activation of Akt (Frauwirth et al, 2002). Akt not only activates the mammalian target of rapamycin (mTOR) pathway, which is a key regulator of translation, but also stimulates glycolysis by increasing glycolytic enzyme activity and enhancing the expression of nutrient transporters, enabling increased utilization of glucose and amino acids (a.a.).

During clonal expansion, T-cells preferentially ferment glucose to meet their energy demands, in spite of having sufficient oxygen to support mitochondrial phosphorylation. This phenomenon is called the Warburg effect (Warburg, 1956) and is shared with cancer cells.

Glycolysis is much less efficient in generating ATP than oxidative phosphorylation, but this anabolic metabolism leaves amino acids, fatty acids and lactate untouched, all of which may be used as “building blocks” to produce biomass and proliferate more efficiently (Fox et al, 2005).

During proliferation, T-cells undergo several differentiation processes, where they will become “armed effector” T-cells. These cells will now be able to synthetize all the proteins required for their specialised function as helper or cytotoxic T-cells. They will also be able to synthesize the

appropriate arsenal of specialised effector molecules when they encounter specific antigen on target-cells, without the need for co-stimulation. This is particularly important in cytotoxic CD8T-cells which must be able to recognise and respond to a cell infected with a virus, whether or not it produces co-stimulatory molecules. It is however also important for effector function of CD4 T-cells, as they must be able to activate B-cells and macrophages that have taken up antigen, even if they have too little co-stimulatory activity to activate a naïve CD4 T-cell.

The quick expansion upon antigen stimulation and effector function differentiation is followed by a contraction phase, where the majority of the antigen specific T-cells undergoes controlled apoptosis to maintain homeostasis, characterised by a stable CD4/CD8 ratio (Rocha et al, 1989). Not all effector T-cells undergo apoptosis; a small fraction undergoes further differentiation into memory cells as previously described.

Differentiation of CD4 T-cells

Originally CD4 T-cells were divided into two major subsets: (i) the T helper 1 (Th1) cells, expressing a T box transcription factor (T-bet) that controls the expression of interferon γ (IFNγ) when induced by IL-12 (Szabo et al, 2000); and (ii) the Th2 cells, expressing trans-acting T-cell-specific transcription factor (Gata-3) that controls expression of IL-4 (Mosmann & Coffman, 1989).

Naïve CD4 T-cells become immature effector T-cells (Th0) after responding for the first time to a specific peptide MHC class II complex. This response triggers IL-2 production, which leads to proliferation. Th0 cells share the characteristics of both Th1 and Th2 and have the potential to become either of them under further appropriate stimulation.

Th1 cells exert their immune function through the activation of macrophages, by inducing B-cells to produce opsonizing antibodies and by inducing the proliferation of CD8 T-cells and their differentiation into memory cells (Bourgeois et al, 2002), therefore protecting the host against intracellular infections, including viruses and toxoplasma (Abbas et al, 1996; Aliberti et al, 2004). Th2 cell, on the other hand, exert their function through the activation of B-cells, inducing antibody class switch, and through the activation and differentiation of eosinophil's (Abbas et al, 1996), leading to the host's protection mainly against nematodes (Finkelman et al, 2004).

The Th1/Th2 paradigm served the purpose to understand the basic principles in cellular specification and gene regulation, but failed to explain a great deal about immunity and autoimmunity. The discovery of regulatory T (Treg) cells, another CD4 lineage with immunosuppressive functions expressing the master transcription factor forkhead box P3 (FoxP3), shed some light into the role of CD4 T-cells in preventing auto-aggression.

The concept of self-tolerance is long known to scientists. Back in 1906, Paul Elrich's observation that goats could produce antibodies against blood components of other goats but not to their own, led him to postulate the concept of self-tolerance.

Throughout the years, several lymphoid cells subsets have been suggested to, under certain circumstances suppress or modulate the immune response. However, up to date only one dedicated type of T-cells has been shown to be capable of modulating immune responses actively: the Treg cells. Tregs where first described by Sakaguchi et al in 1995 as being a population of CD4 T-cells expressing the IL-2 receptor α chain (CD25) (Sakaguchi et al, 1995). These FoxP3 expressing cells are responsible for maintaining dominant self-tolerance and immune homeostasis, via the potent anti-inflammatory cytokine IL-10 (Annacker et al, 2001). In the absence of Tregs the host develops chronic T-cell mediated autoimmunity and immunopathology (Sakaguchi et al, 2010).

The *FoxP3* gene was found to be essential for Treg function only latter, in humans that suffered a loss of function mutation of this gene in the X chromosome. These patients suffered from an early lymphoproliferative autoimmune disease called polyendocrinopathy, enteropathy, X linked (IPEX) (Wildin et al, 2002).

Further knowledge about the function of FoxP3 was obtained using a mouse model that had long before been discovered: the *scurfy* mouse. These mice were characterized by a spontaneous mutation in 1949 at the Oak Ridge National Laboratory and provided William L. Russell with the tools for the discovery, years later, of how sex determination takes place. This mutation on the X chromosome proved to be once again very useful as in 2003 it was discovered that it affected the *FoxP3* gene. Studies in both *scurfy* mice and human IPEX patients led to the finding that FoxP3 is specifically expressed in Tregs and that it controls its development and function (Fontenot et al, 2003; Hori et al, 2003; Khattri et al, 2003).

Although the suppressive role of Tregs is not established in all *in vivo* responses, it has been shown that *in vitro* Tregs mediate their function in two different ways, the direct suppression

of responding T-cells and myeloid cells and the Inhibition of APC or innate immune system cells.

The direct suppression of responding T-cells and myeloid cells

Tregs may inhibit the function of responder cells, mediating cell cycle arrest, through the production of suppressor cytokines (i.e. IL-10, TGF-β, IL-35). They may also compete for the available IL-2 through their high levels of IL2Rα (CD25). The deprivation of IL-2 leads to cell death of the effector cells through bim-mediated apoptosis, controlling the amount of effector cells at a given time. Activated Tregs may also act as activated cytotoxic CD8 T-cells in the way that they are able to bind and directly kill effector cells through granzyme-mediated apoptosis. Finally, within the T-cell targeting, Tregs may also lead to effector cell cycle arrest through expression of Galactin-1 or other unknown molecules which in contact with effector cells induce cell cycle arrest (Shevach, 2009).

Inhibition of APC or innate immune system cells

Tregs can also exert their functional role by interacting with APC. The mechanism of action has been extensively described and is mediated through the downregulation of CD80 and CD86 major costimulatory molecules of APCs following direct binding with CTLA-4. They may also prevent dendritic cells maturation by binding MHC II of immature dendritic cells expressing LAG-3, resulting in a decreased antigen presentation. Also, through CD39 surface receptor, Tregs can catalyse extracellular ATP, which is an indicator of tissue damage and triggers inflammatory response on DCs. Finally, Tregs can inhibit immature DCs from binding to effector cells, by prolonged binding to the immature DCs via Nrp-1 molecule. Conversely, all of these interactions can be used to suppress responder T-cells (Shevach, 2009).

Tregs have higher affinity for the self TCR. This affinity falls, obviously, bellow the negative selection threshold but is believed to be responsible, along with the co-stimulatory molecule CD28, for the expression of FoxP3 through activation of numerous TFs (i.e. NFAT, NF-κB and AP-1) (Josefowicz & Rudensky, 2009). It is currently believed that through this high affinity TCR signalling, CD25 expression is increased ($CD25^{hi}$), augmenting the responsiveness to IL-2, which ultimately leads to the expression of FoxP3 (Burchill et al, 2008; Lio & Hsieh, 2008). For Tregs to endure the high affinity TCR signalling they require potent anti-apoptotic signals. The signal

transducer and activator of transcription 5 (STAT5) and bcl2 are two known molecules that are implicated on Treg survival (Burchill et al, 2008).

The complexity of helper CD4 T-cell subsets continued to increase with the discovery in 2005 of cells that selectively expressed IL-17 and RORγt. These cells were termed Th17 due to their expression of IL-17 and were classified as a new subset since they didn't express T-bet or GATA-3, the transcription factors that define the Th1 and Th2 lineages. (Harrington et al, 2005; Park et al, 2005). It took, once again, mice models and the study of autoimmunity to identify these cells. These models were the experimental autoimmune encephalomyelitis (EAE) and Collagen-induced arthritis (CIA). Both these models were believed to be Th1 autoimmune disease models, until the discovery that CD4 effector T-cells producing IL-17 and not IFN-γ, were responsible for the disease, undermining the importance of Th1 cells in the induction and maintenance of chronic inflammatory disease (Zhu et al, 2010).

Differentiation of the Th1 lineage is mediated through STAT1 signalling, which is induced in response to the production of IFNγ by Th1 mature effector cells or of IFNγ, IL-27 and IL-12 by innate immune cells (Pflanz et al, 2002). STAT1 signalling induces the expression of T-bet in naïve CD4 T-cells, which upregulates IL12R and supresses GATA-3 (Ouyang et al, 1998). This inhibits Th2 at the same time that it promotes Th1 development.

Conversely, the expression of IL-4 by Th2 mature effector cells induces STAT6 signalling, which upregulates GATA-3 expression and blocks IL-12R expression on naïve T-cells (Ouyang et al, 1998). This inhibits Th1 at the same time that it promotes Th2 development.

This Th1/Th2 paradigm fails to predict Th17 function in chronic inflammation diseases. Th17 cells express IL17, a T-cell derived pro-inflammatory molecule. Both EAE and CIA models were rescued in IL-23 deficient mice (Cua et al, 2003; Murphy et al, 2003). IL-23 was shown to promote Th17 activation state (Aggarwal et al, 2003). The role of Th17 cells in pro-inflammatory diseases sparked the interest in its study and quickly led to the discovery of a duality in its function. In humans, upon stimulation by *C. albicans* or *S. aureus* Th17 cells produce either IFN-γ or IL-10 (Zielinski et al, 2012), supporting its protective role in immunity against these pathogens.

This duality of function is also illustrated by the effect of targeting Th17 cells in patients with Crohn's disease, which resulted in the exacerbation of the disease symptoms in the gut. These results show that multiple subsets of Th17 have distinct specialisations and while some have pro-inflammatory functions, others might have a regulatory role (Marwaha et al, 2012).

The complexity of the CD4 T-cell differentiation program has been further highlighted by the recent characterisation of three new subsets: Folicular helper T-cells (Tfh), Th9 and Th22. Tfh reside in B-cell folicules and seem to be essential for high-affinity isotype switching and B-cell memory (Vinuesa et al, 2005). The Th9 subset was identified *in vitro* by treating Th2 cells with TGF-β (Veldhoen et al, 2008). Originally IL-9 was thought to be a Th2 cytokine and, although controversial, recent findings showed that the PU.1 transcription factor is a lineage-determining factor required for the acquisition of a Th9 phenotype, distinct from the Th2 subset (Chang et al, 2010). Th9 is known to express pro-allergic chemokines. Finally, the Th22 subset has been identified on psoriasis patients. So far, this subset has only been identified in humans and has been shown to home to the skin by expressing CCR4, CCR6 and CCR10, where it has an important role in inflammatory skin disorders (Duhen et al, 2009). Although the existence of some of the subsets is still controversial, these results demonstrate the flexibility of helper T-cells phenotypes, which is much greater than originally anticipated (Nakayamada et al, 2012).

Differentiation of CD8 T-cells

CD8 T-cells of both humans and mice were subdivided in subsets analogous to their CD4 counterparts, the cytotoxic T-cell 1 (Tc1) and 2 (Tc2) subsets. Historically CD8 T-cells were regarded as a homogeneous population of cytotoxic cells, characterised by the production of a restricted small array of cytokine. However, recent studies revealed their true cytokine production potential.

The Tc1 subset expresses IFNγ and not IL-4, while the Tc2 subset expresses IL-4, TNFα and IL-2 and not IFNγ. Similarly to their CD4 counterparts, IFNγ and IL-4 are the CD8 lineage-determining cytokines responsible for the differentiation of the Tc1 and Tc2 subsets, respectively, while actively inhibiting the differentiation of the alternative lineage (Croft et al, 1994; Sad et al, 1995). The requirements for type 1 or type 2 commitment of CD8 T-cells appears to be similar as for CD4 T-cells, which is IL-12 and IL-4, respectively.

In addition to the classical role of CD8 T-cells in mediating cytotoxicity towards infected cells, these cells are also able to balance Th1/Th2 responses *in vivo* (Holmes et al, 1997; Noble et al, 1998). Tc1 and Tc2 cells express different chemokine receptors and home differently to epithelium but even so, both subsets share the same cytotoxic activity (Cerwenka et al, 1999).

As previously stated, naïve CD8 T-cells are primed by APCs in secondary lymphoid organs. The exact location where this contact occurs, in the context of either virus or parasite infection models, was recently demonstrated using multiphoton-based intravital microscopy (IVM). It appears that migrating naïve CD8 T-cells interact with virus-infected cells just below the lymph node subcapsular sinus (SCS), in an area called peripheral interfollicular region (Hickman et al, 2008; John et al, 2009). The entry of T-cells into the draining lymph node is done via the HEVs as previously stated. This is why it was thought that CD8 T-cells would encounter antigen near the HEVs rather than on the SCS region, which is a peripheral location in the LN. Shortly after infection, as soon antigens are detected in the lymph nodes, the CD8 and DCs quickly migrate to the peripheral regions of the T-cell zone near the SCS region, where pathogens and antigens arrive through the afferent lymph vessel (Hickman et al, 2008; John et al, 2009). Naïve CD8 T-cells are preferably primed by DCs rather than the antigen rich macrophages. These DC also migrate to the same zone and acquire antigens either by infection of the infected cells or by cross-presentation, which was first demonstrated in 1976 with the discoveries of Michael J. Bevan (Bevan, 2006). It explains how a DC is capable of processing exogenous antigens acquired through phagocytosis of dying cells and process them through MHC class I presentation pathway to initiate cross-priming of CD8 T-cells. Figure 7 illustrates the internal organisation of a peripheral lymph node and the homing processes.

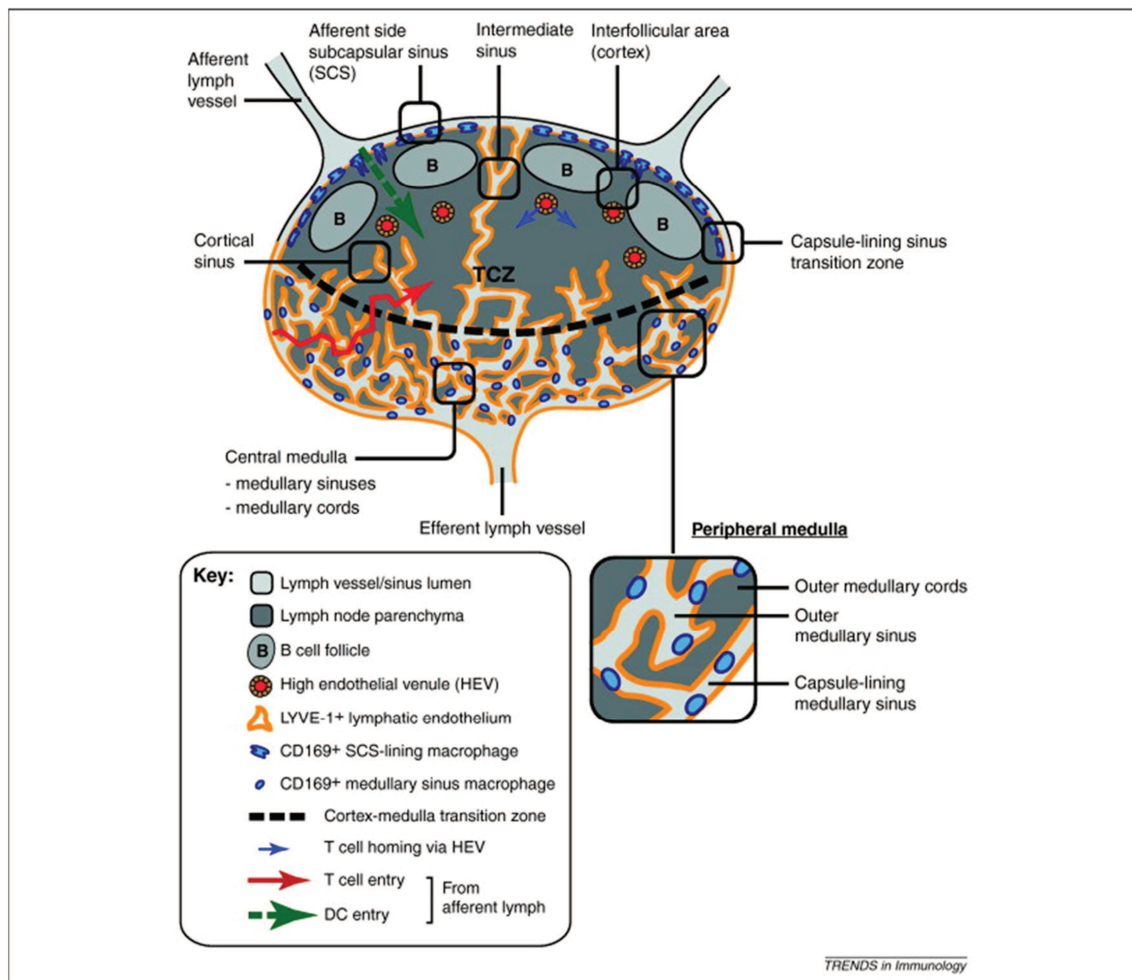


Figure 7: Representation of a mouse peripheral LN homing.

Naïve T-cells home to the LN deeper paracortical T-cell zone (TCZ) from the blood circulation via HEVs (blue arrows) and from the efferent lymph via accumulation on capsule-lining and outer medullary sinus, after which they enter the LN parenchyma and progress through the medullary cords into the TCZ (red line). Activated DCs home to the LN via efferent lymph and accumulate on the SCS of the efferent side of the lymph node, after which they migrate to the TCZ. Image from (Forster et al, 2012).

After infection, DCs are known to express chemokines such as CCL3, CCL4 and CCL17 that will attract cognate $CCR5^+$ or $CCR4^+$ naïve CD8 T-cells, facilitating priming (Castellino et al, 2006; Semmling et al, 2010).

Antigen primed CD8 T-cells become a heterogeneous population of effector T-cells (Gerlach et al, 2010). These are called the short-lived effector cells (SLEC) and are this way designated as most of them will die as soon as the infection is cleared. However, during primary response to pathogen, the balance between CD4/CD8 is greatly shifted towards CD8 T-cells. Similarly to the

CD4 T-cell response, a small population of the antigen primed CD8 T-cells will differentiate into memory precursor effector cells (MPEC).

Naïve CD8 T-cells are stimulated via antigen presentation and co-stimulated by CD28. This leads to clonal expansion and the concomitant acquisition of the effector or memory phenotype. Activation of CD8 T-cells is particularly effective, when a third co-stimulatory signal is delivered by the professional APC: the DCs. This third signal is mediated by cytokine production, including IL-12, IL-21 and the type I IFN-α and IFN-β (Mescher et al, 2006), and induces clonal expansion of the antigen-primed CD8 T-cells. These cytokines are induced via a CD40-dependent interaction with CD4 T-cells or via the engagement of Toll-like receptors (TLRs) by pathogen products. The second signal molecules CD80 and CD86 are also upregulated via these signalling as is the first signal, antigen presentation MHC class I molecules. These DCs able to give the three signals to CD8 T-cells are called activated matured DCs (Mescher et al, 2006). A DC that does not encounter foreign antigen presents self-antigen resulting in tolerance induction and is called an immature DC.

Naïve T CD8 cells differentiate upon antigen presentation by DC. Within 24 hours they express IFNγ and display a high diversity on the expression levels of TNF-α and IL-2 even before the first-cell division (Beuneu et al, 2010), suggesting that CD8 T-cell fate might be determined before the first-cell division. On the other hand, other studies point towards differentiation being decided at the first-cell cycle. The first division of CD8 T-cells after antigen priming segregates proteossome in an asymmetric manner resulting in different levels of the transcription factor T-bet on the two daughter cells which ultimately determines CD8 T-cell fate (Chang et al, 2011).

Despite this controversy, the diversity associated with CD8 T daughter cells, clearly demonstrates that rapid expansion is key to the immune response. No other cell subset of the immune system has proven to expand faster than CD8 T-cells, with a maximal rate of *in vitro* division following activation of approximately 6-8 hours. As the techniques evolved and these studies have been conducted *in vivo*, the time of cell division has been estimated to be of 4 hours, half of the originally anticipated (Yoon et al, 2007). The mysteries on CD8 T-cells still remained largely unexplored as some years later, another *in vivo* study by the same group drew a new limit of newly activated CD8 T-cell division to as short as 2 hours (Yoon et al, 2010). According to our group's previous publications, following *in vivo* immunization, naïve CD8T-cells have a lag time before division of around 27 hours while their memory counterpart only have a 12 hours lag time. This reduction of the lag time is attributed to a higher mRNA

counts in resting memory cells. Also, about 60% of memory cells undergo two or more divisions within 44 hours following immunization and one day later 70% of memory cells have undergone six or more divisions revealing an impressive cycling ability (Veiga-Fernandes et al, 2000). Memory CD8 T-cells proliferate faster than naïve CD8 T-cells to make sure that a reinfection process is dealt with swiftly. This increased proliferation has been shown to correlate with a different state of G0/G1 arrest. Naïve CD8 T-cells in G0/G1 arrest have lower amounts of D cyclins, Cdk6 and Cdk2 than their memory counterparts. They also have higher levels of the inhibitory $p27^{Kip1}$ protein (Veiga-Fernandes & Rocha, 2004).

Independently of the real division time and the kinetics of the division, the amazing cycling ability of CD8 T-cells was correlated to a shortened G1 phase brought upon by elevated levels of a hyper-phosphorylated form of the tumour suppressor retinoblastoma (Rb) protein (Yoon et al, 2010).

B lymphocyte lineage commitment in the bone marrow

B-cells, not unlike the T-cells, must undergo the rearrangement of 3 Ig heavy chain (HC) gene elements (V,D,J) and undergo (V,J) recombination on the light chain (LC). A successful recombination of both chains is required to express a B-cell receptor (BCR). HC recombination occurs first on the pro-B-cells and LC recombination follows on the pre-B-cells. On large pro-B-cells, the heavy chain associates with a Vpreβ chain. This pre-BCR will transmit survival, proliferation and differentiation signals via a heterodimer constituted of Igα and Igβ. After proliferation the small pre-B-cells exit the cell cycle and re-undergo LC rearrangement. The newly rearranged LC proteins associate to the HC proteins forming the BCR, which is expressed on immature B-cells. Immature B-cells also undergo both positive and negative selection steps. After both selection processes, they will finally be able to exit BM and migrate to the periphery where additional selection will allow them to become mature B-cells capable of antigen recognition.

IL-7R signalling is known to be essential for commitment, proliferation and survival of B-cell progenitors through the activation of several signalling pathways i.e. JAK/STAT, Src, PI3K/Akt and MAPK/Erk (Corfe & Paige, 2012). Other important receptors for the development of B-cells are cKit, whose ligand is SCF and Flt-3 whose ligand is Flt-3L.

Back in 1991, mice B lineage cells were very elegantly divided in six fractions, A to F (Hardy et al, 1991). Fraction A ($B220^+$ $CD43^+$ $CD24^-$ $BP\text{-}1^-$) consists of the most undifferentiated B lineage cells, where Ig genes are still on germline configuration. These cells come directly from CLP and are called pre-pro B-cells. Fraction B ($B220^+$ $CD43^+$ $CD24^+$ $BP\text{-}1^-$) and C ($B220^+$ $CD43^+$ $CD24^+$ $BP\text{-}1^+$) have increasing D-J rearrangement of HC but no V-DJ rearrangements. Both these fractions compose the pro-B-cells (early and late respectively). Fraction D ($B220^+$ $CD43^-$ $CD24^+$ IgM^-) has already rearranged DJ genes of the HC and undergoes V-DJ recombination. Also on the LC V-J recombination starts. Fraction D composes the pre-B-cells. Fraction E ($B220^+$ $CD43^-$ $CD24^+$ IgM^+) has higher percentage of LC V-J gene recombination and expresses IgM. The authors gave it the name of newly generated B-cell. Finally Fraction F ($B220^{high}$ $CD43^-$ $CD24^+$ IgM^+) was given the name of mature B-cells and differs from fraction E only by the highest intensity of B220 expression.

To this day, this study continues to prove accurate though some details may be added. On the pre-pro-B stage, the germline configuration of both IgH (heavy chain) and IgL (light chain) is due to a low expression of *Rag1* and *Rag2* (Igarashi et al, 2002). On the pro-B stage, RAG expression is sufficient to initiate DJ_H recombination. At this stage the cells are able to express Igα and Igβ as well as the surface calnexin, constituting the pro-BCR (Kurosaki et al, 2010).

The accessibility of the V_H gene for rearrangement is thought to require the IL-7 cytokine and the TF Pax5 as well as a high expression of *Rag 1* and *Rag2* (Chowdhury & Sen, 2004; Jung et al, 2006). The regulation of *Rag* genes in B-cells but not T-cells was recently shown to depend on Foxp1.

Pro-B-cells arrive at the pre-B-cell stage when they are able to express a pre-BCR. Pre BCR is expressed on the cell surface once the HC genes have successfully undergone V-DJ recombination. Pre-BCR is composed of a transmembrane μHC (mIgμ), the surrogate LC (λ5 and VpreB), Igα and Igβ. Signalling through the pre-BCR triggers clonal expansion, allelic exclusion of the Ig HC and further differentiation (Martensson et al, 2007). At this point, cells no longer are dependent on IL7, which allows them to move away from the direct contact with stromal cells. After the initial clonal expansion, pre-B-cells stop dividing and become small pre-B-cells, coinciding with the silencing of the surrogate LC genes (Kurosaki, 2000).

The accessibility of the Igκ locus is enhanced after pre-BCR signalling, allowing its recombination. The IgM BCR can finally be synthetized and expressed on the cell surface (Geier

& Schlissel, 2006). These cells have now a BCR and are called immature B-cells, which will undergo positive and negative selection before exiting the BM to the blood stream.

Positive selection in B-cell lymphopoiesis is regulated by CD19. CD19 is a membrane bound molecule that is recruited to BCR clusters following antigen recognition and is known to amplify the BCR signal. CD19 was shown to be essential for early B-cell activation following ligand stimulation (Depoil et al, 2008). CD19 lowers BCR threshold activation in association with both CD21 and CD81; reviewed in (Tedder et al, 1997). CD19 expression starts at the pro-B-cell stage and a 2,5 fold increase in its expression is correlated with the transition from immature B220^{low} B-cell to a mature B220^{high} B-cell in mouse BM (Sato et al, 1996). In the absence of CD19 RAG proteins are not supressed, continuing to undergo receptor editing, failing to overcome positive selection (Diamant et al, 2005).

Haematopoietic differentiation and proliferation relies strongly on cell division. These cells possess unique systems of both promoting and arresting cell cycle to control homeostasis. Mature T and B cells have a remarkable division potential after antigen stimulation. This potential to react and proliferate is one of the most important aspects of the immune responses. Both hematopoietic and mature blood cells require therefore a strict control of their cell cycle checkpoints and the ability to quickly alternate between cell cycle arrest to rapid proliferation and back again.

Cyclins in cell cycle checkpoints

To understand cell cycle origins one has to go back in time, maybe not as far as 1665 when Robert Hooke first termed “cell” apparently because it reminded him of a monk’s tiny walled compartment as revealed in the book “the man who knew too much” (Inwood, 2002) but to Walther Flemming’s publications in 1882 “cell substance, nucleus and cell division”. Flemming was the first to describe cell division in animals and thus coined the term Mitosis (Paweletz, 2001).

In 1953, coinciding with the year of Watson and Crick’s discovery of the structure of the DNA, came another great discovery that set the model through which we see cell cycle phases today. Howard and Pelc divided cell cycle occurring in onion root-cells into four phases with distinct lengths after finding that DNA duplication occurred only in a period of time that corresponded to the middle of the cell cycle. G1 gap phase during which the cell prepares for

the DNA replication, S phase was the phase where DNA replication occurred and G2 is the second gap phase during which the cell prepares for the division process that comes in the mitosis (M) phase. Thus was born the concept of cell cycle (Howard & Pelec, 1953). One year later, Lajtha showed the same results in an *in vitro* study of human BM cells (Lajtha et al, 1954).

The next great challenge that scientist set out to unravel were the events which occurred at each of these phases of the cell cycle.

A couple of experiments cannot go unmentioned here. The first is Pardee's discovery of the feedback mechanism by which *E. coli* bacteria was able to control the biosynthesis of no more pyrimidines than necessary for growth. The end product, pyrimidine, would decrease the production of the first enzymes of the pathway. This ground-breaking discovery was published in two back to back papers in the journal of biological chemistry (Pardee & Yates, 1956a; Pardee & Yates, 1956b). The second experiment was the famous PaJaMo experiment in which, the three scientists, Pardee, Jacob and Monod, whose junction of the two first letters of the name composed the word PaJaMo, laid down the foundations that would lead to the discovery of mRNA. Inserting a plasmid from a "male" donor bacteria to a "female" recipient bacteria via conjugation, the scientists hoped to rescue the female bacteria, a mutant *E. coli* incapable of producing β-galactosidase. The remarkable discovery that the rescue was almost immediate, within a minute after gene transfer, not only showed that the transfer of molecular information from gene to protein was very quick, but also that the initial constitutive production of the enzyme was possible because the conditions within the recipient bacteria were sufficient for its production. However, in order for the enzyme production to continue an inducer of β-galoctosidase was indispensable (Pardee et al, 1959). This experiment provided the first insights into the mechanisms behind gene expression control. Both the Pardee's feedback mechanism and the PaJaMo experiment contributed to François Jacob and Jacques Monod great discovery, in 1961, of the operon theory, a model that explains the mechanism which controls the readout of the genes in prokaryotic cells, reviewed in (Yaniv, 2011).

The PaJaMo experiments had yet another great impact in molecular science, as it was one of the foundations for the discovery of messenger RNA (mRNA). The almost immediate expression of the enzyme following conjugation generated a need to explain the link between DNA from the Z^+ gene and its protein product β-Galactosidase. It was Francois Gros that in 1961 stepped up to fill the gaps between these experiments and several others that suggested

a RNA intermediate between DNA and protein production, coining the term mRNA (Gros et al, 1961).

By the late 1960's it was known that growth factors were necessary for cells in G1 phase to transit to the S phase of the cell cycle. In 1974, Arthur Pardee contributed once again with a major finding to cell cycle. He postulated the existence of a restriction point (R point) in the G1 phase that represented commitment to full DNA replication, a point where growth factors would no longer be required for the cell cycle to complete (Pardee, 1974). The molecular mechanisms underlying the R-point switch remain unconcluded. The R point was thus the first landmark within G1 phase to map if cells switched between the quiescence state and the proliferative state at one or several points in the cell cycle.

The understanding of how eukaryotic cell cycle is regulated began with studies in saccharomyces mutants. These mutants were found to have distinct-cellular and nuclear morphologies. The remarkable discovery that forever changed how we see cell cycle was that a mutation in a particular gene reproduced consistently the same morphology (Hartwell et al, 1973). Then started a step by step mapping of the cell cycle in an attempt to understand which gene preceded which on the regulation of the eukaryotic cell cycle. Perhaps the most remarkable of these findings was Paul Nurse's discovery of a network of genes that regulated entry into mitosis, whose main component is the cyclin-dependent kinase 1 (Cdk1), previously known as Cdc2 (Nurse, 1975).

Another milestone of eukaryotic cell cycle was undoubtedly Tim Hunt's discovery of cyclins in 1983. In sea urchin eggs, stored maternal mRNA is maintained inactive until fertilization, by mechanisms that were back then unknown. In an attempt to figure this out, Hunt and Evans looked into the pattern of protein synthesis after fertilization. They found a pattern of proteins that where highly synthetized, then virtually disappeared during cell division, only to reappear periodically, hence the name "cyclin" (Evans et al, 1983).

The general idea might seem straightforward nowadays, but one has to go back to a time when no one dared to think of proteolysis as a tool to control cell cycle, to understand why the first submission of Tim Hunt's paper on cell resulted in the following reviewer's comment "This is wild speculation, based on faulty logic".

Following the original discovery of cyclins, A and B (Evans et al, 1983) that associated to a single kinase subunit, Cdk1 (Nurse, 1975), and undergo proteolysis mediate by ubiquitin to exit mitosis (Glotzer et al, 1991), remarkable progress was made unfolding a vast array of different

cyclins which regulate different Cdks activity in the control of cell-cycle progression. The yeast model along with the yeast genome project has been crucial in identifying different cyclins (Andrews & Measday, 1998).

Resulting from the major accumulation of knowledge over the last few decades came a generally accepted notion of mammalian cell cycle which I will try to sum up before focusing in the main theme of the thesis, the D cyclins.

Quiescent-cells are currently referred to as being in a G0 phase. This is the only phase added to Howard and Pelc first division of the cell cycle and it can also be considered as an extended G1 phase, illustrating how accurate their prediction was. Not all cells enter the G0 phase and when they do, it is a direct consequence of absence of growth factors or nutrients.

The cell cycle begins in G1 phase, when the cell analyses extracellular stimulus and decides whether to commit to divide or to become quiescent. From the moment the decision is made, the so called restriction point (R point) (Pardee, 1974), the cell cycle will only be dependent on intracellular events.

The passage through the stages of the cell cycle is controlled by sequential activation and inactivation of Cdks. The activation Cdks, as the name implies (cyclin dependent) is mediated by the temporal expression and binding of cyclins. The Cdk/cyclin complex is further regulated by the transient binding of Cdk inhibitors (Cdkis).

In the G1 phase, the D cyclin family provides this link between mitogens and the core cell machinery. The presence of adequate nutrient levels and growth factors triggers a signal transduction pathway that results in the expression of D cyclins. The D cyclins form active holoenzyme complexes with Cdk4 and Cdk6, which are capable of phosphorylating the pocket protein family members, Rb, Rb-like protein 1 (p107) and Rb-like protein 2 (p130) (Sherr & Roberts, 2004). The Rb protein is a potent inhibitor of cell proliferation that unlike cyclins isn’t marked for degradation at different steps of the cell cycle. Instead, the Rb is a stable protein controlled by phosphorylating and dephosphorylating events that effect its molecular conformation, determining its active and inactive states. This type of regulation allows for a very flexible and complex array of conformational changes on particular cellular contexts. Different phosphorylation profiles result in different affinities for the substrates which ultimately translate into the so called active and inactive states. The Rb protein influences several processes of cell growth and differentiation through transcription regulation (Zhang et al, 2012). The role it plays in transcription during the G1/S phase transition of the cell cycle is

the most documented. The description of changes underwent by retinoblastoma during G1/S transition were first described in 1989 (Buchkovich et al, 1989; Chen et al, 1989), posterior to its implication, through mutational inactivation, with a genetically predisposed eye tumour which gave it the name retinoblastoma. Back then retinoblastoma was a prototype biological model for the study of recessive oncogenes and the recessive mutation was assigned to the q14 band of human chromosome 13 (Friend et al, 1986). Since this discovery, two other homologous proteins were discovered in humans and mice, p107 and p130 constituting along with Rb, the pocket protein family (Baldi et al, 1996; Cobrinik et al, 1993; Mayol et al, 1993).

In the hypophosphorylated form, Rb, p107 and p130 have a high affinity for E2F transcription factor family members as well as the chromatin remodelling complexes SWI/SNF, Sin3B and histone deacetylase 1 (HDAC1) (Gunawardena et al, 2007; Zhang et al, 2000). The binding of Rb, p107 or p130 to these proteins results in transcriptional repression and an arrest in G0/G1 phase of the cell cycle, which accounts for the tumour suppression activity of the pocket proteins (David et al, 2008). There are six members of the E2F family of TF that have different affinities for each of the pocket protein family (Dyson, 1998). Both p107 and p130 bind to chromatin-bound E2F at gene promoters to exert their repressive function, while Rb only binds E2F transcription domain in the nucleoplasm (Stengel et al, 2009).

The transcriptional repression is released, in response to mitogen signalling, by activated cyclin D/Cdk complexes that hyperphosphorylate the Rb proteins. hyperphosphorylate Rb proteins lose their growth suppressive abilities, by conformational changes, resulting in the release of E2F transcription factor family members and associated chromatin remodelling complexes, which are responsible for the expression of several genes required in G1 for cell cycle progression (Stengel et al, 2009; Weinberg, 1995). The Rb Cdk dependent phosphorylation sites that result in conformational changes which release the transcription domain of E2f have been thoroughly studied and two phosphorylation sites that result in two diferent conformational changes have been identified: (i) S608 phosphorylation causes a flexible large loop within the pocket domain (RbPL) to bind to the pocket domain mimicking and competing with E2F transactivation domain. (ii) T373 phosphorylation induces a global conformational change by interdomain association, which allosterically inhibits E2F transactivation domain binding.

The cyclin D/Cdk phosphorylation function is inhibited by the INK4 family of Cdk inhibitors. The INK4 family members specifically bind to Cdk4 and Cdk6, therefore preventing the binding of the D cyclins to their Cdk4 partners. Cyclin D-Cdk4/6 active holoenzyme complex requires

binding the Cdk2 inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ proteins. These proteins contain a nuclear localization signal (NLS) that might explain how cyclinD1/Cdk4 migrate to the nucleus to phosphorylate the Rb protein (Sherr & Roberts, 1999). The association of cyclin D to $p27^{Kip1}$ and $p21^{Cip1}$ will therefore determine the levels of Cdk2, freed from its repressors, available in the cell (Cheng et al, 1999).

As Cdk2 is released from its inhibitor, it can complex with cyclin E, whose expression is upregulated by E2F family transcription factors (Geng et al, 1996). Cyclin E/Cdk2 complex further phosphorylates the Rb proteins on additional sites (Zarkowska & Mittnacht, 1997) leading to their complete inactivation (Harbour et al, 1999; Lundberg & Weinberg, 1998). Hyper-phosphorylated the Rb proteins releases more effectively E2F transcription factors, promoting the expression of genes that drive initiation and progression of the S-phase as well as providing a positive feedback loop for their continued expression (Geng et al, 1996). Of note, E2F transcription factors are responsible for the upregulation of both cyclin E and cyclin A family members. As cyclin E, cyclin A also binds to Cdk2 and together with cyclin E/Cdk2 complexes, they maintain the levels of $p27^{Kip1}$ low, through phosphorylation-triggered proteolysis (Malek et al, 2001).

In addition to Rb protein, cyclin E/Cdk2 complexes also phosphorylate several other targets, including (i) $p27^{Kip1}$ and $p21^{Cip1}$ proteins, marking them for ubiquitin mediated proteolysis (Bornstein et al, 2003); (ii) Smad3 protein, a signal transducer of transforming growth factor-β (TGF-β) family signalling which is known to inhibit-cell cycle progression (Matsuura et al, 2004) and cyclin E expression (Geng et al, 1996); and (iii) $p220^{NPAT}$, which together with E2F transcription factors, is responsible for the tenfold increase of histone mRNA in S phase, when compared to basal G1 phase (Zhao et al, 2000).

In conclusion, through G1-phase, the cell switches from a mitogen-dependent to a mitogen independent state and commitment to divide is established. While initiation of G1-phase requires the activation of cyclin D-Cdk4/6 complexes, progression through G1-phase and transition to S-phase requires the assembly and activation of Cdk2/ cyclin E complexes.

At this point in the G1 phase the cell manages sufficient Cdk2 activation and the inactivation of both Cdk2 repressors and Rb proteins, switching the cell from a mitogen-dependent state to a mitogen independent state, no longer relying on cyclin D/Cdk4 or 6 complexes, which suggests that cyclin E is the major downstream target of cyclin D. This hypothesis is supported by Geng et al mouse model, where cyclin E was knocked into *cyclin D1* (*CCND1*) locus and rescued cyclin

D1 mouse deficiency (Geng et al, 1999). The mechanisms behind Pardee's 1974 famous R point theory have apparently been unfolded. However, one has to consider other possibilities: with the exception of *cyclin E*, no other cyclin has been knocked into *CCDN1* locus to assert if a different phenotype would manifest. It is possible that this data limits to demonstrate the redundancy of different cyclins in the cell cycle.

Cyclin E as an abrupt decay in early S phase following ubiquitin mediated degradation by the ubiquitin protein ligase complex SCF^{Fbw7} pathway. Both Cdk2 and glycogen synthase kinase 3 (GSK-3) are required to phosphorylate cyclin E to mark it for proteasomal degradation (Welcker et al, 2003).

Cyclin A expression starts during late G1 phase and when bound to Cdk2, it is responsible for the phosphorylation of numerous substrates from the preassembled replication initiation complexes, inducing the beginning of the DNA replication during the S phase once it has accumulated to a certain level (Coverley et al, 2002).

Other than its role in DNA synthesis, cyclin A/Cdk2 holoenzyme is also known to regulate the activation of the cell division cycle protein 25 family (Cdc25) (Mitra & Enders, 2004). Cdc25 is a family of phosphatases which have a dual specificity since they are able to act on both tyrosine and serine/threonine residues. The Cdc25 family, in both humans and mice, is composed of three members: Cdc25A, Cdc25B and Cdc25C (Kakizuka et al, 1992; Sadhu et al, 1990). Besides, these three genes are able to generate alternative splicing isoforms. In humans, Cdc25A has two variants and both Cdc25B and Cdc25C have 5 variants each. These variants and isoforms play distinct roles on different stages of the cell cycle and have distinct subcellular compartmentalisations. Cdc25A is located in the nucleus through the cell cycle, while Cdc25B and Cdc25C alternate their location in and out of the nucleus throughout the interphase (Ferguson et al, 2005). Cdc25A is phosphorylated and its phosphatase ability is activated on the transition from G1 phase to S phase in a Cdk2/cyclin E dependent way. Cdc25A in turn generates an auto-amplification loop by dephosphorylating and activating Cdk2 (Hoffmann et al, 1994). The promoter of *Cdc25A* gene is activated by STAT3 in cooperation with Myc (Barre et al, 2005). Cdc25B and Cdc25C are phosphorylated and their phosphatase capacities are activated by Cdk1/cyclin B complexes in the G2/M phase transition. These proteins also generate a positive feedback loop by dephosphorylating the Cdk1 inhibitor, p34cdc2, activating the kinase ability of the Cdk1/cyclin B complexes (Ferguson et al, 2005; Hoffmann et al, 1994). In HeLa cells, a cell line isolated from human cervical cancer cells, Cdc25B is expressed at low levels during interphase and its expression, on the cytoplasm, peaks during

the prophase. This peak in expression was correlated to the triggering of centrosomal microtubule nucleation, essential for a successful mitosis, while Cdc25C expression is maximal during interphase, where it is suggested to regulate G2/M events (Gabrielli et al, 1996). The activation of Cdk1 complex by Cdc25B or Cdc25C is made by dephosphorylating two residues on the catalytic pocket of Cdk1. Before Cdc25 mediated activation, Cdk1 is kept in an inactive form by the kinases Wee1 and Myt1, which are responsible for the phosphorylation of the above mentioned residues on the catalytic pocket, preventing immature initiation of mitosis by disabling ATP binding and catalytic activity of Cdk1 (Ferrell et al, 2009; Mueller et al, 1995). Cyclin B binds to Cdk1 to form an active holoenzyme, whose role in overcoming G2 and committing to mitosis gave it the name Maturation-promoting factor (MPF). Cyclin binding of Cdk1 is known to facilitate its phosphorylation activating its kinase activity (Solomon et al, 1992).

The MPF factor is closely regulated both by positive and negative feedback mechanisms. On one hand, MPF not only stimulates its own production, but also activates Cdc25B and Cdc25C, which in turn activate the preMPF complex, which is MPF with inactive Cdk1 subunits. On the other hand, MPF indirectly induces its own destruction by promoting the degradation of cyclin B, via the ubiquitin pathway, inactivating the Cdk1 subunit (Novak & Tyson, 1993). These regulatory events ensure that when a critical threshold of MFP is reached, the cell proceeds through mitosis. In the end of mitosis, the MPF is rapidly inactivated, reverting to a state where virtually no cyclin B is detected and Cdk1 is inactivated. To allow mitosis progression, cyclin A also has to be degraded during the prometaphase. Although the mechanisms leading to cyclin A degradation remain largely elusive, some recent findings have highlighted the acetylation patterns of the N-terminus region, which were shown to condition ubiquitin mediated degradation of cyclin A (Mateo et al, 2010).

The variation of the expression of the different members of Cdc25 family is associated to checkpoint bypasses and genetic instability (Lavecchia et al, 2010). Overexpression of Cdc25A and Cdc25B variants are observed on several human cancers, most important of which are primary breast tumours, where it is associated with poor survival rates (Cangi et al, 2000). Cdc25A and Cdc25B phosphatase inhibitors have been developed and are being used in clinical trials with promising results (Lavecchia et al, 2010).

This overview of the cell cycle is ultimately a very simplified model. There are many substrates for the different cyclin/Cdk complexes already described and probably many others still unknown. Also, studies with knockout mice reveal that the absence of an important Cdks or

cyclins might be compensated or somehow overcome (Malumbres & Barbacid, 2009). In the study of lymphopoiesis, the cyclins responsible for the initiation of the cell cycle have a prominent role and for that reason, D cyclins will be further depicted in this introduction.

The D cyclins Family

D cyclins, along with their associated kinases, are the first regulators of the cell cycle. Cyclin D-Cdk4/6 complexes are responsible for the translation of the extracellular mitogen signals to the to the cell machinery, promoting G1 phase progression and prime cells for DNA replication on the S phase via the Rb protein pathway.

The cyclin D family is composed of three highly homologous members in both human and mice. These three members are cyclin D1, the first to be discovered (Motokura et al, 1991), cyclin D2 and cyclin D3 (Xiong et al, 1992). All three members have equal ability to phosphorylate the Rb protein and are thought to be redundant. This substrate-specific phosphorylation was reported to be dependent on a unique site located within the B domain of Rb protein that binds to the LXCXE motif found in D type cyclins. Mutation analysis of this motif in D cyclins were reported to lead to the loss of cyclin D/Cdk4/Rb complexes *in vivo* (Dowdy et al, 1993; Ewen et al, 1993). However, contradictory results were obtained *in vitro*. It was suggested that in the absence of the B domain, the Rb protein can be phosphorylated in a C domain by cyclin D1/Cdk4 complex independently of the LXCXE motif (Grafstrom et al, 1999; Pan et al, 2001; Pan et al, 1998).

More recently another group has found that mice lacking the LXCXE motif in cyclin D1 don't share any of the abnormalities of cyclin D1 knock-out mice in growth, retinal development or mammary gland development (Landis et al, 2007).

CCND1 shares 62% and 51% similarity with *cyclin D2* (*CCND2*) and *cyclin D3* (*CCND3*), respectively. *CCND2* and *CCND3* share among themselves 62% similarity (Musgrove et al, 2011). The highest homology between the three D cyclins is on a sequence motif termed cyclin box, reaching over 70%. This cyclin box is known to mediate Cdk and CKI binding and is conserved among different species (Nugent et al, 1991).

Of note, in human but not in mouse, there are two isoforms of cyclin D1. The canonical cyclin D1 is a 295 a.a. protein, whereas the second isoform is a smaller protein of 274 a.a., named

cyclin D1b. This shorter version results from alternative splicing, which generates a protein where the first 240 a.a. are similar to the canonical cyclin D1, but with a different C-terminal region. The carboxy-terminal region of cyclin D1 is known to code for the PEST domain, responsible for the ubiquitin mediated degradation of the protein that is not present on cyclin D1b (Knudsen et al, 2006). The PEST domain is rich in proline, glutamine, serine and threonine, a trade mark of proteins with rapid turnover. The threonine residue near the C terminus (T286 in cyclin D1) targets the molecule for ubiquitin mediated degradation (Alt et al, 2000).

Mutation analysis of this PEST domain in cyclin D1 revealed additional insight. T286A mutant is stable and constitutively located in the nucleus for it cannot be marqued, via phosphorylation of T286, for ubiquitin mediated degradation. K112E mutant has impaired ability to under certain condition bind Cdk4 and sequester $p27^{Kip1}$ and $p21^{Cip1}$ proteins (Musgrove et al, 2011).

Despite all the homologies between these family members, there is also a motif that is restricted to cyclin D1 that might explain some of its unique functions. It is a LLXXXL motif able to bind a LXXLL motif, present in steroid receptor co-activators (SRCs).

The homology of the human D cyclin family domains as well as its ligand affinity is resumed on figure 8.

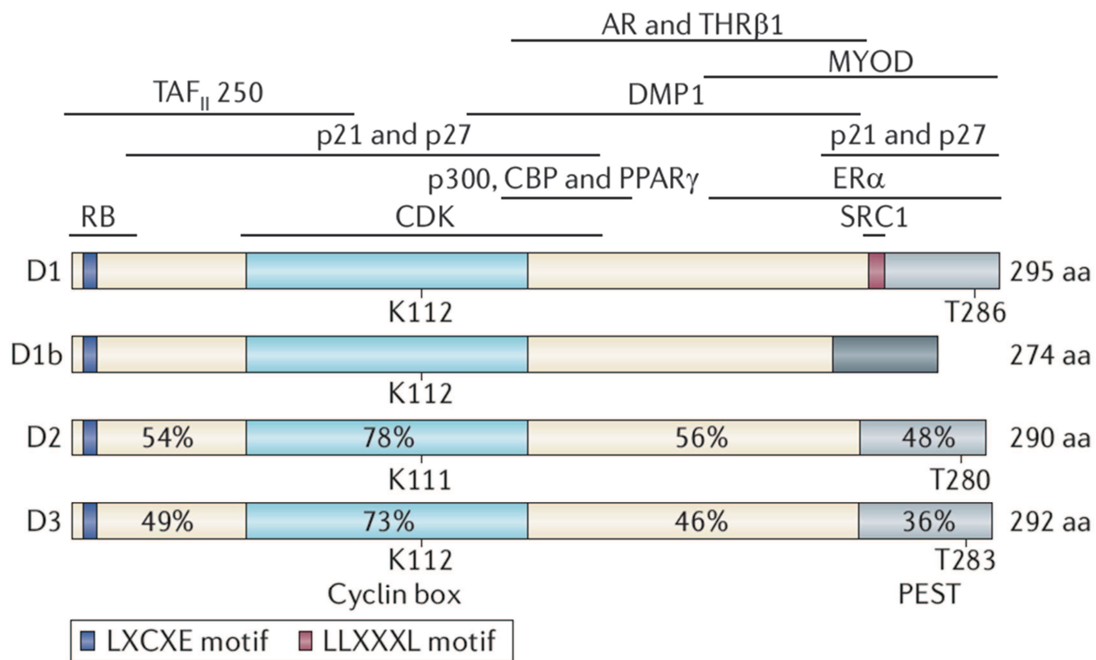


Figure 8: D cyclin family homology and binding domains.

The Human D cyclin family, D1, D1b, D2 and D3. Cyclin box domain is highlighted in blue and the PEST domain is highlighted in grey. Also highlighted are the LXCXE and the LLXXXL motifs. The cyclin box and the PEST domain along with the regions in between have a percentage written in them, this is the homology relative to cyclin D1.The cyclin length in a.a. is displayed on the right and the position of each domain is accurately placed according to the sequence length. Both lysine (K112 or 111) and threonine (T286, 280 or 283) are also highlighted for their relevance in mutation analysis previously stated. Between the cyclin box and the PEST domains, there is a poorly conserved region which in cyclin D1 is known to interact with transcription factors. On the top of the image there are the known interaction domains whose location is indicated by a line. These binding sites are refered to cyclin D1 only since the majority of the literature focus only on this cyclin. Some of the interaction donains that have been well documented on the other D cyclins is the P21 and P27 shared by all D cyclins and the AR and PPARγ shared by cyclin D3. Image from (Musgrove et al, 2011).

Legend of the binding domains of cyclin D1: RB – retinoblastoma protein; TAF_{II} 250 - transcription factor II B; P21 and P27 - Cdk2 inhibitors $p21^{Cip1}$ and $p27^{Kip1}$; p300 – CREB-binding protein co-activator; CBP –CREB-binding protein; PPARγ - Peroxisome proliferator-activated receptor γ; DMP1 - Dentin matrix acidic phosphoprotein 1; AR - androgen receptor; THRβ1 - thyroid hormone receptor β 1; MyoD - myogenic differentiation factor D; ERα – estrogen receptor α; SRC1 - steroid receptor co-activator 1.

As mentioned above in the T CD8 differentiation chapter, the faster proliferation rates correlated to a shorter G1 phase (Yoon et al, 2010). This observation is not only important for CD8 T-cells but to all mammalian cells, as G1 is thought to be a major control step for cell proliferation.

D cyclins bind Cdk4 (Matsushime et al, 1992) and Cdk6 (Meyerson & Harlow, 1994) and are capable of *in vivo* phosphorylating the Rb protein. Activation of both Cdk4 and Cdk6 occurs during mid-G1 phase, prior to Cdk2 activation. Interestingly, cyclin D2 and D3 but not D1 were shown to complex *in vitro* with Cdk2 (Ewen et al, 1993) which normally complexes with cyclins E and A. To date, the holoenzymes cyclin D/Cdk4 or cyclin D/Cdk6 are the only cyclin D complexes known to phosphorylate and functionally inactivate the Rb protein and the Rb related pocket proteins p107 and p130.

Cyclin D1/Cdk4 or 6 holozyme complex is inactivated by Cdk-inhibitors (CKIs) of the INK4 family. This family is composed of four proteins, p16INK4a (Serrano et al, 1993). p15INK4b (Hannon & Beach, 1994), p18INK4c (Guan et al, 1994; Hirai et al, 1995) and p19INK4d (Chan et al, 1995; Hirai et al, 1995), all of which are composed of multiple ankyrin repeats that bind specifically to Cdk4 and 6 and not to other Cdks. The induction of INK4 promotes Rb dependent-cell cycle arrest in some conditions, such as in senescent-cells (Gil & Peters, 2006).

D cyclins, unlike other cyclins, are synthetized as long as growth factors stimulation persists. During cell cycle they oscillate only moderately, peaking near G1 to S phase transition. When mitogens are withdrawn, they are rapidly degraded independently of the cell cycle phase. As mentioned, the deprivation of growth factors before overcoming the R point results in failure to enter S phase while it seems to have no effect on cell cycle afterwards (Sherr, 1994).

The three members of the D cyclin family share their function of phosphorylating retinoblastoma. However, they are believed to be dominantly expressed in different lineages (Matsushime et al, 1991; Pagano & Jackson, 2004; Sherr & Roberts, 2004). For example, cyclin D1 expression has been described *in vitro* to be high in undifferentiated multipotent-cells of embryonic origin and both cyclin D2 and D3 expression is upregulated upon differentiation (Bryja et al, 2008). The differential expression of D2/D3 cyclins was also linked to B-cell development and although several links have been made, it remains largely speculative (Pokrovskaja et al, 1996). What is well known, and was confirmed in this study, is that cyclin D1 expression in mouse is higher in some lymphocyte precursor cells and is lower in mature lymphocyte subsets. In humans, cyclin D1 has the most restricted pattern of expression while cyclin D2 exhibits the most whispered expression in a variety of haematopoietic and non-haematopoietic tissues (Cheshier et al, 1999; Metcalf et al, 2010).

Cyclin D1

Within the D cyclin family, cyclin D1 was the first described to link growth factor signalling to the cell machinery, back in 1991 when they were still called cyclin-like (CYL) proteins in the mouse (Matsushime et al, 1991). Cyclin D1 expression is rapidly induced at the beginning of the cell cycle via the ras GTPase signalling. Ras protein transmits mitogenic signals from the cell surface receptors to the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, a protein kinase cascade (Marshall, 1995). This pathway stimulates the expression of AP-1 proteins. AP-1 DNA binding proteins are homodimers and heterodimers constituted from basic region-leucine zipper (bZIP) proteins from several transcription factor subfamilies, i.e. Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun (c-Jun, JunB and JunD), Maf (c-Maf, MafA, MafB, MafG/F/K and Nrl) and ATF (ATF2, ATF3, B-ATF, JDP1 and JDP2). The AP-1 homo or heterodimers recognize either 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-response element (TRE) or a cAMP-response element to assert their transcription regulation role. The AP-1 proteins belonging to the Jun group have been reported to have a strong role in the transcription regulation of *CCND1*. c-Jun is a strong inducer of *CCND1* transcription and conversely, JunB is known to repress *CCND1* transcription (Shaulian & Karin, 2001; Shaulian & Karin, 2002). c-Jun is able to bind *CCND1* promoter inducing its expression not only through the above mentioned response elements but also through the E2F-responsive site. c-Jun plays and important role in both the expression of E2F1 and E2F2 that bind E2F response elements on *CCND1* promoter, and on the expression of their dimerization partners, DP1. E2F family of TF and DP1 play an important role not only on the expression of *CCND1* but also several other cell cycle genes containing E2F-responsive sites (Shen et al, 2008). Beside c-Jun, c-Fos also plays a major role in regulating *CCND1* promoter. Unlike Jun members who can either homo or heterodimerize, Fos members are only able to form heterodimers with Jun members (Shen et al, 2008). Another AP-1 member with crucial importance in the regulation of *CCND1* Promoter is ATF-2. ATF-2 has been described to be essential to bind the c-AMP response element on *CCND1* promoter, either in the homodimer form or hetorodimerized with c-Jun, where it induces *CCND1* expression following stimulation by estrogen, hepatocyte growth factor/scatter factor (HGF/SF) or regenerating gene product (Reg), all of which are able to phosphorylate ATF-2, inducing its activity (Castro-Rivera et al, 2001; Recio & Merlino, 2002; Sabbah et al, 1999; Takasawa et al, 2006).

Besides *CCND1* transcriptional control by stimulation of AP-1 proteins, Ras activation is also capable of increasing *CCND1* expression via formation of a complex on the Sp1 response

element on the promoter region of *CCND1*. This complex is formed by Sp1, nuclear factor-κβ (p50) of the NF-κβ family and p107 following the activation by nerve growth factor (NGF) (Marampon et al, 2008). Sp1 is also able to activate the promoter of *CCND1* via binding to B-Myb and complexing with it also on the Sp1 response element of the promoter region. This mechanism is active at the G1 phase, before cyclin A/Cdk2 phosphorylation of B-Myb. B-Myb also controls cyclin A1 expression by binding a Sp1 response element (Bartusel et al, 2005).

Rac 1, a small GTPase in the heregulin beta 1 (HRG) mitogenic signalling in known to be involved breast tumourigenesis via the MEK/ERK pathway following activation by ErbB2, ErbB2, EGFR and PI3K (Yang et al, 2006a). Rac-1 activation is also responsible for inducing *CCND1* expression and $p21^{Cip1}$ which can account for the effect of HRG on promoting cancer cell proliferation. HRG also activates p50 that similarly is responsible for *CCND1* activation (described above) (Yang et al, 2008). It has also been suggested that Rac mediated induction of *CCND1* requires activation of the NF-κβ pathway, while ERK induction of *CCND1* is independent (Klein et al, 2007).

Certain cytokines are also known to stimulate *CCND1* expression through the JAK-STAT signalling pathway. Upon cytokine receptor signalling, STAT TF are translocated to the nucleus. CCND1 is known to be activated by IL3 and IL6 via STAT3 and STAT5 (Leslie et al, 2006; Matsumura et al, 1999; Mishra & Das, 2005).

The promoter region of mouse *CCND1* was cloned and sequenced (GenBank accession number AF212040). This promoter contains a TRE, a cAMP-response element and a NF-κB response element (Eto, 2000). These three response elements, as well as the remaining structure are fairly similar in mouse, human and rat. Also, they lack the canonical TATA-box or TATA like sequences. In humans, the initiation sequence (Inr) is similar to that of the adenovirus major late promoter and has been first described as starting +251 upstream of the ATG (Philipp et al, 1994). A Inr-like element was also described on both mouse and rat (Eto, 2000).

The only major differences between the human promoter region and that of mouse and rat are the presence of: (i) a butyrate response element (BRE); (ii) an enhancer element or serum response element (SRE); (iii) and of a second E2F binding motif. Of note, in mouse there is only one Sp1 motif directly upstream the Inr, while there are two in both rat and humans (Eto, 2000).

The promoter region of cyclin D1 in mouse, rat and human is illustrated on figure 9 A:

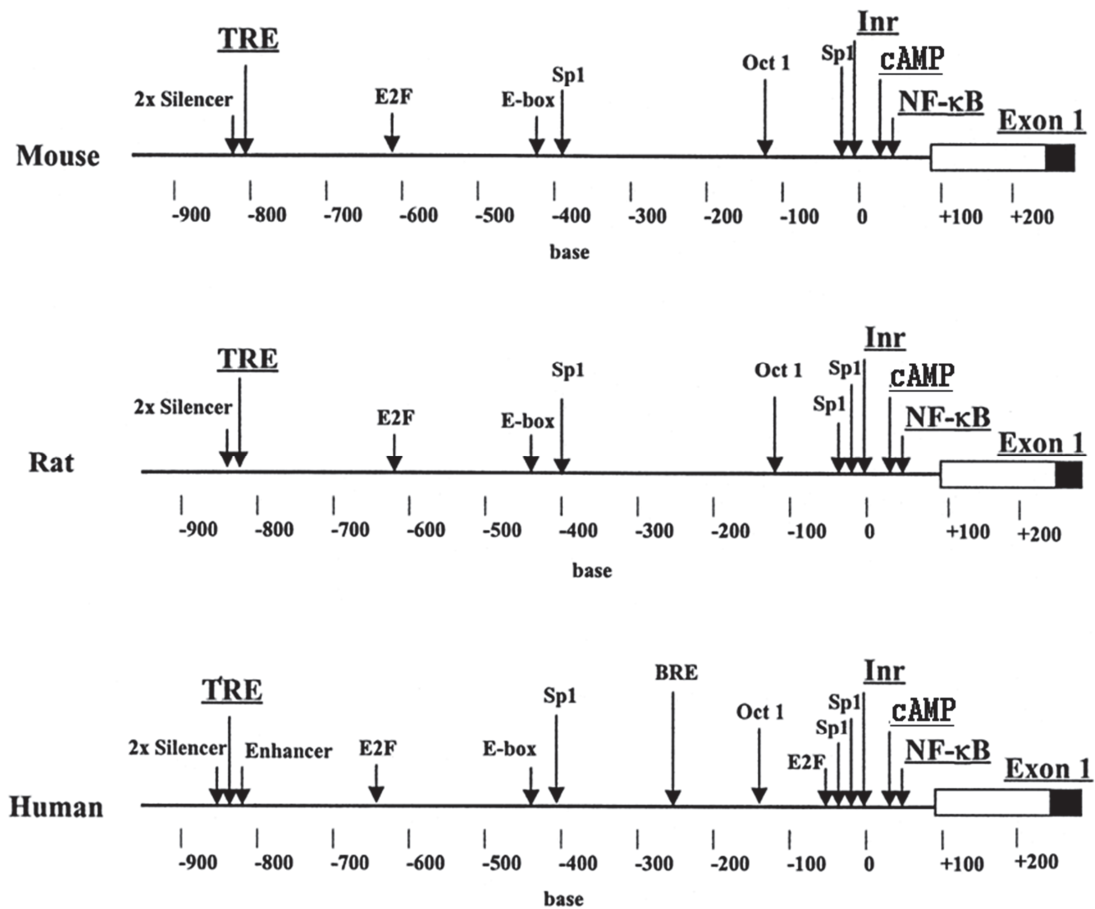


Figure 9 A: Cyclin D1 promoter region on mouse, rat and human.

The position +1 is assigned to a putative initiator (Inr) region on the human cyclin D1. Legend of the abbreviations: NF-κB - NF-κB response element; cAMP – cAMP response element; TRE – TPA response element (AP-1 binding site); BRE – butyrate response element; Enhancer – enhancer element or serum response element. There are also binding sites for E2F, E-box and Sp1. Image adapted from (Eto, 2000).

Cyclin D1 transcription regulation has been described in great detail to better understand its role in both cell cycle initiation and oncogenic transformation. Based on recent advances and bioinformatics tools, a new schematic representation of CCND1 promoter as well as a representation of the complex networks of its regulation was made with greater detail and can be seen on figure 9 B.

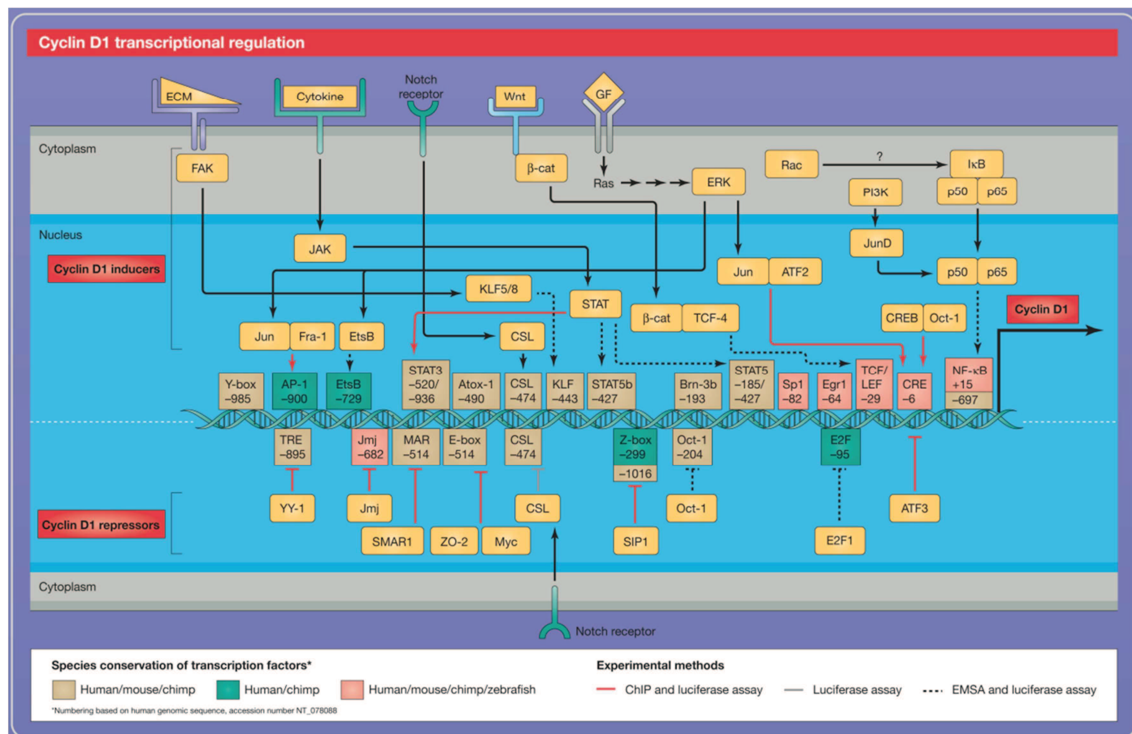


Figure 9 B: Cyclin D1 promoter region on mouse, chimp, zebrafish and human.

In this image, some new response elements of the CCND1 promoter have been added and also the complex network of activators and repressors are represented on a simple diagram with different colour for the different organisms and methods by witch they were detected. It is visible that on different conditions. Legend of the abbreviation: ATF – activating transcription factor; β-cat – β-catenin; Brn-3b – brain-specific homeobox/POU domain protein 3b; ChIP – chromatin immunoprecipitation; CSL – CBF-1/suppressor of hairless/LAG-1; CRE – cyclic AMP-response element; CREB – CRE-binding protein; ECM – extracellular matrix; Egr1 – early growth response protein 1; EMSA – electrophoretic mobility shift assay; ERK – extracellular signal-regulated kinase; FAK – focal adhesion kinase; GF – growth factor; JAK – janus-activated kinase; Jmj – jumonji; KLF – Kruppel-like factor; LEF – lymphoid enhancer-binding factor; MAR – matrix attachment region; NF-κB – nuclear factor-κB; PI3K – phosphoinositol 3-kinase; SIP1 – SMADinteracting protein 1; SMAR1 – scaffold matrix-associated region-1; STAT – signal transducers and activators of transcription; TCF – ternary complex factor; YY-1 – yin and yang 1; ZO-2 – zona occludens-2. Image from (Klein & Assoian, 2008)

Cyclin D1 protein has a very rapid turnover and its half-life has been determined to be under 30 minutes (Diehl et al, 1998; Diehl et al, 1997). While its transcription is assured as long as mitogenic signals are available, it has been argued that even in *in vitro* models, during the S phase cyclin D1 must be degraded, marking the passage of one cell cycle to the other. Afterwards, the rapid accumulation of cyclin D1 in the G2 phase booth commits and speeds the entry on the second cell cycle, but the forced expression of cyclin D1 prolongs the duration of the S phase (Yang et al, 2006b). Nuclear export of cyclin D1 has been attributed to the phosphorylation of a C terminus residue Thr-286 by GSK-3β. The GSK-3β is excluded from the nucleus during G1 phase and gains access to the nucleus during the S phase (Diehl et al, 1998).

Both cyclin D1b isoform and cyclin D1-T286A mutant do not have the Thr-286 residue and therefore remain in the nucleus throughout the cell cycle. Although this might prolong the S phase as mentioned above, it promotes oncogenic transformation (Hao et al, 2011).

D1 cyclin and cancer

Historically, cyclin D1 was found in humans in an attempt to identify a putative Bcl1 oncogene located on human chromosome 11q13. This candidate oncogene, then named PRAD1, was suggested to be a cyclin, whose overexpression might play an important role in the development of various tumours with abnormalities in 11q13 (Motokura et al, 1991). At the same time, another group simultaneously identified cyclin D1 through its ability to complement G1 cyclin loss in *S. cervisae* (Xiong et al, 1991).

Since this time, several groups have studied the role of cyclin D1 as a human oncogene. Cyclin D1 locus is known to be amplified in specific tumour types. Figure 10 summarizes some of these known deregulations in cancer:

Mechanism of deregulation	Tumour type	Frequency
Amplification and overexpression		
CCND1 amplification	Head and neck squamous cell carcinoma	26–39%
Cyclin D1 overexpression	Head and neck squamous cell carcinoma	20–68%
CCND1 amplification	Non-small-cell lung cancer	5–30%
Cyclin D1 overexpression	Non-small-cell lung cancer	18–76%
CCND1 amplification	Endometrial cancer	26%
Cyclin D1 overexpression	Endometrial cancer	40–56%
CCND1 amplification	Melanoma	0–25%
Cyclin D1 overexpression	Melanoma	30–65%
CCND1 amplification	Pancreatic cancer	25%
Cyclin D1 overexpression	Pancreatic cancer	42–82%
CCND1 amplification	Breast cancer	15–20%
Cyclin D1 overexpression	Breast cancer	50–70%
CCND1 amplification	Colorectal cancer	2.5%
Cyclin D1 overexpression	Colorectal cancer	55%
Chromosomal rearrangement and overexpression		
CCND1: IGH translocation t(11;14)(q13;q32)	Mantle cell lymphoma	>90%
Cyclin D1 overexpression	Mantle cell lymphoma	>90%
CCND1: IGH translocation t(11;14)(q13;q32)	Multiple myeloma	16%
Cyclin D1 overexpression	Multiple myeloma	30–50%
Splice variants and transcript aberrations		
3' UTR rearrangements, microdeletions or point mutations	Mantle cell lymphoma	4–10%
Cyclin D1b overexpression	Breast cancer	22%*
Cyclin D1b overexpression	Prostate cancer	27%*
Mutations affecting nuclear export and proteolysis		
Cyclin D1 T286R; δ266–295	Oesophageal cancer	4%
Cyclin D1 P287S; P287T; δ289–292	Endometrial cancer	4%
FBXO4 S8R, S12L, P13S, L23Q, G30N and P76T	Oesophageal cancer	14%

Figure 10: Cyclin D1 implication on human cancer.

Cyclin D1 deregulation is believed to play an oncogenic role on several known tumours. This table resumes some of those tumour types, the mechanism by which cyclin D1 is deregulated and the frequency of each particular tumour type that has cyclin D1 deregulation compared to the total population of patients. Image adapted from (Musgrove et al, 2011)

Legend: FBXO4 - F-box 4; IGH - immunoglobulin heavy chain locus; UTR - untranslated region; *CCND1 – cyclin D1* gene.
*Cyclin D1b overexpression without overexpression of full-length cyclin D1.

The fact that Cyclin D1 is much more often implicated as an oncogene than other D cyclins, suggests that this cyclin may have unique functions. Indeed, cyclin D1 is thought to directly bind transcription factors or coactivators, including Histone acetyl transferases (HATs) and HDACs to regulate transcription and epigenetic modifications which play important roles in human cancer metabolism (Fu et al, 2004). Cyclin D1 but not cyclin D2 or cyclin D3 was also associated to other oncogenes such as *neu* and *ras* that act on *CCND1* promoter upregulating its expression on mice breast cancer models and are exclusively dependent on cyclin D1 for malignant transformations. Therefore Cyclin D1 connects the Neu-Ras pathway to the cell machinery (Yu et al, 2001).

However, despite all the evidence of cyclin D1 overexpression in different types of cancers, over-expression of the wild type cyclin D1 has failed so far to promote oncogenic transformation even in *in vitro* systems (Alt et al, 2000; Quelle et al, 1993; Resnitzky et al, 1994). It seems that the regulation of cyclin D1 availability in the nucleus is somehow maintained through temporal regulation of nuclear export. Indeed the T286 mutant, previously described as being constitutively located in the nucleus, is able to induce a transformed phenotype in cultures murine fibroblasts (Alt et al, 2000). The T286 mutant was never found in humans, but there is a spontaneous mutation of amino acids 266 to 295 of cyclin D1 that was detected on esophageal cancer. This mutant was also found to be constitutively in the nucleus (Hao et al, 2011). Furthermore, the splicing isoform of cyclin D1 which lacks exon 5, where T286 is located, has also been implicated in human cancers (Knudsen et al, 2006; Lu et al, 2003).

Cyclin D1 function on cancer cells is not limited to conventional retinoblastoma phosphorylation. Cyclin D1 has also been shown to play a role in the DNA network repair. By binding RAD51, a recombinase that assists in DNA repair of double strand breaks by homologous recombination (Jirawatnotai et al, 2012), Cyclin D1 helps its recruitment to DNA damage sites by binding BRCA2, another well-established component of homologues recombination. The reduction of Cyclin D1 leads to an increased susceptibility of cells to radiation both *in vitro* and *in vivo*, revealing that even on retinoblastoma negative cells, Cyclin D1 plays other important roles in cancer cells (Jirawatnotai et al, 2011). The association of cyclin D1 as a mediator of damaged DNA repair is at first glance contradictory to its association in cancers, since DNA repair prevents mismatches produced during DNA replication. The increased frequency of errors in target genes is often associated with carcinogenesis (Masuda et al, 2011). On the other hand, DNA repair ensures cancer cells to survive longer and to be more resistant to genotoxic therapy (Kuntz & O'Connell, 2009; Pontano et al, 2008; Yan et al, 2012).

DNA damage activates GSK3β that, as described above, phosphorylates cyclin D1 on T286 making it for nuclear export and ubiquitin mediated degradation (Diehl et al, 1998; Pontano & Diehl, 2009). GSK3β is in turn inactivated by Akt to allow continuation of the cell cycle after DNA damage repair. Of note, T286 phosphorylation is exclusive of cyclin D1 suggesting that this D cyclin might have a unique role in cell cycle arrest following radiation induced DNA damage. Cyclin D1 T286 phosphorylation is dependent on ataxia-telangiectasia mutated (ATM) signalling following gamma-irradiation double strand break (Choo et al, 2009; Pontano et al,

2008) and on ataxia-telangiectasia and Rad3-related protein (ATR) signalling following ultraviolet (UV) irradiation (Hitomi et al, 2008). The ATM and ATR signalling pathways ensure complete duplication of the genome on unperturbed cell cycle and halt replication in the case of DNA damage by inhibiting S-phase kinases (Shechter et al, 2004). Besides these two signalling pathways, cyclin D1 was also shown to be phosphorylated at T286 following DNA damage by direct interaction with the F-box motif of F-box only protein 31 (FBXO31). FBXO31 expression is activated by ATM in response to not only gamma-irradiation but also general genotoxic stress (Santra et al, 2009). Cyclin D1 degradation is mediated through the destruction box, a consensus RxxL sequence. This destruction box is subject to degradation by the E3 ubiquitin ligase, anaphase-promoting complex. This anaphase promoting complex is constitutively associated to cyclin D1/Cdk4 complex but only cleaves it following ionizing radiation. With cyclin D1 destruction, $p21^{cip1}$ is released from cyclin D1/Cdk4 complex and inhibits Cdk2 leading to G1 arrest until DNA damage is resolved (Agami & Bernards, 2000).

These different models of cyclin D1 degradation following different aggressions probably reflect distinct models that might be present in particular cell types, illustrating an important role of cyclin D1 degradation for cell cycle arrest following DNA damage in a complex network of interconnecting molecules.

To further elaborate on this elaborate network, it is of importance to mention some proteins that intervene in the response to DNA damage. NF-κB is activated in response to DNA break in an ATM mediated way (Barre et al, 2010). STAT3 is activated in response to DNA damage mediated by topoisomerase I inhibition, in the G2 phase, and is responsible for the upregulation of several DNA repair genes (Courapied et al, 2010; Vigneron et al, 2008). On the same DNA damage conditions, both STAT3 and E2F1 where complexed with p21*warf1*. These complexes were recruited to the promoters of *myc* and *cdc25A*. Bothe these genes play major roles in DNA damage transcriptional repression (Vigneron et al, 2006).

These 3 molecules, NF-κB, STAT3 and E2F1 besides the role in DNA damage, also interact with cyclin D1 resulting in an additional potential network of regulation. STAT3 transcriptional activity is inhibited in a Cdk independent way by cyclin D1 (Bienvenu et al, 2001) but on the other hand, is responsible for inducing *CCND1* expression following mitogen signalling (Vigneron et al, 2008). NF-κB induces *CCND1* transcription but is in turn down-regulated by cyclin D1 (Rubio et al, 2012). Finally, cyclin D1 is known to bind to the promoter region of *E2F1* where it should regulate gene transcription (Casimiro et al, 2012), and on the other hand, E2F1 is known to inhibit promoter activity of *CCND1* (Watanabe et al, 1998).

So far cyclin D1 was successfully implicated in the DNA repair of cancer cells. It remains to be demonstrated however its effect on DNA repair on normal cells not subjected to UV or gamma-irradiation and to demonstrate whether cyclin D2 and cyclin D3 are able to bind RAD51 and BRCA2 and be recruited to DNA damage sites like cyclin D1.

The role of Cyclin D1 in DNA repair is independent of Cdk. This independent role helps to explain the importance of cyclin D1 in Rb negative cancers (Jirawatnotai et al, 2011).

Cyclin D1 Cdk independent role – “transcriptional function”

As mentioned above, cyclin D1 unique role as an oncogene separated it from the other D cyclins ever since its discovery. The ability to directly modulate cellular transcription factors (figure 9) in a Cdk independent fashion has been shown throughout the years. Some of the first findings to be published of a Cdk-independent role of Cyclin D1 in transcription were the regulation of the Dentin matrix acidic phosphoprotein 1 (DMP1), a myb-like transcription factor, which has to be down-modulated for cells to enter the S phase (Hirai & Sherr, 1996; Inoue & Sherr, 1998), the role in activating the estrogen receptor (ER) and the role in inhibiting muscle differentiation by inhibiting the myogenic differentiation factor, MyoD (Gu et al, 1993; Skapek et al, 1996). In the years to follow further involvements of cyclin D1 in transcription regulation were identified.

Cyclin D1 interacts directly with the ligand-binding domain of ER enhancing its transcription. This plays an important role in both the development of breast cancer and on the proliferation and differentiation of normal breast-cells in pregnant mice (Neuman et al, 1997; Zwijsen et al, 1997).

By being able to bind both ER and SRCs, Cyclin D1 acts as an alternate route of coactivator recruitment to ER , establishing a direct role for cyclin D1 in regulation of transcription (Zwijsen et al, 1998).

Cyclin D1 was also involved, along with cyclin D2, in the Cdk independent inhibition of transcription factor if the myeloblastosis family, v-Myb but not of c-Myb. Cyclin D1 an D2 bind to both c-Myb and v-Myb through the DNA binding domain, the same domain where Cyclin D1

binds to DMP1. Interaction with v-Myb inhibits its transcriptional activity but does not affect its DNA binding or nuclear transport (Ganter et al, 1998).

Soon to follow was the discovery that Cyclin D1 is able to inhibit androgen receptor (AR) transactivation. Cyclin D1 was hypothesized to directly form a complex through the carboxyl-terminal LXXXLL motif with the androgen receptor as part of a negative feedback loop (Knudsen et al, 1999; Reutens et al, 2001).

Cyclin D1 is known to inhibit the activity of thyroid hormone receptor β 1 (THR-β1) (Musgrove et al, 2011), although the THR-β1 role in inhibiting cyclin D1 expression by binding its promoter has been studied in much greater detail due to its tumorigenic potential (Garcia-Silva & Aranda, 2004; Porlan et al, 2008).

The Peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor which loss of function is involved in human cancer (Sarraf et al, 1999) is also inhibited by cyclin D1 both via the Rb pathway and by a Cdk independent pathway (Wang et al, 2003).

Another transcription factor which Cyclin D1 is able to regulate is the specificity protein 1 (Sp1), a transcription factor that controls the expression of several genes essential for early development. Sp1-mediated transcription is inhibited via Cyclin D1 binding to the transcription factor II B (TFIIB) complex largest subunit, $TAF_{II}250$. This binding is mediated by the first 100 aminoacids of Cyclin D1 and is not mediated through the LXCXE Rb-binding domain. The $TAF_{II}250$/cyclin D1 complex blocks the formation of $TAF_{II}110$/ $TAF_{II}250$ complex essential for Sp1-mediated transcription (Adnane et al, 1999; Shao & Robbins, 1995).

Another striking discovery was the ability of Cyclin D1 to repress STAT3 activation by a negative feedback loop. This inhibition is done by directly binding the STAT3 activation domain in the nucleus, marking it for nuclear export by dephosphorylation (Bienvenu et al, 2001). The studies of cyclin D1/STAT3 complex progressed further and it was found that upon interaction with STAT3, cyclin D1 is able to bind to the P21warf1 promoter, obstructing the promoter in a way the histone acetylase CREB-binding protein (CBP) and RNA polymerase II are unable to initiate transcription. Altogether these findings illustrate examples of both negative and positive feedback regulation as p21warf1 functions as an inhibitor of Cdk activity in G1 and G2 (Bienvenu et al, 2005).

Cyclin D1 is also able to repress the basic helix-loop-helix transcription factor, BETA2/neuroD. This repression is mediated by the binding the C-terminal domain of the coactivator p300

which is then recruited to BETA2. This repression may serve to prevent secretin gene transcription from occurring in relatively immature epithelial progenitor cells (Ratineau et al, 2002).

The domain where Cyclin D1 interacts with the above proteins is schematized in figure 8:

Cyclin D1 is suggested to regulate the accessibility of promoters by binding proteins such as $TAF_{II}250$ and P/caf which mediate acetylation of histones and other proteins and acclaims the need for chromatin immunoprecipitations experiments to reveal if cyclin D1 associates with transcriptional regulators on DNA or transiently in the nucleus (Coqueret, 2002).

Several years later, the development of a Flag- and haemagglutinin- tagged cyclin D1 in the mouse permitted to do a chromatin immunoprecipitation coupled to DNA microarray (ChIP-chip) to determine the binding sites of Cyclin D1 through the genome. Cyclin D1 was found to occupy the promoters of abundantly expressed genes. Among the over 900 hits of Cycline D1 binding to promoter regions in close proximity to transcription start sites, six regions of extreme interest were confirmed to correspond to the promoter regions of transcription factors NF-Y, STAT3, CREB2, ELK1, ZNF423 and CUX1 . Additionally using the Cyclin D1 KO model, other genes were proven to have their expression altered such as *Notch-1* (further detailed below), *Id3*, *Id1*, *Meis2* and *Tcf4*. Cyclin D1 was shown to bind the transcription regulatory elements of all these genes (Bienvenu et al, 2010).

Another genome-wide ChIP sequencing of cyclin D1 revealed that besides a role in transcription regulation, Cyclin D1 is also able to induce chromossomal instability (CIN) (Casimiro et al, 2012). Up to date, it was believed that this was a feature unique to cyclin E (Spruck et al, 1999). This genome-wide ChIP revealed 2840 putative promoter binding sites for Cyclin D1 of both promoter-proximal and distant elements, up to 10kb and beyond. Further analysis was confined however to those of under 2kb distance to the promoters and the top hits where Ctcf, Zfx, Sp1, Mizf, estrogen receptor R1, E2f1, Creb1 and Hif1α/Arnt (Casimiro et al, 2012).

Recently, Cyclin D1 was described as a co-repressor of NF-κB. As mentioned previously, the cyclin D1 promotor contains a NF-κB response element (Eto, 2000). Inhibition of NF-κB by cyclin D1 is is done by binding its coactivator RAC3. Of note, one of the target genes of the transcription factor NF-κB is Cyclin D1. Therefore NF-κB leads to cyclin D1 expression which will induce NF-κB down-regulation by a feedback mechanism (Rubio et al, 2012).

Notch-1/Cyclin D1 pathway

Notch-1 has been implicated in leukemia as a gain of function was observed in over 50% of all T-cell acute lymphoblastic leukemia (T-ALL) patients (Weng et al, 2004). An interplay between Notch-1 and Cyclin D1 was suggested to occur in human breast cancer cells expressing Her2/neu epidermal growth receptor (ErB2), where the induction of Notch-1 was demonstrated to occur via cyclin D1 (Lindsay et al, 2008). Notch-1 was also shown to control Cyclin D1 expression through Akt signalling pathway (Guo et al, 2009). Therefore both these studies combined indicate that a positive feedback mechanism may be involved in the expression of both Notch-1 and Cyclin D1.

Cyclin D1 was demonstrated to promote *Notch-1* promoter acetylation by directly binding to its promoter and recruiting CBP histone acetyltransferase. In the absence of cyclin D1 a decrease in histone acetylation of Notch-1 in the developing retina is registered. Notch-1 plays a crucial role in retinal development and the phenotype of mice with Notch-1 conditionally ablated in the retina closely relates to that of Cyclin D1 deficient mice. Also, overexpression of cyclin D1 leads to a twofold increased *Notch-1* expression. Most importantly, the post-natal injection of retroviral construct containing notch intracellular domain into subretinal space of cyclin D1 deficient mice attenuated the knockout phenotype by increasing retinal progenitor cell proliferation (Bienvenu et al, 2010).

Transgenic expression of the intracellular domain of *Notch1* ($N1^{IC}$) in mice potentiates the development of mammary tumours. Crossing them with Cyclin D1 $^{-/-}$ or Cyclin D1 $^{+/-}$ mice leads to the abolishment or great reduction of mammary tumours formation. This implies that Cyclin D1 is required to generate high number of tumour-initiating cells. Partial decrease of Cyclin D1 levels by RNA interference in $N1^{IC}$ mice was sufficient to severely impair the growth and mammosphere-forming potential of tumour cells, as well as to increase their apoptosis (Ling & Jolicoeur, 2012).

It is surprising that, considering the importance of D1 cyclin in regulating Notch-1 expression and the importance of Notch-1 in development in general and in particular in haematopoietic and T-cell differentiation (Radtke et al, 2010), Cyclin D1 deficient mice were reported to have no defects in T-cell lineage (Sicinski et al, 1995).

When studying by Western Blot the kinetics of the up-regulation of D cyclins after the activation of T lymphocytes with anti-CD3 MoAbs, the laboratory had found that the D1 cyclin was up-regulated before the other two D cyclins, suggesting that D1 could have a role in the initiation of G1/S transition in T lymphocytes. This finding, together with the preferential association of D1 mutations with lymphoid cancers, as well as its association with Notch-1 lead us to review a possible role of this cyclin in haematopoiesis. For that purpose, Dr Sicinsky kindly provided us the previously described “D1 deficient mice” (Sicinsky et al, 1995). When we initiated this breeding from heterozygous crosses, we could not obtain a single homozygous adult mouse, what forced us to review the breeding conditions. When finally we studied the first two homozygous mice, we found that they were totally different. One had virtually no thymus. The second was anomaly big, and had a hypertrophic thymus. These findings were so perplexing that we decided to continue this study, in spite of the difficulties it involved.

Annex to Materials and Methods

Over-expression of exons 4-5

To obtain a vector leading to the over expression of the exons 4-5, we designed a construct by using two primers to clone this exon.

FORWARD PRIMER

ACT	CAATTG	GCC	ATG	GTG AAG TTC ATT TCC AAC CC
1	2	3	4	5

In this forward primer we added 3 bases (1) in 5' to provide protection of the restriction site MunI (Aka MfeI) (2), even though we will be using blunt edge cloning in a plasmid before restriction assays. We also added a GCC into generate a perfect Kosac site (3). Finally the ATG from the initial exon 4 was slightly modified to have a full protein, we added a G to do a new ATG, this way bypassing the question of which of the two in frame ATG does the ribosome choose (4). As the ribosome starts reading from the beginning of the sequence, we want it to be stuck in our newly added ATG. This way we keep the in frame GTG (Valine) (5) which by starting with a G will complete the Kosac sequence, making it a perfect and unfaultable start point.

As a first step, the PCR will have 20 complementary bp, on which we have 9 (G+C) making a 58ºC melting temperature. We'll have 15 un-complementary bases that are 5'.As a second step, we will have 35 complementary bp, with a G+C content of 16, maintaining the melting point at 58ºC.

REVERSE PRIMER

TG CGA GAT GTG GAC ATC TGA	GCC	TACGTA	TCT
1	2	3	4

The reverse cloning primer starts with a 3'End sequence of cyclin D1 ending in the TGA (1). Following, a GCC was added to have some bp between the TGA and the restriction site. To give a 60º melting point, since the total length is of 32 bp, we opted to have any 3 G+C bases (2). The restriction site added is SnaBI (3) and following it, TCT was added to maintain the ACT added in the forward primer.

Forward primer (35pb) - ACTCAATTGGCCATGGTGAAGTTCATTTCCAACCC
Reverse primer (32bp) – AGATACGTAGGCTCAGATGTCCACATCTCGCA

Primers were produced by eurogentec and purified by method RP-Cartridge – Gold.

RNA was extracted from activated CD8 cells.

To clone D1 exons 4-5 Lymphocites were extracted from lymph nodes (axillary, brachial, inguinal and mesenteric) and depleated for CD4(GK1-5), CD19(1D3), 6B2(B220) and Ter119 (ter 119) (all homemade antibodies) with anti rat Dynal® beads from Invitrogen according to manufacturer's instructions. The control Purification was performed by staining CD8(53.6.7), CD4(RM 4-5), IgM (polyclonal) from BD Pharmingen. These cells were cultured in vitro for 24 hours with purified αCD3(1452C11) at a concentration of 1ug/ml on a volume of 1M cells/ml at 37ºC, 5%CO2, and the efficiency of activation was tested by the expression of CD69(H1.2F3) from BD Pharmingen. Dead cells were removed in a Ficol (from Cedarlane) gradient, the RNA extracted using RNeasy Plus kit from Quiagen following manufacturer's instructions, and retrotranscribed with MuLV from Applied Biosystems using the GeneAmp RNA PCR kit.

The cDNA obtained from these steps was then used as template for the PCR with the cloning primers described above. The PCR was performed using the high fidelity proof reading Pfu Turbo Polymerase from Stratagen.

PCR protocol:

(1X) 94ºC 5min
(5X) 94ºC 10sec, 68ºC 30 sec (touchdown PCR -2ºC per cycle), 72ºC 1min
(35X) 94ºC 10sec, 58ºC 30 sec, 72ºC 1min
(1x) 72ºC 10 min
Forward primer – 20pmol
Reverse primer – 20pmol
cDNA template – 100ng
PFU turbo DNA polymerase (stratagen #600250) – 5U
Cloned Pfu DNA polymerase reaction buffer (stratagen #600250) – 5uL
DNTP (applied biosystems, UK; Warrington, N8080260) – 50nmol (12,5 nmol each)
UltraPure™ DNAse/RNAse-Free Distilled Water (Gibco 10977-035) – add until 50 uL of final volume reaction

Ater amplification the PCR product was run in an agarose gel to confirm purity and to purify the amplified band using Promega Wizard® PCR Clean-Up System following user instructions. The purified product was quantified and subcloned in a blunt vector pCR®-Blunt II-TOPO® and then amplified using Zero Blunt® TOPO® PCR Cloning Kit with One Shot® TOP10 Chemically Competent *E. coli* following manufacturer's instructions

The amplified vector was the purified using QIAprep Spin Miniprep Kit, following manufactures instructions and was sequenced using applied biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit and pre designed primers for the plasmid according to manufacturer's instructions.

The amplified and sequenced vector was then then restricted with both MfeI and SnaBI restriction enzymes from new-england bioabs and cloned into pMiev using new-england biolabs T4 ligase according to manufacturer's instructions. Upon confirmation of the sequence, pMiev with the truncated *CCND1* (*tD1*) exons 4-5 and empty vector were amplified using QIAfilter Plasmid Maxi Kit.

PlatE virus packaging cells were grown in (Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (High Glucose) with 10%FCS + pen/strep respectively at 10U/ml and 10ug/ml and b2-mercaptoetanol diluted to final concentration $2x10^{-5}$. and also supplemented with both selection antibodies, 1ug/ml puromycin and 10ug/ml blasticidin.

PlatE cells were seeded 2×10^6 cells in a 60 mm culture dish without antibiotics overnight and then pMiev (empty and cloned with cyclin D& exons 4 and 5) was transfected with Invitrogen's lipofectamine LTX according to manufacturer's instructions. The medium was changed after 24h and the viral supernatant collected two days after, filtered and used directly for the infection of HSC.

LSK cells from mice treated with 5FU were stained and sorted as described in the manuscript. Were cultured for 1h with the cocktail of cytokines (50ng/ml muSCF, 50ng/ml TPO, 100U/ml IL-6, 10 ng/ml IL-11, 5ng/ml Flt3-L, all from R&D systems) in 24 well plates at a concentration of 100 thousand cells per well, washed and incubated with the viral supernatant containing 5mg/ml protamine sulphate and undergone spinoculation for 45 min at 1800 rpm and 37ºC on 24 well plates coated with Takara's RetroNectin®.

After infection the cells were incubated 37ºC, 5% CO2, for one additional hour with the virus supernatant before being washed and injected I.V. (intravenous)(25.000/mouse) into irradiated (600 rad 24 minutes with homogenizer) RAG mice preventively treated with neomycin or plated.

The mice were studied 3 weeks after injection, after being injected I.P. (intraperitoneal) twice a day with BrdU. The cells were then sorted for $EGFP^+$ prior to the fixation protocol described in the manuscript. We sorted $EGFP^+$ cells because the fixation protocol used to visualize BrdU

staining lead to the loss of EGFP preventing the identification of infected cells.

Generation of D4-D5 deficient mice

To investigate the role of these two exons, we wished to generate mice which expressed D1 exons 1-3 but lacked exons 4-5. Since the D1 deficiency may be lethal, we generated conditional deficient mice by introducing two LoxP sites flanking the 4th exon (figure 11). A LoxP site, short for locus of x-over P1, is a small sequence where a small sequence of 8 base pairs (bp) is flanked by two palindromic 13 bp sequences. These two sites are known targets of site specific recombination (SRR) for the CRE recombinase of phage P1. The CRE/LoxP recombination system has long been described and used to promote site specific recombination in *in vivo* mammal cells (Sauer & Henderson, 1988). The expression of CRE recombinase mediates the excision between the two LoxP sites (floxed region).

The LoxP sites were knocked in to flank exon 4, having a LoxP site on intron 3 and another on intro 4. Following CRE mediated recombination, Exon 4 should be excised completely leaving the intron 3 donor site and intron 4 acceptor site free to be spliced by the spliceosome. Exon 3 coding sequence ends with a tyrosine residue followed by a G bp. Exon 5 starts with three in frame bp. What happens when exon 3 splices to exon 5 is that exon 5 gets out of frame. This strategy was design to conditionally delete exons 4 and 5 while maintaining the expression of exons 1-3. To maintain the stability of the mRNA after splicing it was important to keep the splice acceptor site of exon 5, otherwise RNA decay was likely to occur. The amino acids added by exon 5 out of frame start to exon 3 after recombination are GLPPCLPGTD (figure 11). This sequence was blasted and no homology to other proteins was found on mice background.

This strategy was designed to allow the stable expression of exons 1-3, since both splice acceptor site and the poly adenylation sites of exon 5 are not modified what should guarantee mRNA stability. However there is still a risk that the change in the stop codon could cause the mRNA to be unstable. These mice were generated by the ISc at Strasbourg.

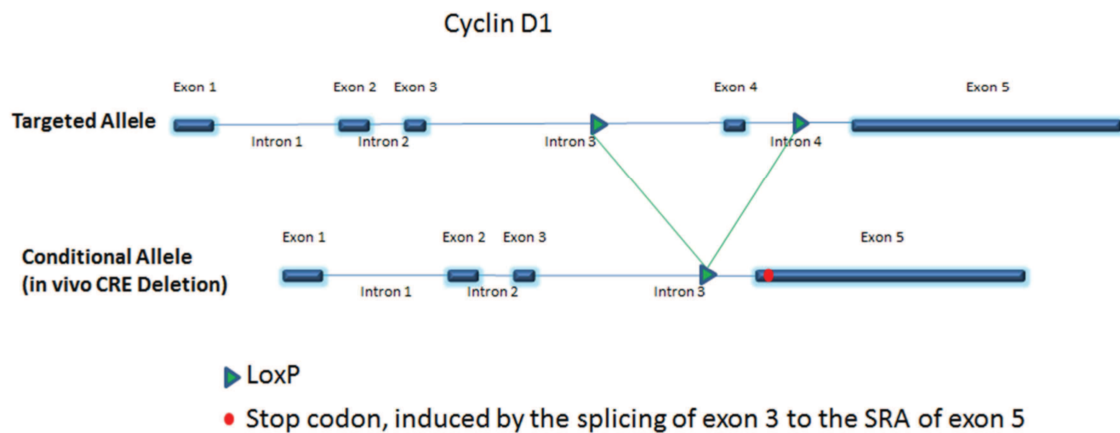


Figure 11: Strategy for the creation of floxed exons 4-5 cyclin D1 mice.

Two LoxP sites were knock into the introns 3 and 4 of cyclin D1. Expression of CRE recombinase leads to the excision ox exon 4 and the introduction of an early stop codon on the now out of frame exon 5.

The selection of mice expressing CRE under different promoters

We crossed these mice with two mouse strains expressing CRE respectively under the Vav1 promoter, and the hCD2 promoter (a kind gift of A. Potocnik). The expression of CRE in these mice was revealed when they were crossed with rosa-lox-stop-lox EGFP, and is shown in the Figure 12. Thus while Vav1 CRE is already expressed in HSC, in hCD2-CRE CRE is first expressed in a fraction of CLPs, TN1 and TN2 thymocytes but in all TN4, DP and mature T-cells.

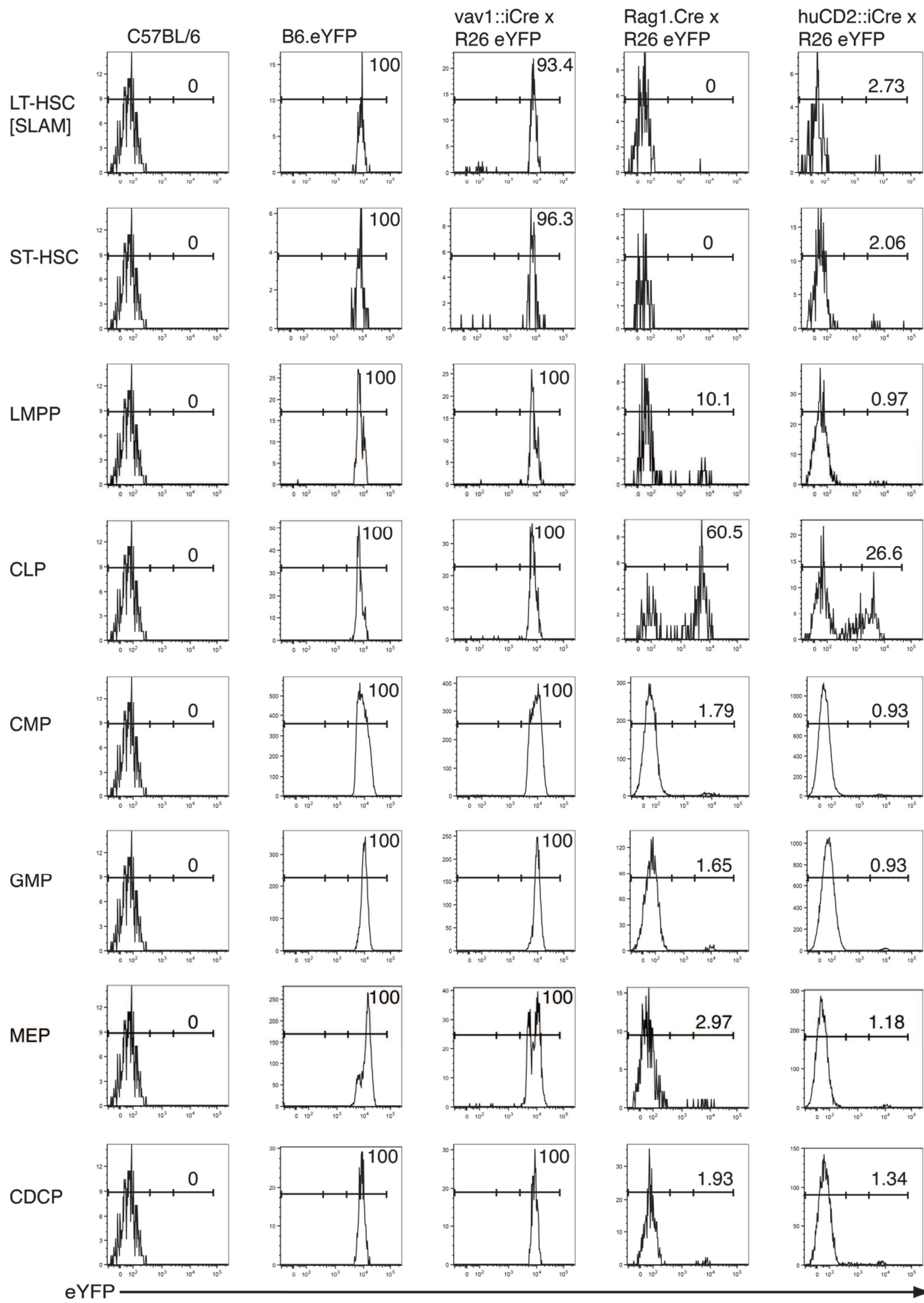


Figure 12: hCD2, Vav1 and *Rag1* medieted expression of CRE in different haematopoietic compartments

Mice with a floxed stop eYFP (enhanced yellow florescent protein) under the control of Rosa 26 (R26) promoter will express eYFP as soon as the stop codon is excised. Each row represents a different haematopoietic compartment and the expression of CRE in that compartment is assessed by eYFP expression on the Y axis. Here we see that Vav is expressed very early on and that hCD2 promoter is almost not expressed on any of these compartments, for it starts its transcription at TN1 to TN2 stages only. Using these different promoter allow us to assess the importance of 4-5 at different levels. As a last assay, it is also possible to cross floxed mice with rosa26-CRE mice to express ubiquitously CRE (by the courtesy of A. Potocnik).

Supplementary Results

The majority of our results are described in the annexed manuscript Here, we go through them again, to simplify reading by putting the figures in the sites they are included in the manuscript Besides, we have additional data describing negative or preliminary results that will be described in the heading Additional Results

Cyclin D1 knockout mice model

To study the role of Cyclin D1 protein in lymphopoiesis we used mice deficient for Cyclin D1 cyclin box kindly provided by Dr. Sicinski (Sicinski et al, 1995). These mice were created by inserting a neomycin cassette, deleting the coding portion of exon 1 and exons 2 and 3 but sparing exons 4 and 5. The neomycin cassette was inserted using an *EagI* restriction site, 4 bp upstream the ATG codon. As can be seen on figure 9, the entire promoter region along with the putative initiator region is left untouched. Exons 1 to 3 were chosen because they code for the cyclin box domain. This domain is responsible for the Cdk interactions on all cyclins of all eukaryotes (Xiong & Beach, 1991). By deleting the cyclin box, a full knock out phenotype would be expected, since this domain is thought to be essential for cyclin function. Also, a full knock out would have been too ambitious since the five exons of cyclin D1 are spread over 7 kb on the mouse genome. Figure 13 A depicts full cyclin mRNA. The size of intron 3 acts as a barrier for the deletion of exons 4 and 5 by substitution. Figure 13 B contains a schematic representation of how exons 1-3 of cyclin D1 were knocked out by substitution.

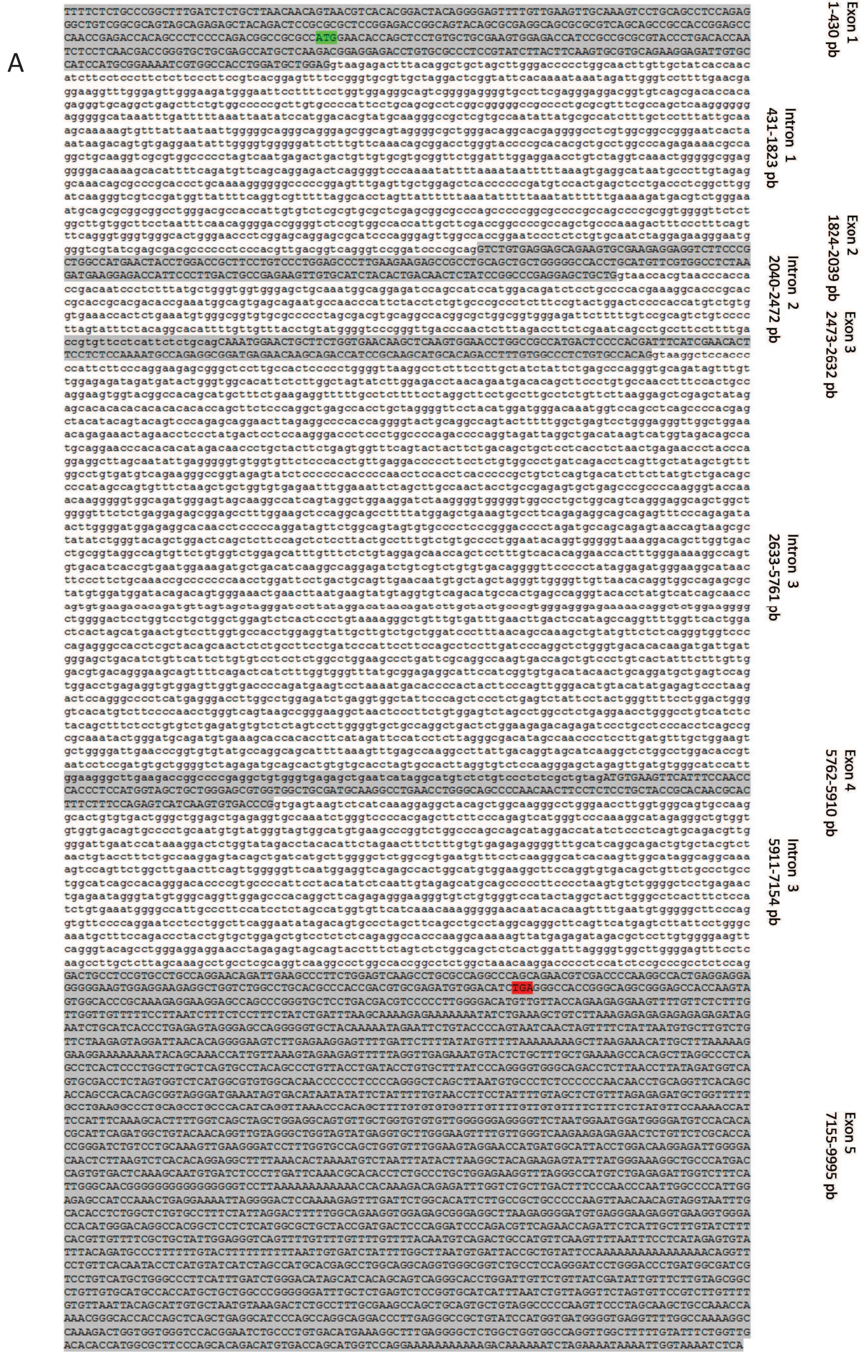


Figure 13: *CCND1* and Cyclin D1 deficient mice

(A) *CCND1* mRNA sequence. The exons are highlighted in grey, the start and stop codons are highlighted in green and red respectively. (B) Deletion of exons 1 to 3 by insertion of a neomycin cassette. Exon 1 is only partially removed, starting a few base pairs upstream the ATG codon. (B) Is adapted from (Sicinski et al, 1995).

Cyclin D1 knockout mice and different phenotypes

Phenotypes at thymic level

The thymus of cyclin D1 knockout mice ($D1^{-/-}$), from heterozygous cross, described in materials and methods section, were studied at 4 weeks of age. Surprisingly, these knockout mice had very different phenotypes regarding thymic populations. The thymus of each individual $D1^{-/-}$ mouse was screened (figure 14 A) for the expression profiles of CD4 and CD8 in lineage positive cells (top graphics), the expression profiles of CD44 and CD25 in lineage negative cells (middle graphics) and finally the expression profiles of CD24 and CD117 (cKit) within $CD44^{+}$ $CD25^{-}$ (TN1) (lower graphs). The expression profiles on these three stainings of each individual $D1^{-/-}$ mouse could be separated in three different groups.

Group I mice showed very few $CD4^{+}CD8^{+}$ (DP) cells and the TN populations were found to be mostly constituted by the early $CD44^{+}CD25^{-}$ (TN1) and few $CD44^{+}CD25^{+}$ (TN2) progenitors. Some mice even lacked TN2 cells altogether (figure 15). In most mice, progenitor cells got blocked in the transition from TN2 to $CD44^{-}CD25^{+}$ (TN3), never fully loosing CD44 marker. The TN1 compartment is also abnormal since the $CD24^{+}$ $cKit^{+}$ (ETP) were virtually absent suggesting that the differentiation block may precede thymus seeding, either by a failure of precursor homing from the BM or by a block in BM differentiation of progenitors. The low frequency of ETP suggests the latter (figure 14 A lower graphs).

Group II mice thymocyte populations showed a decrease on the DP compartment and a consequent enrichment in TN populations. The block in TN differentiation occurs latter than group I in the TN3 to $CD44^{-}CD25^{-}$ (TN4) stage. The TN3 compartment fully lost CD44 expression and TN2 compartment showed almost normal levels, revealing that the block at this stage was fully overcome. The TN1 compartment was increased comparing to wild type mice and the ETP compartment was present although its cellularity was less than half of that of a wild type mouse. The TN1 compartment was also less than half of that of group I mice, supporting the idea that TN differentiation block occurs latter (figure 14 A middle graphs).

Group III mice appeared to have a normal TN thymocyte population distribution. Comparative analysis of TN1 compartment showed an increase on ETPs in comparison with wild type mice (figure 14 A lower graphs).

The analysis of thymic cellularity of each individual mouse also revealed a considerable heterogeneity. This could be explained by the different groups created. Thymic cellular counts

were plotted within the three designated groups (figure 14 B). Group I mice had the lowest-cellularity counts ranging from under 10^6 cells to slightly over 10^7 cells. Group II displayed moderate thymus atrophy with cellularity varying between 10^7 cells up to 10^8 cells. Finally, group III mice were subdivided within two groups according to the thymic cellularity alone. A group IV was created to encompass those mice who displayed a thymic hyperplasia with cellularity over $4x10^8$ cells. The remaining mice of group III mice (group III on figure 14 B) had normal thymic cellularity rounding the 10^8 cells.

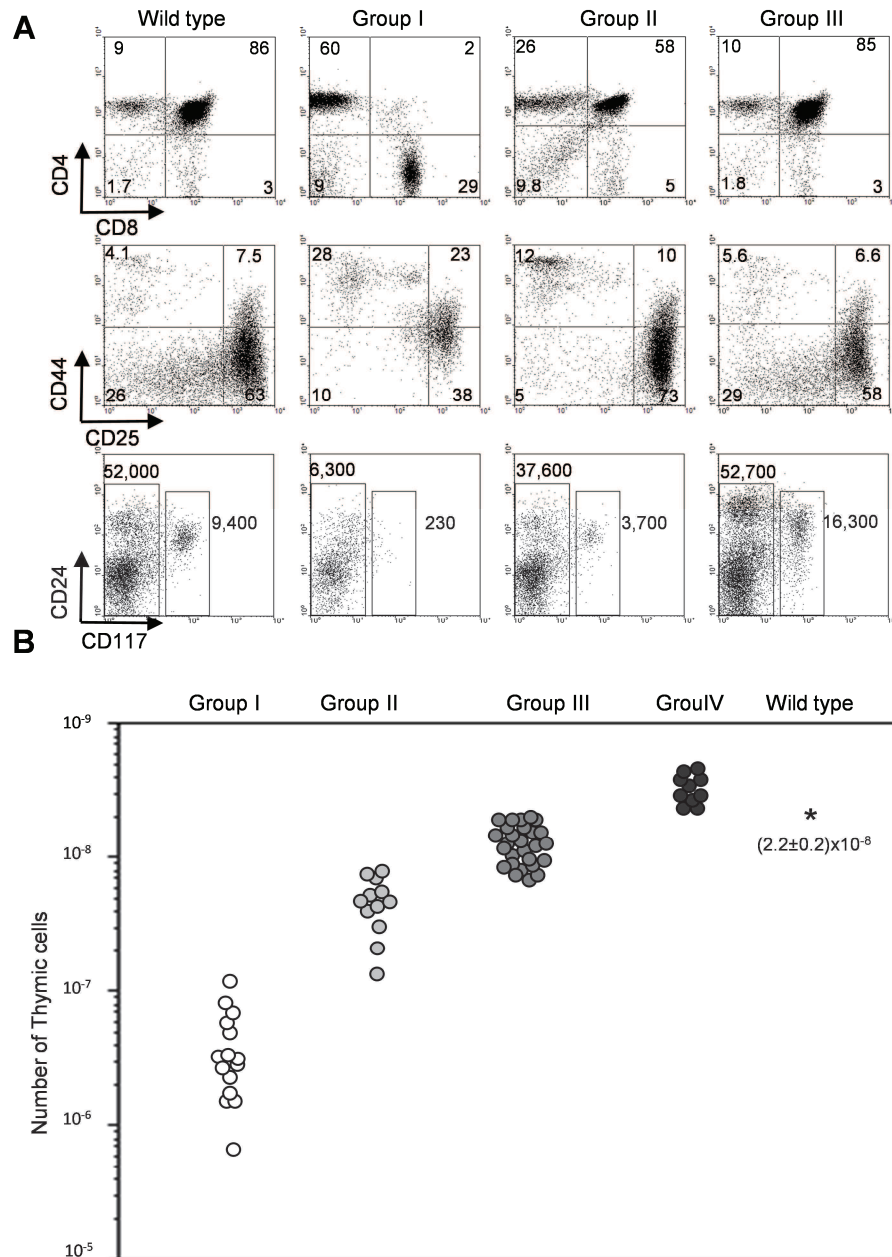


Figure 14: Thymocytes from cyclin D1-box deficient mice.

Thymocytes from individual cyclin D1-box deficient mice were studied and grouped according to the different phenotype observed. (A) Thymocyte population distribution. Results show representative profiles of mice belonging to each group. Upper graphs: CD4/CD8 profiles within $CD19^{-}TER119^{-}Gr\text{-}1^{-}$ populations. Middle graphs: CD44/CD25 profiles within triple negative (TN: $CD3^{-}CD8^{-}CD4^{-}CD3^{-}CD19^{-}TER119^{-}Gr\text{-}1^{-}$) subsets. Lower graphs: TN1 subset was further subdivided by the expression of CD24 and CD117 (cKit). $CD117^{+}$ which are CD24 intermediate compose the ETP population. Numbers show the number of cells/thymus. Mice were analysed at four weeks of age and results are from one mouse from each Group, representative of 15 (Group I) 12 (Group II) and 22 (Group III) mice. (B) Total yields. Each point represents one individual mouse. An additional group, group 4 can be differentiated by thymic cellularity alone within the group 3 mice of (A). Groups 1 to 3 also have a distinct thymic cellularity although there is some overlap. They were divided in groups according to (A).

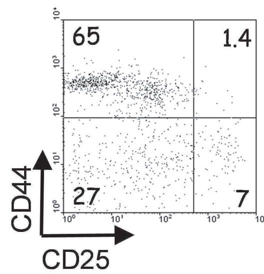


Figure 15: TN population of an individual group I mouse

Thymocytes from individual cyclin D1-box deficient mouse was plotted as in figure 14 A. In some rare cases the blockage observed in TN populations of group I mice is so severe that there is a total absence of the TN2 compartment.

Phenotypes in BM progenitors

B-cell lineage precursors

Since our data on thymocyte subpopulations suggested that differentiation blocks could have origin in the BM precursor cells, we also analysed BM subpopulations cells.

Interestingly enough, the three arbitrary groups we had previously defined in the thymus compartment correlated with an equivalent heterogeneity in the B-cell lineage precursors in the BM (figure 16 A).

As such, Group I mice were characterised by having an almost complete absence of $B220^{low}$ $CD43^{-}$ pre-B-cells. Most $B220^{+}$ $CD43^{+}$ pro-B-cells were $CD24^{-}$, functional assessment of B lineage precursors in adult mice BM established that most early B lineage progenitors express CD24 (Mojica et al, 2001). The absence of CD24 expression reveals therefore a major block in pre-proB differentiation which is the earliest stage of B-cell differentiation.

Group II mice showed a partial pre-proB block and a reduction in pre-B-cells, but the expression of CD24 had been rescued to almost normal levels.

Group III mice appeared to have a normal B-cell lineage precursor's population.

Haematopoietic precursors

Since both T-cell and B-cell differentiation appear to be severely affected, we next studied if the block in BM differentiation was before T or B-cell lineage commitment.

The results were striking as they revealed that group I mice had few common lymphocyte progenitor cells Lin^- $IL7R^+$ $CD117^+$ $Sca\text{-}1^+$ (CLPs): less than a third of those in wild type mice. Interestingly enough, group II mice also had a severe deficiency, albeit to less extent, on these very early progenitors that give rise to T-cells, B-cells and natural killer (NK) cells (figure 16 B).

In contrast, the haematopoietic stem cells Lin^- $CD117^+$ $Sca\text{-}1^+$ (LSKs) were not affected. Surprisingly, the analysis of these earlier BM precursors revealed that even group III-IV mice were very abnormal. Both CLP and HSC were increased as compared to normal mice (figure 16 B).

These results show that elimination of the D1 cyclin box may have two opposite effects. It can block HSC differentiation thus constituting the most precocious block in lymphoid lineage differentiation described so far. Alternatively, it can promote HSC, CLP and ETP generation.

Intrigued by the pre-natal and peri-natal death rates of $D1^{-/-}$ mice, we studied mice two weeks after birth. These studies revealed that in early stages of mice development all mice exhibit a lymphopenic phenotype accompanied by a severe depletion of the LSK compartment as well as a considerable depletion of myeloid and erythroid lineage cells (figure 16 C). Both these phenotypes could account for the high mortality rates in homozygous new-borns. The depletion of both myeloid and erythroid lineage cells is not observed on 4 week old mice (data not shown).

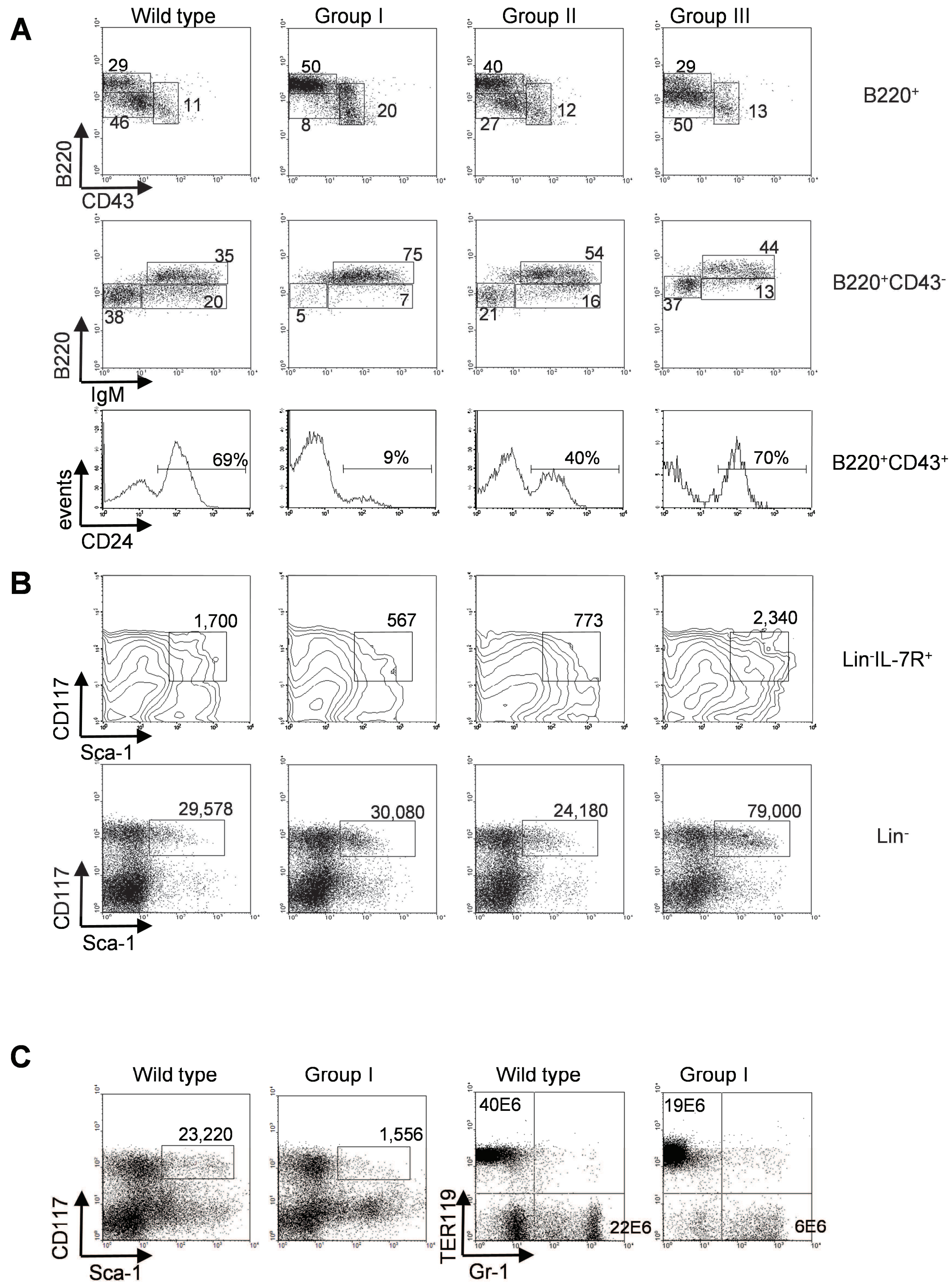


Figure 16: Bone marrow cells from cyclin D1-box deficient mice.

(A, B) Mice were four weeks old. Results are from one mouse from each Group, representative of 15 (Group I) 12 (Group II) and 18 (Group III) mice (A) B lineage cells of wt mouse and of group 1 to 3 cyclin D1-box deficient mice. The percentage of cells of each sub-population is shown. (B) Lineage negative (Lin$^-$: CD3$^-$CD4$^-$CD8$^-$CD19$^-$TER119$^-$Gr1$^-$Mac-1$^-$NK1.1$^-$) progenitors analysed by their expression of IL-7R, Sca-1 and CD117 (cKit). Results show the numbers of cells recovered/bone (2 femurs+ 2tibias) within each subtype in four weeks old mice. (C) Analysis of Lin$^-$ (left graphs) and erythroid and myeloid cells (right graphs) in 9 days old mice. Numbers represent yields/bone.

Phenotypes at peripheral lymphocyte pools

The changes orchestrated by the absence of cyclin D1 cyclin box in the haematopoietic compartment also translate into the peripheral lymphocytes. We studied a pool of brachial, axillary, inguinal and mesenteric LN on individual $D1^{-/-}$ mice. Correlating to the data on group I mice in the thymus and BM, group I peripheral LN showed a very limited cellularity (figure 17 A). These LN presented structural defects, being very small and translucent. Group II albeit also having a greatly reduced lymphocyte count on the peripheral LN, had three fold more cells than group I. Group III mice had a cell count of approximately 10^7 on these LN, representing one fourth of the cell pool on wild type mice.

Group I mice low cellularity on the LN can be accounted by a deficient homing to these organs. As described in the introduction, both CD62L and CCR7 play a crucial role in LN homing. CD8 T-cells were studied on peripheral LN and plotted against CD62L and CCR7 (figure 17 B). The results show that on group I there is a total absence of CCR7 which explains why in these mice the LN were devoided of cells. Group II and group III mice have restored the expression of CCR7 but its expression is halved compared to the wild type control.

In both group II and group III mice, 50% of the CD8 T-cells are positive for CD62L (figure 17 B). Curiously enough, group I mice have approximately 75% of CD8 T-cells positive for CD62L, which is closer to the averaged 85% on wild type mice.

Taken together, both the reduced CCR7 and CD62L expression on group II and group III are likely to explain the low cell count on LN, since in the spleen the lymphopenia of group II and group III mice is much less severe (data not shown).

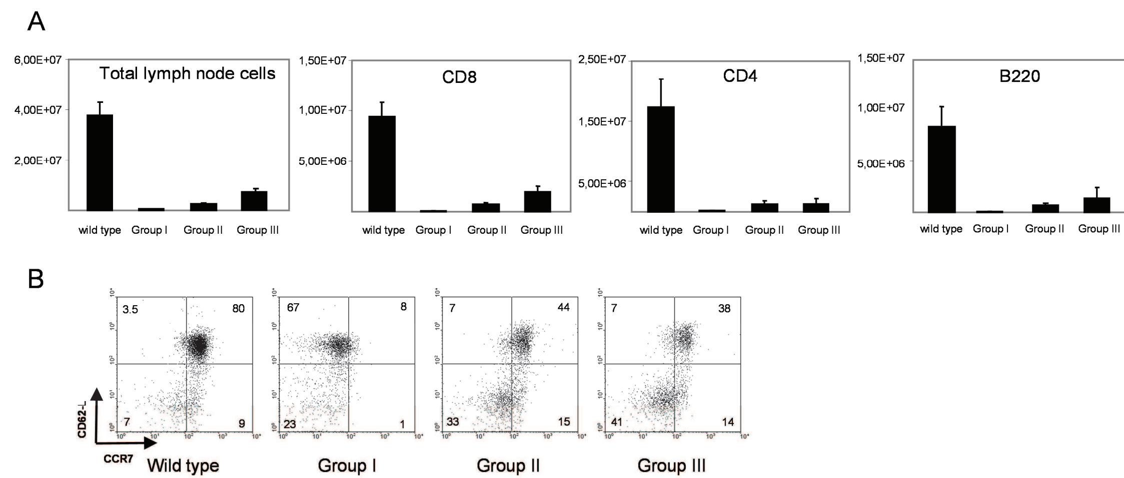


Figure 17: Lymphocyte counts of D1$^{-/-}$ in peripheral LN and CCR7 expression

Peripheral LN cell population of 4 week old mice. Results are from individual mice studied in the same experiment and correspond to a pool of the same LNs in each mice Group. The same results were obtained in 5 other experiments. (A) Absolute numbers of total, CD8^{+}, CD4^{+} and B220^{+} cells of D1$^{-/-}$ in comparison with wild type mice. (B) Expression profiles of CCR7 and CD62L on CD8 T-cells.

Ablation of D1 cyclin box and cell division

Cyclin D1 cyclin box mediates interaction with Cdk4/6 which in turn is involved on G1 to S transition following exogenous stimulation. To study the effect of the removal of this cyclin box, we have undertaken a series of *in vivo* division rates analysis (figure 19). The results were most striking as they revealed that group I mice was virtually devoid of cycling cells in all lymphoid compartments. This lack of division explains why lymphoid lineage cells do not differentiate in these mice since it has been estimated that for the transition from TN2 to DP state a progenitor cell undergoes a series of 8 to 10 divisions during 3 to 4 days (Penit et al, 1995; Petrie et al, 1990). In contrast, group II and group III had a marked increase on cell cycling in almost all of the studied lymphoid compartments, as shown by BrdU incorporation (figure 19). Curiously group II showed the most marked increase in the frequency of cycling cells, indicating that their reduced precursor frequencies were compensated by an increase in the division rates in an attempt to populate the reduced haematopoietic niche. The increased precursor frequency and division rates of group III mice, could explain why D1 deficient mice eventually develop a lymphoid hyperplasia. Interestingly enough, the ablation of cyclin D1 box, whose role in the cell cycle should be redundant, seem to on one hand block cell division, leading to a reduced HSP population and as seen before lymphoid lineage differentiation arrest at a pre T, B and NK cell commitment phase, which requires cell division and on the

other hand, it can be associated with an increase in cellular division rates leading to increased HSC like LSK's and CLP's of group III mice (figure 16 B). In the case of group II it can even have both phenotypes simultaneously, leading to an accelerated cell cycle in one hand and on the other reducing some precursor cell types.

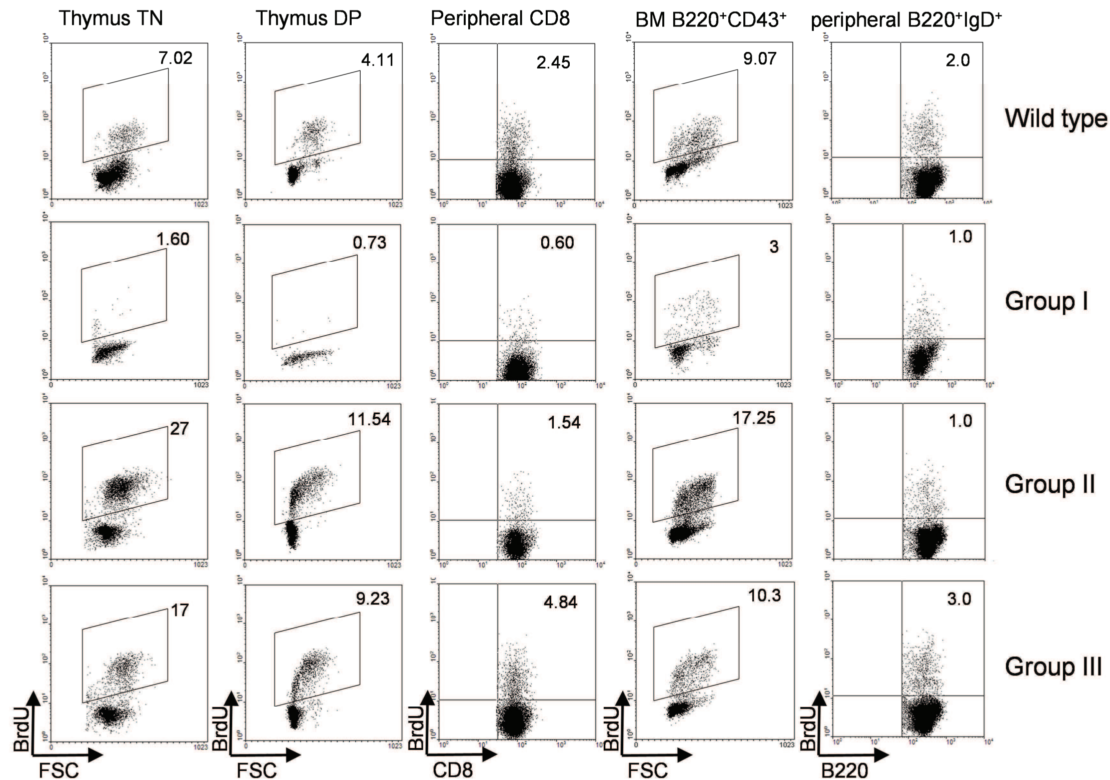


Figure 18: BrdU incorporation in D1-cyclin box deficient mice.

Results show the percentage (%) of cells in S phase in the thymus and BM after a 2h BrdU pulse and the BrdU incorporation in peripheral T and B-cells in mice receiving BrdU, 2 injections/day spaced by twelve hours, during three days. Results are from one experiment out of 4 experiments giving the same results.

Cyclin box deficient mice breading

The majority of homozygous cyclin D1 box deficient mice ($D1^{-/-}$) are described to die within the first month (Sicinski et al, 1995). Female $D1^{-/-}$ have an impairment on breast development during pregnancy thought to be induced by inadequate levels of progesterone and esterogen (Sicinski et al, 1995). Also male $D1^{-/-}$ have an impairment on spermatogenesis and a reduced sperm count. Taken into consideration these phenotype conditions, the crossing of cyclin D1 box deficient mice has to be performed between heterozygotes.

The statistical results preformed over 200 $D1^{-/-}$ mice from heterozygous crosses revealed that contrary to what was previously suggested (Sicinski et al, 1995), we do not find the progeny to

be 25% $D1^{-/-}$, but rather 16 to 14%, suggesting embryonic lethality which could be explained by the severe anaemia observed in young group I mice (figure 14 C). We also found that the percentage of $D1^{-/-}$ slightly decreases from the first litter to the third litter (figure 19 A). The homozygous $D1^{-/-}$ mice were categorized within the four different groups previously described and the distribution on each group was compared between first and third litter (figure 19 B). The results reveal a striking difference between the first and third litter. The mortality of mice under 1 week of age (prior to PCR testing) doubles. Also, the generation of group I mice are four fold reduced on the third litter. Reversely, the generation of group II and group IV mice doubles. The successful study group I mice requires therefore constant renovation of the breeding pairs.

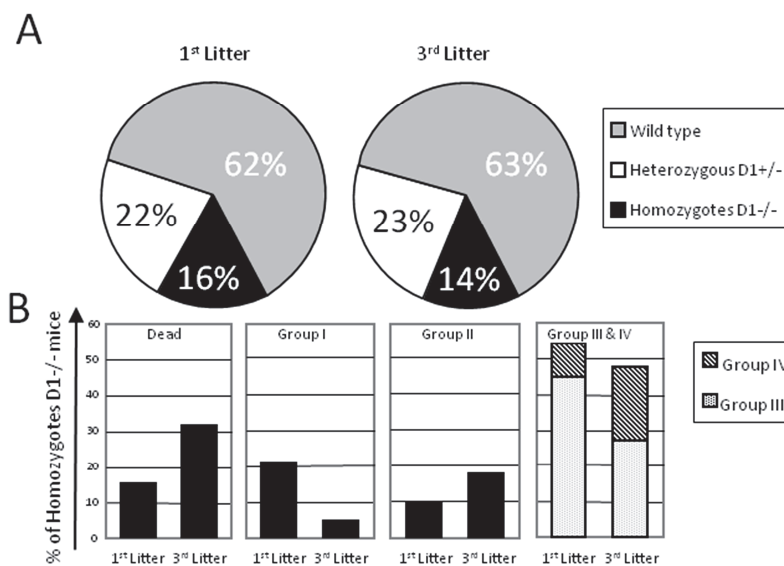


Figure 19: The offspring of heterozygous D1-box deficient mice.

(A) Relative proportion of homozygous ($D1^{-/-}$), heterozygous ($D1^{+/-}$) and wild type mice ($D1^{+/+}$) in the first and third litter (cross I and cross III). Results are from the analysis of 684 mice (B) Mortality and group distribution within the homozygous ($D1^{-/-}$) cohort on cross I and III. Results are from the analysis of 200 homozygous mice.

Expression of a truncated *CCND1* may explain the different group phenotypes

It remained unclear why we were observing these different phenotypes, so a study of the full *CCND1* mRNA was performed. No traces of coding exon 1 or of exons 2 and 3 were found on homozygous cyclin D1 knockout mice. Shockingly, the presence of a truncated *CCND1* mRNA corresponding to exons 4 and 5 was found on group II, III and IV mice. Group I had a total absence of this truncated *CCND1* mRNA. These results indicate that while the function of D1

cyclin box may be redundant, the expression of its putative regulatory domain coded by exons 4-5 would be fundamental for haematopoietic differentiation.

If this was the case, the elimination of the full cyclin D1 protein should reproduce the phenotype of group 1 mice.

Cyclin D1 as a master cyclin D regulator

The fact that Cyclin D1 was playing such a major role on lymphopoiesis continued to elude us. Cyclin D2 is known to be the D cyclin predominantly expressed on lymphoid cells (Cheshier et al, 1999). Also cyclins D2 and D3 should compensate for the absence of cyclin D1, since it is believed that individual D cyclins have largely redundant functions, their different impact in organogenesis being determined by their different tissue distribution (Pagano & Jackson, 2004; Sherr & Roberts, 2004). With this in mind we proceeded to study cyclin D family expression on the thymus and peripheral T-cells of these mice (figure 20). Strikingly, our results showed that in the absence of the regulatory domain coded by exons 4-5 of *CCND1* only trace values of cyclins D2 and D3 were found, highlighting a role for cyclin D1 as master D cyclin regulator. This further supports the profound aplasia and arrest in lymphoid lineage division in the absence of this truncated Cyclin D1 protein. In contrast, in the context of activation, cells expressing truncated *CCND1* mRNA expressed higher levels of *CCND2*, *CCND3* and truncated *CCND1* (*tD1*) than wild type mice, what could explain their higher division rates and the progressive development of lymphoid hyperplasia in mice expressing *tD1*. This compensatory effect of cyclins over-expression could be attributed to homeostatic proliferation, explaining why group II mice had higher proliferation counts (Ernst et al, 1999; Goldrath & Bevan, 1999). On group I mice, although activation induced CD69 protein up-regulation, on a similar fashion as in group II and III (not shown), *CCND2* and *CCND3* expression remained virtually inexistent. Considering the absence of all D cyclins on group I mice, it is easy to imagine that lymphoid cells would be unable to respond to exogenous differentiation signals.

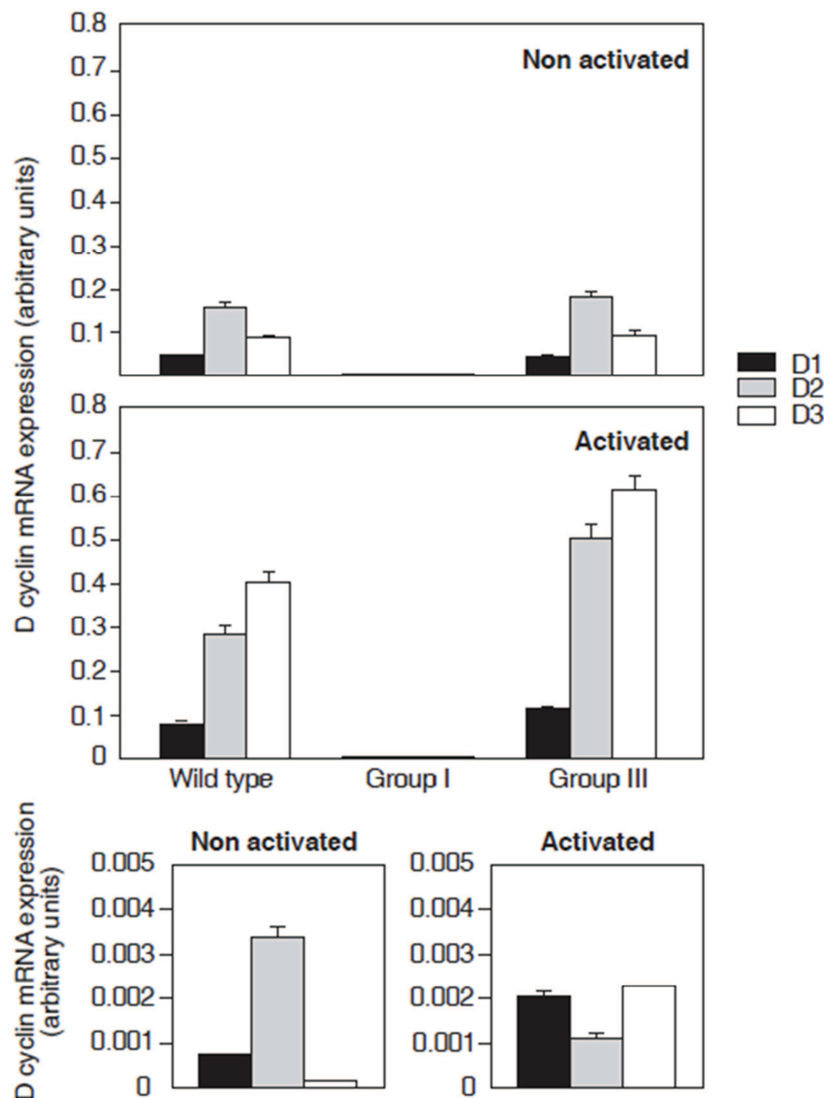


Figure 20: Quantification of D family cyclins mRNA expression in D1-cyclin box deficient mice.

Homogeneous populations of thymus and peripheral naïve CD8 T-cells were stimulated *in vitro* with anti-CD3 Abs and studied 12h later. Results show the levels of *CCND1* (D1), *CCND2* (D2) and *CCND3* (D3) transcripts before and after activation relative to the *RPII* housekeeping gene. *RPII* housekeeping gene expression levels were similar in all mouse Groups. D1 primers amplify the exons 4-5 only. On the bottom of the image it is highlighted the expression of the three cyclins on group I mice, using a scale spanning lower values. These results are representative of three independent experiments.

Cyclin distribution on different populations

D cyclins are known to be differentially expressed in mammalian cells (Matsushime et al, 1991) and *CCND1* was suggested to be highly expressed in undifferentiated cells (Bryja et al, 2008). However the expression of the three cyclins was never evaluated on the different lymphoid lineages. We set to evaluate this expression on both new-borns, 9 day old mice and adult mice. The expression of *CCND1* in new-born mice was high on the haematopoietic cells from the BM

(LSK), on the first stage of thymic precursors (TN1) and on the first stages of B-cell progenitors (Pre-Pro B and Pro B). In the rest of the populations it was virtually absent except for the last stage of Pre B-cells differentiation where some expression was rescued (figure 21 A). These results may imply that Cyclin D1 expression is necessary on the first stages of lymphopoiesis and that its role is latter taken by other D cyclins. Also in the adults, *CCND1* is only mildly expressed on these cells (figure 21 B), suggesting that cyclin D1 role may be of crucial importance on the establishment of lymphopoiesis rather than on mature cells. This idea coadunate well with the group I lymphopoiesis failure and the following rescue by compensated mice that at some point manage to express a truncated form of *CCND1* which should be sufficient for its early fundamental role in lymphopoiesis.

CCND3 seems to be stably expressed at low levels in all populations but the DP and CD4 in new-born mice (figure 21 A). On adult mice, although DP also express the highest registered level of *CCND3*, it is very low all around (figure 21 B). Finally, in new-borns *CCND2* is the most expressed cyclin mRNA on all but the DP population (figure 21 A) and on adults, *CCND2* is almost the unique cyclin mRNA expressed on all populations except on DP, pro-B-cells and on the latest stage of Pre-B-cells where there is only limited expression of any D type cyclin mRNA (figure 21 B).

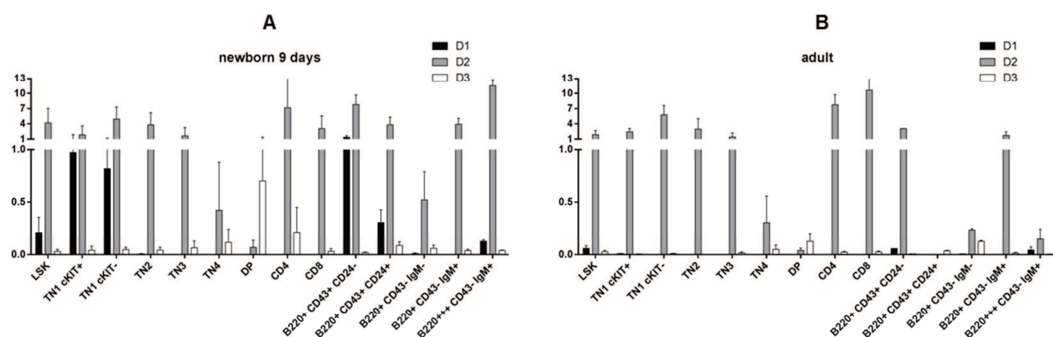


Figure 21: Expression pattern of D cyclins in different sorted cell subsets

The expression of the three D cyclins: *CCND1* (D1), *CCND2* (D2) and *CCND3* (D3) were compared by quantitative PCR analysis. On the x axis from left to right, LSK cells from BM, following lineage negative TN1 to TN4 thymocyte precursors, following DP, CD4 and CD8 cells from thymus, following pre-pro-B-cells B220^{+} CD43^{+} HSA^{-}, pro-B-cells B220^{+} CD43^{+} HSA^{+}, and the three stages of Pre-B-cell development in order of differentiation B220^{+}CD43^{-}IgM^{-}, B220^{+}CD43^{-}IgM^{+}, B220^{+++}CD43^{-}IgM^{+} from BM. On the Y axis, the relative quantification normalized to *28s* ribosomal RNA housekeeping gene. The Y axis was divided in two segments so that the very highly expressed genes could be seen without distorting the scale. The first segment 0 to 1 and the second segment 1 to 11. These numbers are relative to the expression of 28s in these cells, being 1 the actual level of 28s in the cells. Results are representative of two independent experiments.

Full *CCND1* mRNA interference

To address the hypothesis that *CCND1* exon 4 and 5 coding sequence was responsible for the early block in the differentiation observed in group I mice, we set out to eliminate full cyclin D1 protein by RNA interference both *in vitro* and *in vivo*. For this purpose we generated several lentiviral constructs expressing EGFP and small hairpin RNAs (shRNA) either nonspecific, as a control, or complementary to *CCND1* mRNA. Jurkat tumour cell line infected with these lentiviral constructs reached 66% efficiency (figure 22 A) and infected cells in one of the constructs led to the selective deletion of 90% of *CCND1* expression without changing the expression of either *CCND2* or *CCND3* (figure 22 B).

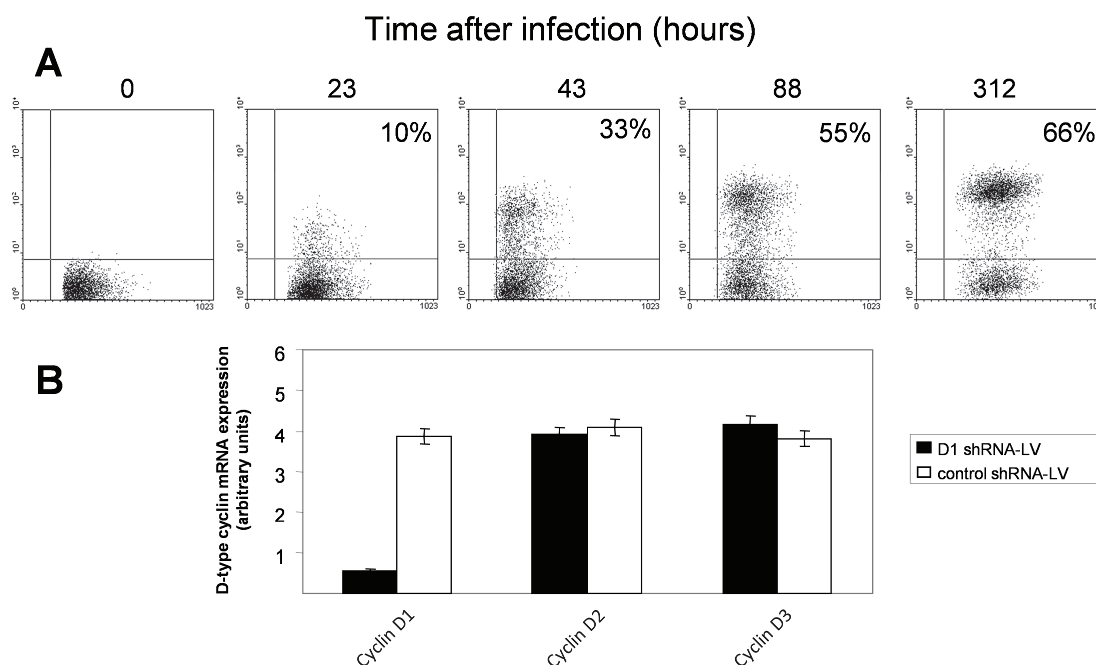


Figure 22: Efficiency and specificity of *CCND1* mRNA interference.

Jurkat-cells were transduced with control or *CCND1* (D1) shRNA-LV-EGFP. (A) Kinetics of EGFP expression on Jurkat cells after transduction. (B) Quantification of D family mRNA levels in EGFP+ cells, two weeks after infection. These results are representative of three independent experiments.

In vivo and *in vitro* shRNA assays in T and B-cell development

Infected LSKs followed *in vivo* (figure 23 A) or cultured *in vitro* (figure 23 B) in appropriate conditions, showed that the deletion of the full *CCND1* mRNAs fully reproduced group I phenotypes both *in vivo* and *in vitro*. On *in vivo* conditions, irradiated RAG mice were injected with infected LSK cells. $EGFP^+$ cells were studied 4 months after injection. *In vitro* cultures were made either on OP9 delta 4 stromal cells, expressing the notch ligand delta 4 to study T-cell development, or on the OP9 stromal cells to study the development of B-cells. $EGFP^+$ cells where then studied through time to assess B and T-cell potential.

T-cell development: (i) *in vivo*, the thymus DP compartment was reduced, arguably not at group I level but rather group II. The TN differentiation revealed a block at the TN2 differentiation stage replicating the group I phenotype (figure 23 A upper graphs); (ii) *in vitro*, LSKs cultured with OP-9 delta-4 cells, revealed a TN differentiation block even more marked, as neither TN2 cells nor DP thymocytes were generated (figure 23 B upper graphs), replicating the most severe phenotypes of group I mice shown on figure 15.

B-cell development: (i) *in vivo*, shRNA led to the accumulation of pre-pro and pro B-cells in the BM. These $B220^{+}$ $CD43^{+}$ cells are the less differentiated B progenitors, illustrating an important blockage in early stages of B-cell development. This block also affected the pre-B-cell compartment, it is visible a severe block on the cells expressing lower levels of B220 (fraction D and E as described by (Hardy et al, 1991)) (figure 5 A lower graphs); (ii) *in vitro*, the development of LSK, cultured in OP9 cells, revealed a severe block in B-cell development, as cells were unable to express B220 on days 7 and 14 and although the percentage of cells expressing B220 was rescued on day 21, these cells remained unable to express high levels of B220 (figure 23 B middle graphs), characteristic of the fraction F previously described (Hardy et al, 1991) as mature B-cells. Also, B-cell development was shown to be compromised at its earliest stage, as pre-pro B-cells expression of CD24 was severely hindered even in day 21 (figure 23 B bottom graphs).

These results demonstrate the fundamental role of the D1 protein in lymphoid development and present evidence that group III mice might be able to restore a normal phenotype by expressing a truncated form of cyclin D1 consisting of exons 4 and 5.

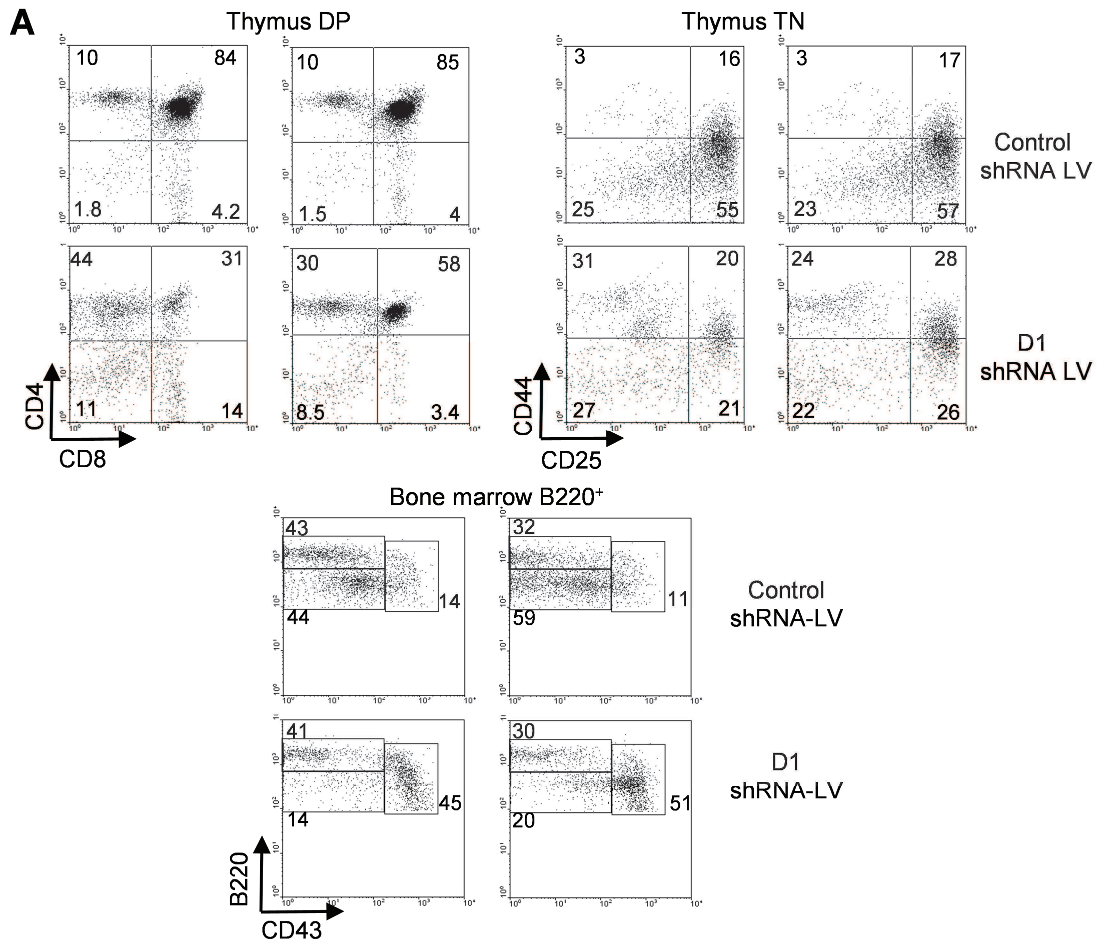


Figure 23 A: Effect of cyclin D1 RNA interference in lymphoid differentiation.

BM LSK cells were infected with cyclin D1 or control shRNA-LV-EGFP. (A) Infected LSKs were injected into lethally irradiated RAG mice and studied two months after. Top graphs, thymic populations; bottom graphs BM populations. Each plot represents an individual mouse. Results show EGFP+ thymocytes and B-cell lineage profiles.

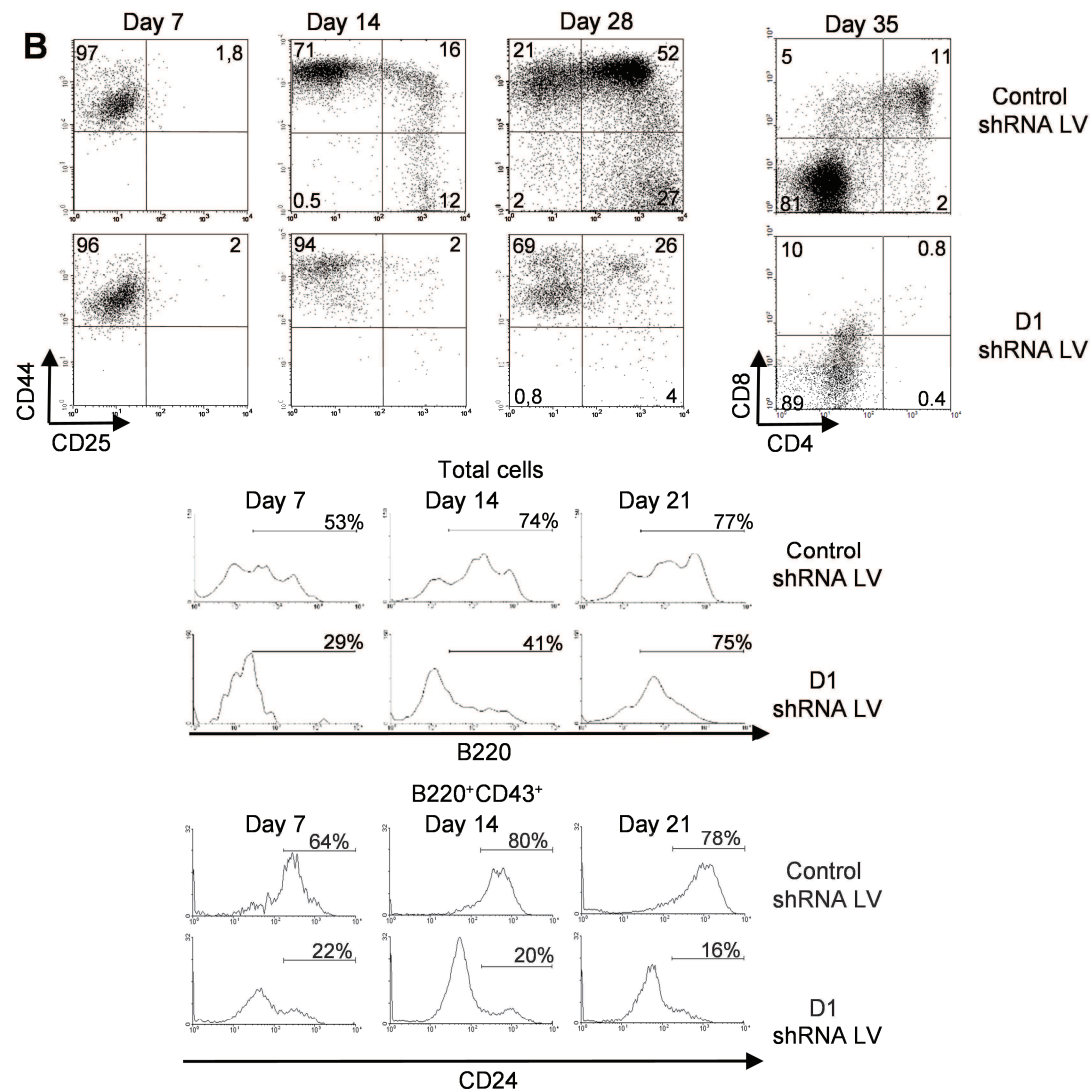


Figure 23 B: Effect of cyclin D1 RNA interference in lymphoid differentiation.

BM LSK cells were infected with *CCND1* (D1) or control shRNA-LV-EGFP. (B) Infected LSKs were cultured *in vitro*, promoting T (OP-9 delta 4) or B/myeloid (OP-9) cell differentiation and studied at different time points. Top graphs, T-cell TN population development on OP-9 delta 4 cells; middle graphs, expression of B220 on total LSK cells cultured on OP9 cells; bottom graphs, expression of CD24 within B220+ CD43+ cells . Results show EGFP+ of T and B-cell lineage profiles. These results are representative of three independent experiments.

Effect of the over-expression of exon 4-5 of the D1 cyclin

Since mice overexpressing exons 4-5 showed increased proliferation rates, we wished to identify if this was due to an homeostatic response, or if the over expression of these exons increased cell division. We set out to produce a lentiviral vector expressing exons 4 and 5 of *CCND1* (*tD1*) to study the influence of its overexpression in cell division rates, lymphoid differentiation and cancer progression in HSCs of normal mice.

A point mutation guanine was added to the start of exons 4 and 5 to produce an ATG codon in frame with the rest of the protein (described in materials and methods). The sequence was then cloned into the pMiev plasmid, under the control of a PGK promoter. This plasmid has an internal control of transfection through the expression of EGFP, allowed by an internal ribosome entry site (IRES) sequence downstream to the PGK promoter and insertion site (Leung et al, 1999).

The plasmid was then used to produce retrovirus using Platinum E (Plat-E) packaging cells (Morita et al, 2000). We started studying the effect of *tD1* in cell growth in mouse fibroblast NIH3T3 cells.

NIH3T3 were infected with approximately 90% efficiency. In these cells, we measured mRNA from *CCND1* exons 4 and 5, which amplify both the endogenous *CCND1* and the *tD1* induced by the PGK promoter. In cells infected with a vector containing this *tD1*, the expression of truncated D1 was around 100 fold higher than in non-infected cells and around 40 fold higher than that of *28s* ribosomal RNA housekeeping gene (figure 22 A).

We also set out to study if the expression of EGFP was stable throughout time and the results indicated that it didn't vary with time (figure 24 B). Also in a 50% mixture of non-infected with *tD1* infected cells or empty Miev vector infected cells, the EGFP expression didn't change with cell passages or freezing (data not shown).

The effect of *tD1* in cell growth was studied by plating serum starved cells in 24 well plates one day after infection and counting the cell number daily (figure 24 C). By allowing the cells to grow to confluence we could both assert the effect on cell growth and on cell survival. The results indicate that at early time points the empty vector infected cells have a slower growth potential than the non-infected cells, visualized by a one day delay in the growth kinetics, probably due to the strain of the viral infection. Cells infected with *tD1* seem to grow slightly slower than the empty vector infected cells showing a two day delay in the growth kinetics which could indicate that the S phase duration is increased as it is the case with *CCND1* overexpression (Yang et al, 2006b). These conclusions were drawn from day 3 and day 4 where a statistical significant difference (p=0.01) is observed between cells infected with the empty vector and the vector containing *tD1*. These differences were calculated by a mean t test on three independent assays. We also studied if the expression of this vector modified the behavior of the NIH3T3 cell line when they reached confluence (day 5). Cells infected with *tD1* did not divide further nor survived longer (figure 24 C).

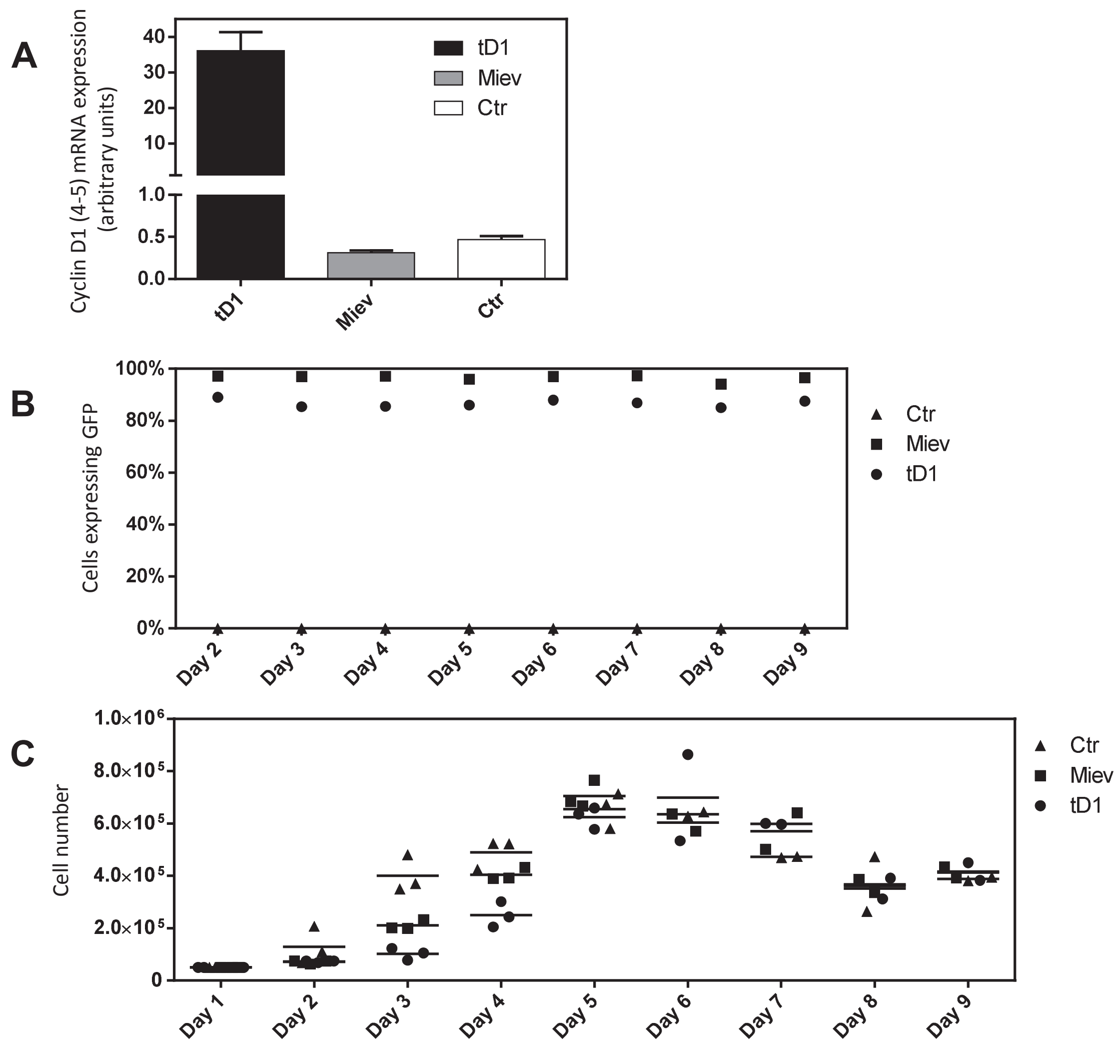


Figure 24: *tD1* expression on NIH3T3 cells

NIH3T3 cells were infected with retrovirus containing either an empty vector (Miev) or a truncated form of *CCND1* consisting of exons 4 and 5 (*tD1*). As a control non infected cells were also used (Ctr). (A) mRNA of exons 4 and 5 of cyclin D1 was analyzed by qPCR in the three populations. On *tD1* infected cells both the endogenous *CCND1* and *tD1* from the inserted vector are amplified illustrating an almost 100 fold increased expression. (B) The percentage of EGFP expressing cells is an internal control for viral infection. It is constant through the 9 days of the experiment and is around 90% for *tD1* and near 100% in Miev in all independent wells. (C) Cells from independent infections were cultured at 50 thousand per well on day 1 after infection and overnight serum starvation (0.2%). Each dot is an independent well and is plotted against the cell number on the YY axis. On day 3 and 4 statistical significant difference (p=0.01) between *tD1* and Miev can be observed.

To study the effect of this *tD1* in lymphopoiesis, LSK cells were harvested from mice injected intraperitonealy (IP) with 5-fluorouracil (5FU). The objective of this treatment was to kill all the cycling cells. The diminished compartment of LSK cells rapidly proliferates after the treatment in order to repopulate the destroyed lymphoid compartment. By doing this, the LSKs

compartment will be enriched within the total population, they will synchronize their cell cycle and they will be actively dividing. LSK cells are a primary cell type, hard to infect even with retrovirus. Cycling cells have the best infection efficiency, maximizing the infection potential of these cells. As it can be seen in figure 25, day 7 has the best percentage of cycling cells, reaching 80% and while both the percentage of the compartment within lineage negative cells and the total cell number is lower than in day 8 after injection, mice have already replenished most of the LSK compartment in day 8 and cell cycle drops considerably. On day 6 the total number of LSKs is too low, making day 7 after injection the best day to harvest LSKs to infect with retrovirus.

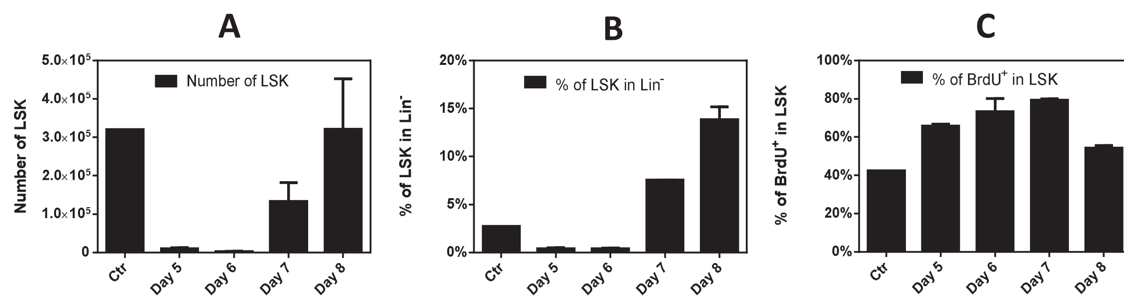


Figure 25: Effects of 5FU treatment in LSKs

Mice are weighted and injected IP with 200ul of PBS containing 0,1mg/g 5FU and studied 5 to 8 days after injection and as a control, a non-treated mouse (Ctr). (A) Total number of LSKs in the mouse. (B) % of LSK compartment within lineage negative cells (Lin^-). (C) % of LSK cells in S phase in the BM after a 4h BrdU pulse. Results are from two independent experiments.

Cells are harvested from 5FU treated mice 7 days after injection, depleted and sorted to isolate the LSK compartment. LSK cells are then infected with retrovirus containing either an empty vector or a vector containing *tD1*. None of the cyclin D1 antibodies tested worked for the *tD1* isoform. Since the only other control of infection available was EGFP and both empty vector and *tD1* express it, to separate the cells we used mice congenic markers, LY5.1 or LY5.2 to differentiate the vector used in transfection. Using this approach we can mix both LSK cells infected with the empty vector and with *tD1* after infection.

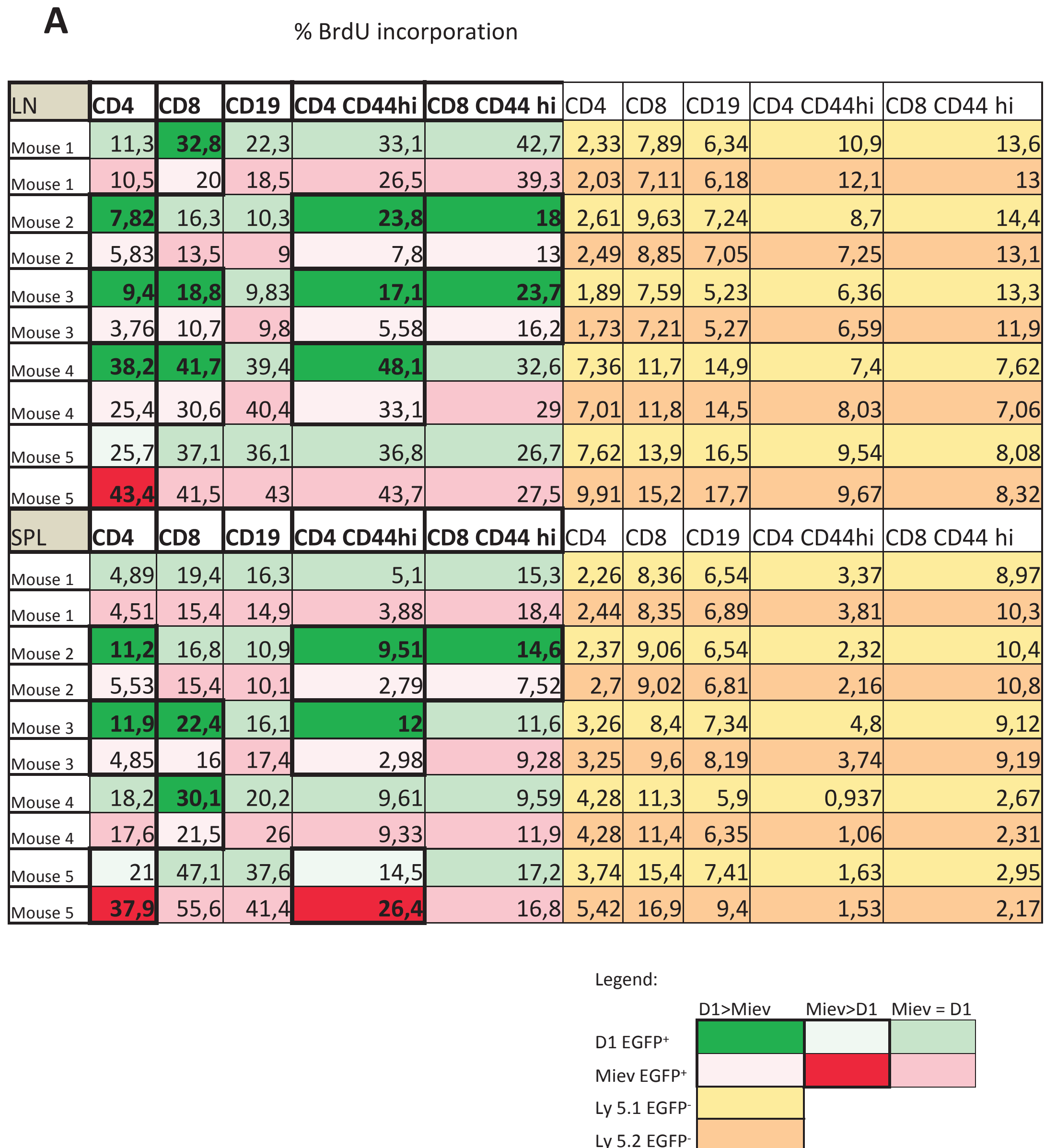
On yellow and orange are the $EGFP^-$ populations that act as internal controls for both the mixing of LSK cells and for the non-infected population. These non-infected cells are differentiated by the expression of the congenic markers Ly5.1 or Ly5.2. The majority but not all of the 5 mice seem to have a little more CD4 and CD8 cycling cells in both LN and SPL of *tD1* expressing cells (figure 26 A), but since the outcome is not regular nor very striking, it is hard to

make conclusions, with the diversity of each individual mice, even if they have the proper internal controls.

Interesting to note is that, while the internal control % of Ly5.1 and Ly5.2 is fairly close to the expected 50%, that is not the case for *tD1* 4-5 and Miev (figure 26 B). This should not be explained by the infection efficiency for the infection control of NIH3T3 had 90% infection efficiency on both vectors. These results raise the doubt on why on one hand both CD4 and CD8 infected with *tD1* have a higher percentage of cells in cycle and on the other hand cells infected with the empty vector seem to accumulate in relation to those infected with *tD1*.

On most mice, Miev seems to have an advantage over the total EGFP population, but what is more unsettling is that the relative quantity of each within $EGFP^+$ changes dramatically from LN to SPL within the same mice. The LN compartment is more devoided of *tD1* than the spleen, suggesting that these mice might have a defect on lymphoid homing caused by the overexpression of *tD1* (figure 26 B). Other than that, the specific population analysis on percentages is too variable within both mice and organs to allow us to take any interpretation of the data.

Comparing the percentages of $EGFP^+$ cells either infected with *tD1* or empty vector (figure 16 C) shows that in most mice, CD4, CD8 and CD4 $CD44^{hi}$ populations have a greater percentage of cells infected with the empty vector than *tD1*. Conversely CD19 and CD8 $CD44^{hi}$ have more *tD1* infected cells than empty vector infected cells. It is interesting to note that while CD19 cells don’t have higher BrdU staining in *tD1* infected cells, they somehow tend to accumulate on this subset. Also, it is yet more interesting that CD8 cells in the context of activation, expressing high levels of CD44 molecule, have more cells in cycle and accumulate in relation to those infected with empty vector. This suggests a role for *tD1* in CD8 T-cell activation.



A % BrdU incorporation

LN	CD4	CD8	CD19	CD4 CD44hi	CD8 CD44 hi	CD4	CD8	CD19	CD4 CD44hi	CD8 CD44 hi
Mouse 1	11,3	32,8	22,3	33,1	42,7	2,33	7,89	6,34	10,9	13,6
Mouse 1	10,5	20	18,5	26,5	39,3	2,03	7,11	6,18	12,1	13
Mouse 2	7,82	16,3	10,3	23,8	18	2,61	9,63	7,24	8,7	14,4
Mouse 2	5,83	13,5	9	7,8	13	2,49	8,85	7,05	7,25	13,1
Mouse 3	9,4	18,8	9,83	17,1	23,7	1,89	7,59	5,23	6,36	13,3
Mouse 3	3,76	10,7	9,8	5,58	16,2	1,73	7,21	5,27	6,59	11,9
Mouse 4	38,2	41,7	39,4	48,1	32,6	7,36	11,7	14,9	7,4	7,62
Mouse 4	25,4	30,6	40,4	33,1	29	7,01	11,8	14,5	8,03	7,06
Mouse 5	25,7	37,1	36,1	36,8	26,7	7,62	13,9	16,5	9,54	8,08
Mouse 5	43,4	41,5	43	43,7	27,5	9,91	15,2	17,7	9,67	8,32
SPL	**CD4**	**CD8**	**CD19**	**CD4 CD44hi**	**CD8 CD44 hi**	**CD4**	**CD8**	**CD19**	**CD4 CD44hi**	**CD8 CD44 hi**
Mouse 1	4,89	19,4	16,3	5,1	15,3	2,26	8,36	6,54	3,37	8,97
Mouse 1	4,51	15,4	14,9	3,88	18,4	2,44	8,35	6,89	3,81	10,3
Mouse 2	11,2	16,8	10,9	9,51	14,6	2,37	9,06	6,54	2,32	10,4
Mouse 2	5,53	15,4	10,1	2,79	7,52	2,7	9,02	6,81	2,16	10,8
Mouse 3	11,9	22,4	16,1	12	11,6	3,26	8,4	7,34	4,8	9,12
Mouse 3	4,85	16	17,4	2,98	9,28	3,25	9,6	8,19	3,74	9,19
Mouse 4	18,2	30,1	20,2	9,61	9,59	4,28	11,3	5,9	0,937	2,67
Mouse 4	17,6	21,5	26	9,33	11,9	4,28	11,4	6,35	1,06	2,31
Mouse 5	21	47,1	37,6	14,5	17,2	3,74	15,4	7,41	1,63	2,95
Mouse 5	37,9	55,6	41,4	26,4	16,8	5,42	16,9	9,4	1,53	2,17

Figure 26 A: *tD1* 4-5 overexpression in lymphopoiesis.

Sorted LSKs were infected with retrovirus containing either the empty Miev vector or the construct containing exons 4 and 5 of cyclin D1, mixed in a proportion 1:1 and injected I.V. into Irradiated RAG mice at 25 thousand cells per mouse. Mice were then studied 3 weeks after, following a 3 day treatment with BrdU pulse. Cells were injected immediately after the infection protocol ended, so EGFP could not be sorted and as a result the majority of the injected cells are EGFP$^-$. (A) LN and spleen (SPL) BrdU incorporation, in percentages, on several mature cells subsets. Green are the EGFP$^+$ cells which were infected with the vector containing *tD1* (exons 4-5) and the red are the EFGP$^+$ cells infected with the empty vector Miev. The separation of the two EGFP$^+$ populations is made through the congenic marker LY. To simplify the reading, the subpopulations found to have a difference greater than 25% between cells infected with *tD1* or empty vector are highlighted. These differences were highlighted by using a darker color on the subsets having >25% BrdU staining and inversely, a lighter colour was used to highlight the subsets with <25% BrdU staining. The results show that in both LN and SPL most CD4 and CD8 cells have an increase of BrdU staining. On CD19 cells no difference was observed. On yellow and orange are both EGFP$^-$ internal controls. As expected there is no difference on any of the subsets expressing different congenic markers.

B

LN	%EGFP	%D1 EGFP+	%Miev EGFP+	%Ly5.1 EGFP-	%Ly5.2 EGFP-
Mouse 1	1,35	21,3	**78,7**	52	48
Mouse 2	4,18	14,9	**85,1**	45,9	54,1
Mouse 3	2,13	9	**91**	50,5	49,5
Mouse 4	1,20	15,7	**83,7**	**35,4**	**64,4**
Mouse 5	2,50	49,9	49	55,6	42,3
SPL	%EGFP	%D1 EGFP+	%Miev EGFP+	%Ly5.1 EGFP-	%Ly5.2 EGFP-
Mouse 1	2,71	42,3	57,7	52	48
Mouse 2	5,93	10,6	**89,4**	43,7	56,3
Mouse 3	2,09	33,2	**66,8**	50,5	49,5
Mouse 4	2,60	19,8	**79,3**	36,3	62,2
Mouse 5	4,60	19,1	**79,7**	55,6	42,3

Figure 26 B: *tD1* 4-5 overexpression in lymphopoiesis.

(B) on the left, %EGFP corresponds to the percentage of total cells Ly5.1 and Ly5.2 that are EGFP^{+} three weeks after infection. Continuing from the left to the right, % *tD1* (D1) and % Miev is the relative percentage of each of those populations within the EGFP^{+} population. Results indicate that in the majority of the mice, Miev infected cells represent the majority of EGFP cells, resulting from a preferential growth over their *tD1* counterparts. On the right. %Ly5.1 and %Ly5.2 are the relative percentages of each of this GFP^{-} control population (internal control for mixture before injection) mouse 4 had a significant disproportion on the control representing a missed mix of the injected population, but taking into account this difference, the previous statement is still valid.

C

LN	%CD8 D1 EGFP+	%CD4 D1 EGFP+	%CD19 D1 EGFP+	%CD8 CD44hi D1 EGFP+	%CD4 CD44hi D1 EGFP+
Mouse 1	18,2	9,35	5,38	**16,6**	2,45
Mouse 1	**31,5**	**39,1**	**19,1**	6,47	**7,9**
Mouse 2	14,6	8,41	**8,41**	9,47	2,71
Mouse 2	**33,1**	**56,2**	5,31	10,7	**19,8**
Mouse 3	7,63	10,1	**10**	**10**	6,26
Mouse 3	**30,4**	**60,4**	2,05	7,19	**18,5**
Mouse 4	41,3	10,6	18,8	**11,5**	3,53
Mouse 4	41,6	**27,3**	22,5	7,13	**5,47**
Mouse 5	**57,6**	23,4	13,1	21,6	6,27
Mouse 5	34,5	18,2	**30,8**	16,7	**9,17**
SPL	%CD8 Miev EGFP+	%CD4 Miev EGFP+	%CD19 Miev EGFP+	%CD8 CD44hi Miev EGFP+	%CD4 CD44hi Miev EGFP+
Mouse 1	12,5	12	18,2	3,16	4,36
Mouse 1	9,69	**18,4**	**22,3**	3,63	**11,5**
Mouse 2	22,2	10,5	**12,7**	3,59	4,55
Mouse 2	24,9	**53,3**	8,19	**19,2**	**32,9**
Mouse 3	13	6,64	**17,3**	3,02	2,96
Mouse 3	16,6	**42,1**	8,99	**11**	**23**
Mouse 4	7,74	10	36,7	2,48	4,61
Mouse 4	**10,5**	**16,1**	**48,7**	2,65	**7,52**
Mouse 5	**29,1**	**14,4**	35,6	**15,6**	**4,76**
Mouse 5	4,79	4,19	54	3,25	2,19

Legend:

	D1>Miev	Miev>D1	Miev = D1
D1 EGFP+			
Miev EGFP+			

Figure 26: *tD1* 4-5 overexpression in lymphopoiesis.

(C) In this table, 5 populations of peripheral cells are depicted on LN and SPL and compared for the relative % of *tD1* population (upper row) with the Miev population (lower row) on 5 independent mice. Any difference greater than 25% is highlighted as indicated in the legend. The results illustrate variable, but in overall, both CD19 and CD8 CD44^{hi} *tD1* populations seem to have a relative increase over their Miev counterpart, where the other populations favour Miev infected cells.

Conditional knockout mice for *CCND1* exons 4 and 5:

We have shown that cyclin D1-/- mice have a considerable pre-natal and peri-natal mortality, which we associate to the lack of expression of *CCND1* exons 4 and 5. In order to demonstrate irrefutably the transcriptional role of the C' terminal moiety of this protein we envisaged the creation of a *CCND1* knockout mouse for exons 4 and 5 only.

By deleting specifically these two exons which we attribute all the transcriptional regulation functions responsible for both the lack of expression of other D cyclins and the premature block in haematopoiesis, we hope to generate group I mice phenotype only on the homozygous -/- population.

Since group I mice have such severe phenotype even with the potentially leaky expression of exons 4 and 5, such knockout mice risk to be a lethal. It is also important to consider the neurological and muscular phenotype associated to *CCND1* knockout, particularly on group I mice. To study the influence of exons 4 and 5 of *CCND1* exclusively on the lymphopoiesis, without any background of the development of other tissues, we set out to create a CRE/LOX system in which exons 4 and 5 are floxed and would be excised by CRE recombinase under specific promoters to map out at which key steps exons 4 and 5 of *CCND1* are essential for the development of lymphopoiesis. Allowing exons 1 to 3 to remain after CRE mediated excision would allow us to demonstrate irrefutably the Cdk independent role of C' terminus regulatory domain of cyclin D1.

Crossing the knockin floxed mice with mice expressing CRE under different promoters will allow us to study the importance of this putative transcription regulation site in different key steps of lymphopoiesis. For the moment, we have not obtained homozygous floxed mice expressing CRE under the Vav1 promoter. In contrast, we obtained two homozygous mice expressing CRE under the hCD2 promoter. In both mice, we found a major reduction of the CLPs (figure 27 A), in accordance to the expression of the CRE recombinase in this precursor population. Conversely, we did not had any reduction in the thymus ETPs, and in one of these mice, ETPs were actually quite increased (figure 27 B). If 4-5 has a fundamental role in haematopoiesis we expected to engage compensatory mechanisms and thus sorted ETPs from these mice to verify if they yet expressed the floxed *CCND1* 4-5 exons. Indeed, this was the case, these ETPs did express CRE, but the floxed *CCND1* 4-5 exons had not been deleted. We are presently studying individual ETPs to clarify this behaviour.

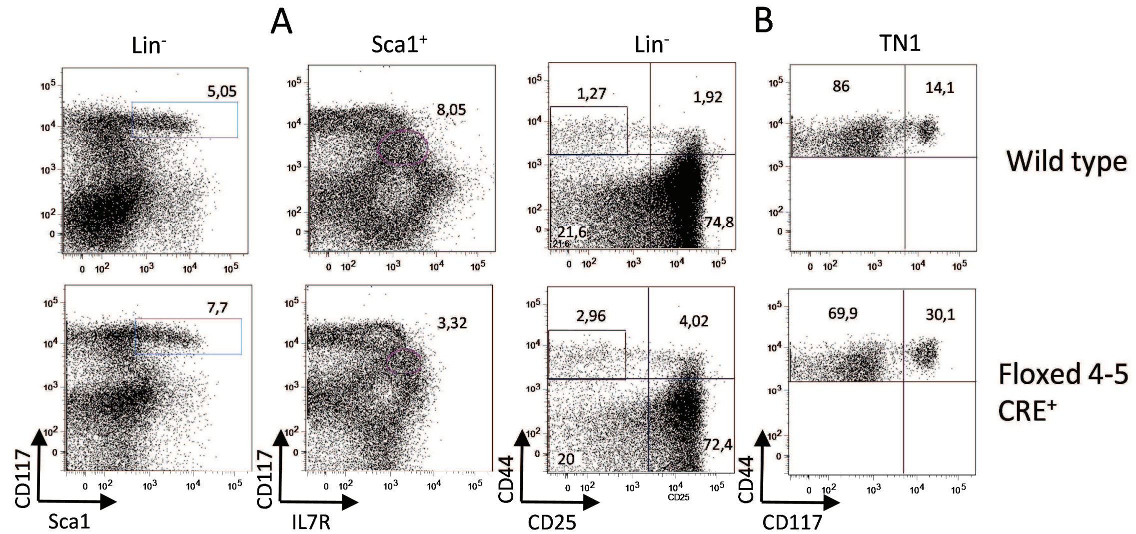


Figure 27: hCD2 promoter mediated ablation of cyclin D1 exons 4 and 5

Wild type (upper graphs) versus conditional *CCND1* exons 4-5 KO (floxed 4-5 CRE+) under the control of hCD2 (lower graphs). (A) On the left the LSK compartment on the BM shows a very slight increase on the *CCND1* 4-5 KO. On the right, the CLP compartment reveals an almost three fold decrease by *CCND1* 4-5 KO. (B) on the left the TN populations in the thymus show a two fold increase of both TN1 and TN2 by *CCND1* 4-5 KO. On the right, the CD117^{+} compartment represents the ETPs. On this particular mice, *CCND1* 4-5 KO seems to be responsible for the two fold increase on the ETP compartment.

Discussion

Overview

Our results show that cyclin D1 plays a fundamental role in early stages of haematopoiesis. In the absence of *CCND1* mice suffer from a severe lymphopenia characterised by the almost complete lack of ETPs, a great reduction of CLP, a blockage on pre-pro B-cell stage and on TN1 to TN2 stage and a total absence of DP cells. Culminating from this blockage, these mice have virtually no mature lymphocytes and the few precursor cells available are virtually non-cycling. Moreover, our data suggests that this defect on lymphopoiesis is mediated the carboxyl terminal moiety of cyclin D1 composed of exons 4 and 5. This end part of cyclin D1 is known for its regulatory functions. In mice deficient for the cyclin box (coded by exons 1 to 3) the expression or not of the last two exons explains the different phenotype of these mice. The above stated deficiencies are observed in mice that aren't able to express *tD1* mRNA, so called group I mice. Within the mice that manage to express *tD1*, group II mice have a transient phenotype characterised by the rescue of basic lymphopoiesis function and homeostatic proliferation, displaying a above normal percentage of precursor cells in the S phase. Group III mice have normal population distributions; except for a hyper-proliferation of ETPs, LSKs and CLPs. Within group III mice an additional group can be defined by a hyper-proliferation of thymocytes, displaying greater than normal cellularity.

The almost complete lack of precursor cells in S phase in group I mice is explained by the lack of not only *CCND1* mRNA expression but also by the lack of *CCND2* and *CCND3*. This demonstrates a master regulatory function of cyclin D1 in regulating the expression of other G1 Cyclins, explaining the non-redundant role of cyclin D1. Conversely, in the context of cell activation, a greater than normal expression of *tD1*, *CCND2* and *CCND3* could account for the observed increased number of cells in cycle, and the eventual development of lymphoid hyperplasia.

Group I mice have a total absence of CCR7 expression, which explains both the limited cellularity on the thymus and on the LN. Group II and III mice repopulate successfully the thymus, regaining normal organ sizes and cell rate but are unable to do so in the LN, since the expression of CCR7 and CD62L in circulating lymphocytes is never completely re-established. Alternatively, the initial lack of *CCND1* might also cause irreparable damage to the LN organ formation, explaining the low cell counts even in group III mice.

Developmental defects of the lymph nodes could be due to several reasons. The lymph node anlage requires cellular interactions between organising and inducing cell populations during

foetal life (Cupedo et al, 2004). Inducer cells are haematopoietic cells that express lymphotoxin (LT), IL-7Rα and CD4 (Chappaz et al, 2010). It has been described that a critical number of lymphoid tissue inducers (LTi) is necessary to prompt lymph node formation (Kim et al, 2000). Given the severe cellular depletion which should be even more restraint in the foetus, it could explain why this defect is observed, as inducer cells are thought to be derived from CLPs (Cherrier et al, 2012), it is possible that D1 deficiency also affects their generation.

Alternatively, even if anelage of lymph nodes is successfully initiated, the circulating T and B cells would be too phew for a correct development.

Moreover, the expression of CCR7 is known to be essential for the development and organisation of secondary lymph nodes (Ohl et al, 2003). The defect on CCR7 expression of group I mice, shown on figure 17 B could therefore also explain this developmental deficiency. The reduced number of cells found even in compensated mice could be explained by the fact that these mice all issue from group I mice earlier during foetal period.

This could present another evidence towards the idea that $D1^{-/-}$ mice have to overcome a the deficiency on expressing *tD1* during their development to become group II and then group III, since in these mice the defects on LN reconstitution could be explained by a deficiency on its development earlier on.

Cyclin D1 effect on T and B-cell differentiation was first studied on 1994 where cyclin D1 was inserted under a SRα promoter. The results obtained were that in young mice (2 week old) there was a slight decrease of both B-cells and CD4 and CD8 T-cells. The adult mice didn't have any difference and were thought to be balanced by homeostatic pressures, by the need to establish an effective immune system (Bodrug et al, 1994).

Comparing *CCND1* expression profile on several cell populations, it is observable that *CCND1* is highest expressed on young mice and within the most immature precursors. This observation taken together with the high pre-natal and peri-natal lethality observed in $D1^{-/-}$ mice suggest that the role exons 4 and 5 play in lymphopoiesis might be both very early on the mouse development and at the level of the most immature cells, explaining why its absence creates such a major blocks in differentiation, the early death of mice and the overexpression of *CCND1* exons 4-5 doesn't result on a visible phenotype. In other words, in concurrence with these results we are inclined to stipulate that cyclin D1 plays a fundamental role early on lymphopoiesis by regulating the expression of other genes, among which *CCND2* and *CCND3*.

CCND2 seems to be of particular importance since its expression levels on lymphocytes are clearly the highest among the D cyclin family.

Compensatory mechanisms

It is remarkable that group I mice can have such a strong phenotype and that group III mice are quasi normal. This suggests a compensation model that would explain why at a given moment if a mouse manages to overcome its deficiency it can harvest enough HSC to start a colonisation of all the niches that would eventually result in the complete occupancy of all the niches by a process of homeostasis. This hypothesis would explain why group 2 mice, which have managed to break the blockage, arguably by the expression of a truncated version of cyclin D1 (*tD1*) composed of exons 4 and 5, have a higher rate of proliferation than the ones from group 3 (figure 18). In other words, by a homeostatic process, the colonisation of free niches would provide a proliferative advantage (Ernst et al, 1999; Freitas & Rocha, 2000; Goldrath & Bevan, 1999) that is not necessarily related to cyclin D1 effect on the cell cycle. Following this train of thought, the advent that would permit or not the transcription of the *tD1* would occur at a given time in the development of a particular mouse, which would then evolve to a group II mouse followed by group 3 and maybe finally evolving into a group 4 mouse. The advent of group 4 mice might be explained by the over two fold proliferation rate of TN and TP populations on group 3 mice (figure 18).

A second hypothesis would be that the event that allows for the named compensation and following expression of *tD1* can however result of a genetic predisposition and each and every group would be completely unrelated, albeit with different levels of success in overcoming the hematopoietic block.

A third hypothesis could be a mixture of both the previous cases, where a group 1 mouse would have a genetic impossibility of expressing *tD1* and that all the other mice would start out as group 2 mice and proceed to group 3 once reconstitution would have been successful.

Of interest to note is that the exons 1-3 *CCND1* knockout mice was generated by the insertion of a neomycin cassette by substitution in a region immediately upstream of the ATG of cyclin D1 (Sicinski et al, 1995). This means that the entire promoter region (figure 9) remained unaltered.

There are three explanations as to why there is leaky expression of *CCND1* exons 4 and 5. (i) starting at the promoter which is unchanged on this KO, there might be transcriptional read-through of the selection marker followed by translation initiation from a downstream ATG (Muller, 1999).

(ii) There might be the use of cryptic promoters. The neomycin cassette is usually excised or at least inserted in antisense to avoid unpredictable effects in the expression of interest and of nearby genes. Neomycin resistance cassette is known to induce incorrect mRNA splicing events and also of affecting the expression of downstream genes through its promoter or cryptic promoter sites from within the neomycin resistance cassette (Haldar et al, 2008; Pham et al, 1996; Ren et al, 2002; Scacheri et al, 2001).

There are also two additional cryptic promoter sites described in human *CCND1*, one in the exon 4 (stronger) and one in the exon 5 (weaker). Both were demonstrated to be active *in vivo* in human cancer cell lines (Terrinoni et al, 2001). The resulting transcription efficiency of these two cryptic promoters was of 10% of the normal *CCND1* transcription. Also mRNA was shown to be correctly polyadenilated and therefore stable. In Humans There is only one possible ATG start site which is in exon 5 and has a strong kozak sequence. Translation from this ATG would generate a small protein of 3,39kDa (MDPKAAEEEEEEEEEVDLACTPTDVRDVDI*). Unfortunately its presence was not tested. Controlling these cryptic promoters are two binding consensus sequences: NF-κB and TP53. Seeing as though the sequences from both human and mice *CCND1* are highly homologous it is expected that these cryptic promoter regions work in the same way. Exon 4 promoter has only one bp different on mouse and the one in exon 5 is exactly the same. These cryptic promoters could not only allow for the expression of exon 5 on $D1^{-/-}$ but could also be active on normal mice, where it may have a regulatory function or compete with canonical *CCND1* for ubiquitin marked degradation, increasing canonical *CCND1* stability. Though in the mouse *CCND1*, this particular ATG is muted, there are two other ATG in frame on exon 4. The first ATG is flanked by an adequate kozak sequence, having a G in position +4 and two C in position -2 and -1. This ATG should therefore not allow transcription slippage. *CCND1* Exon 4 sequence in mouse is the following: AT.GTG.AAG.TTC.ATT.TCC.AAC.CCA.CCC.TCC.ATG.GTA.GCT.GCT.GGG.AGC.GTG.GTG.GCT.GCG. ATG.CAA.GGC.CTG.AAC.CTG.GGC.AGC.CCC.AAC.AAC.TTC.CTC.TCC.TGC.TAC.CGC.ACA.ACG.CAC. TTT.CTT.TCC.AGA.GTC.ATC.AAG.TGT.GAC.CCG. The dots represent the in frame composition.

(iii) aberrant splicing of neomycin cassette could allow the expression of exons 4 and 5 from exon 1 promoter. Although we found on most mice a larger mRNA sequence of approximately

850 bp starting on exon 1 promoter region to exon 5 in some rare mice we found an alternative splicing of the neomycin cassette. The sequencing was as follows: GCTCCGGAGACCGGCAGTACAGCGCGAGGCAGCGCGCGTCAGCAGCCGCCACCGGAGCCCAACCGAG ACCACAGCCCTCCCCAGACGCGCTCGAGGGCCCCTGCAGGTCAATTCTACCGGATGTGAAGTTCATTTC CAACCCACCCTCCATGGTAGCTGCTGGGAGCGTGGTGGCTG. The grey highlighted sequence is all that remains from the targeting vector, the rest having been spliced left exon 1 in close contact with exon 4.

A setback to the theory that exons 4 and 5 are crucial for lymphopoiesis is that the phenotype associated to cyclin D1 deficiency is rescued by substituting it with cyclin E (Geng et al, 1999). While it is known that Cyclin E expression overcomes the need for cyclin D1, playing a downstream role in the cell cycle, the lack of cyclin D1 phenotype could in this case only be explained by a epigenetic process, by which the accessibility of the chromatin region adjacent to *CCND1* locus would be assured by the expression of cyclin E. Another explanation could be that cyclin E has unknown gene regulatory functions that might be redundant to those necessary of cyclin D1 for lymphopoiesis development. Thus by expressing cyclin E under the promoter of *CCND1*, i.e. upon mitogen signalling, cyclin E would ensure cyclin D1 TF regulation role. Finally and perhaps more simple of an explanation would be that when deleting *CCND1* to knock in cyclin E, exon 5 was spared, which could perhaps account for the gene regulation functions the same way as in group II and III mice.

Master regulatory function of *CCND1* exons 4 and 5

It is very interesting to note that in group I mice, not expressing *tD1*, no other D cyclins are expressed. This process cannot be explained by epigenetics since each of the three cyclins is located on different chromosomes, but it suggests a master regulatory role of cyclin D1 on the expression of other D cyclins. While cyclin D1 might not be expressed in high level on mature lymphocytes as is seen on figure 19, it's expression is high on hematopoietic precursors, particularly in young mice. Interesting to note, neonate heterozygous $D1^{+/-}$ mice exhibit a group II phenotype. Together with the severe anaemia displayed on 2 week old group I mice which disappears on week 4, it suggests a particularly important role of cyclin D1 in the first stages of haematopoietic differentiation, explaining why there is such abnormal pre-natal and peri-natal death rates of $D1^{-/-}$ mice.

D cyclins are thought to be redundant on their role to drive G1 to S phase of the cell cycle upon mitogen stimulation. The impact that KO models have on particular organ development is

thought to be determined by tissue distribution (Pagano & Jackson, 2004; Sherr & Roberts, 2004). Although in some organs like for example on testis, D cyclins play a different role on different steps of spermatogenesis (Beumer et al, 2000). In the case of lymphopoiesis, cyclin D1 might play a unique role in the early stages of haematopoiesis and then be replaced by other D cyclins, particularly cyclin D2 which is the highest expressed on lymphocytes. Besides other regulatory functions, cyclin D1 might be able to unblock *CCND2* and *CCND3* transcription in the first stages of haematopoiesis. The absence of all D cyclins could explain the severe differentiation block observed in haematopoiesis. Expressing *CCND2* and *CCND3* might therefore be the first step towards “compensation” of the group I phenotype and the changes in cell cycle observed in figure 16 might be mediated by the overexpression of *CCND2* and *CCND3*, as can be seen on figure 18 in the context of activation.

The fact that cyclin D1 transcription regulation function would be necessary only on early stages of haematopoiesis would explains why its expression is so low on mature T-cells that it’s said to be non-existing on human CD4 and CD8 T-cells (Dunne et al, 2005; Latner et al, 2004). Of note, we find cyclin D1 expression on all haematopoietic cell subsets in mice, even if just trace amounts (figure 21).

CCND1 exons 4 and 5 overexpression

Several hypotheses can be put forward to explain why the transfection of hematopoietic LSK cells with the truncated cyclin D1 did not result in significant changes in the cell progeny (figure 26): *CCND1* gene overexpression is seen in only 15% of the of breast tumours, while the overexpression of cyclin D1 protein is found in 50% of breast tumours (Tobin & Bergh, 2012). This indicates that there is a strong proteomic control of cyclin D1. Of note also is that the effect of overexpression of Cyclin D1 remains dependent on growth factors and fails to induce *in vitro* transformation of cells (Alt et al, 2000; Quelle et al, 1993; Resnitzky et al, 1994). The reason behind this might be due to a nuclear export control and a rapid turnover via ubiquitin mediated degradation (Diehl et al, 1998; Diehl et al, 1997). This mechanism that operates via the phosphorylation of the Thr286 which is present on the truncated cyclin D1 construct may as well tightly control the availability of this protein in the nucleus as it does with the overexpression of wild type cyclin D1 (Alt et al, 2000).

Besides post transcriptional regulations, *tD1* might not be rate limiting and having already wild type cyclin D1 present might prevent us from inferring the actual role of our construct.

Also relevant for this discussion are the technical difficulties behind the setup of the experiment. While EGFP expression on infected LSK cells peeks at 48 hours after infection, it is of crucial importance to inject the infected cells immediately after infection, otherwise they will differentiate in vitro immediately after infection, for these cells begin to differentiate immediately after division. To do this, we have to renounce sorting for EGFP positive cells. By injecting the totality of the cells, we introduce a non-infected population that represents the majority of the cells, since viral infection on these cells is fewer than 10%. This population might create a high selective pressure against the infected cells which have a slightly slower cell cycle entry (figure 24 C).

From these data we conclude that the overexpression of *tD1* does not always increase the division rates of haematopoietic cells from normal mice.

Overexpression of *tD1* on NIH3T3 cells did not result decrease cell cycle time nor cell survival. In another study, overexpressing the human cyclin D1 in mice, which is highly homologues to mice cyclin D1, gave the same results, tumours could not be induced by overexpressing cyclin D1 and although successfully expressed in lymphocytes, thymus, spleen and lymph nodes were all normal in cellularity and population distribution (Lovec et al, 1994).

Cyclin D1 regulatory function

LSK cells transfected with sh-RNA for cyclin D1 show both *in vivo* and *in vitro* a retardation of the haematopoiesis (figure 23). *In vivo*, the thymus DP population was reduced and there was a block of differentiation at the TN2 stage. *In vitro*, cultures with stromal op9 delta 4 cells showed that the TN block was most severe in early time points and that the few TN2 cells present in day 28 would somehow overcome the blockage reaching complete differentiation on day 35. The fact that sh-RNA of cyclin D1 has such a strong effect on the differentiation of thymocytes shows that it's playing a crucial role on their development. As it is shown in figure 19, cyclin D2 is the most expressed cyclin on these cells and even though cyclin D1 acts as a master cyclin on group I mice, the expression of cyclin D2 and D3 is not affected by the sh-RNA (figure 22 B). This suggests that in the complete absence of cyclin D1 the entire D cyclin family

is affected, but in a model where some cyclin D1 is available (sh-RNa only selectively deletes 90% of cyclin D1 mRNA) cyclin D2 and D3 have no transcription problems. It remains unknown why cyclin D1 would be necessary for the transcription of both other D cyclins but this interference models serves a great propose on point out a unique role in haematopoiesis of cyclin D1. Alternatively cyclin D1 might only be necessary to allow the accessibility of *CCND2* and *CCND3* locus in the first stages of haematopoiesis and then no longer control *CCND2* and *CCND3* expression.

As it was depicted on the introduction, cyclin D1 plays an important role in controlling TFs in a Cdk independent way. It is of particular importance to be independent of Cdk since the *tD1* present on compensated mice, which we hypotheses as being responsible for rescuing haematopoiesis does not have the cyclin box which binds to Cdk 4 or 6 (Nugent et al, 1991). Notch-1 is the TF known to be induced by cyclin D1 that would better explain the phenotype observed in group I mice. Notch-1 KO mice are lethal but studies on conditional KO mice for Notch-1 in the retina show a similar phenotype to cyclin D1 KO (Bienvenu et al, 2010). Notch-1 is long known to be essential on the generation of T-cells but it is also important for early B-cell lymphopoiesis (Allman et al, 2002; Pui et al, 1999; Radtke et al, 2010; Santos et al, 2007; Wilson et al, 2001).

Notch-1 is known to control other TF and some of them, i.e. Creb1 and NF-κB are also involved in cyclin D1 transcription regulation (Casimiro et al, 2012; Eto, 2000; Radtke et al, 2010). NF-κB leads to cyclin D1 expression which in turn co-represses NF-κB (Rubio et al, 2012). Cyclin D1 is also known to bind the promoter of Creb1 (Casimiro et al, 2012).

Besides the interplay between TF, cyclin D1 and Notch-1 mutually regulate their expression (Bienvenu et al, 2010; Guo et al, 2009; Lindsay et al, 2008). Notch-1 is required only in a narrow window of time for the development of HSCs (Radtke et al, 2005). Cyclin D1, as figure 19 suggests might also only be required for a narrow window of time. Also disruption of Notch-1 signalling blocks T-cell development before TN2 stage, similarly to group I mice (Radtke et al, 1999; Wilson et al, 2001).

Taken into consideration, *tD1* transcription regulation of Notch-1 might be the so called compensation point that mice have to undergo to unblock haematopoiesis, bridging once again these proteins which are known to share similar effects on the development of retina and oncogenic potential on breast cells (Jadhav et al, 2006; Lindsay et al, 2008; Radtke et al, 2010). Though the effect of Cyclin D1 isn't exclusively the regulation of Notch, since in its

absence, ETPs acquire B-cell potential (Sambandam et al, 2005; Wilson et al, 2001), which dies not happen when cyclin D1 is totally ablated.

The mechanism underlying the control of transcription of other D cyclins might be explained by several hypothetical molecular networks. First, cyclin D1 binds to STAT proteins and inhibits its transcriptional activity. This effect is Cdk-independent (Bienvenu et al, 2001). Cyclin D2 promoter function depends on STAT transcription factors (Friedrichsen et al, 2003). Another Cdk-independent function of cyclin D1 refers to its activation of ER (Estrogen Receptor). This function is exclusive of cyclin D1, not shared by cyclin D2 neither D3 (Neuman et al, 1997; Zwijsen et al, 1998; Zwijsen et al, 1997). ER enhances cyclin D2 promoter activity through the cAMP response element (Kanda et al, 2004). More generally, cyclin D1 interacts with several complexes that remodel the chromatin and allow the recruitment of transcriptional initiation complex (P/CAF, members of the p160 co-activators, NcoA/SRC1a, AIB-1, TAFII250 and GRIP-1). Allowing chromatin accessibility of several genes, including *CCND2* and *CCND3* could very well explain why cyclin D1 might be so important in the first steps of haematopoiesis.

Cyclin D1 nuclear translocation

Conventional cyclin D1 nuclear translocation is described to be dependent on a NLS site present in both $p27^{Kip1}$ and $p21^{Cip1}$ proteins (Sherr & Roberts, 1999). Binding of cyclin D1 to these proteins requires both the carboxy terminal region of the protein and the cyclin box (Musgrove et al, 2011). In order to control gene transcription, *tD1* has to migrate to the nucleus. Thought tD1 might not bind to $p27^{Kip1}$ and $p21^{Cip1}$ proteins, ER, SRC1 and AR all have NLS (Picard et al, 1990). These transcription factors are known to interact with the C’ terminal moiety of *CCND1* (Musgrove et al, 2011) and thus have the possibility to bind tD1 and facilitate it’s entry to the nucleus.

Cyclin $D1^{-/-}$ breading and phenotypes

Subsequent pregnancies changes in phenotype distribution of homozygous $D1^{-/-}$ mice could be explained by two mechanisms: (i) Oocytes of older mice have significantly higher levels of histone acetylation (Akiyama et al, 2006). These high levels of histone acetylation might play an important role in the ability to transcribe exons 4 and 5 of cyclin D1, explaining the four fold

difference in group I mice that are unable to express mRNA of *CCND1* exons 4 and 5. (ii) Several human genetic diseases are known to manifest earlier and with increased symptoms from one generation to the next. This phenomenon is termed genetic anticipation. The reduced lag time for the onset of the diseases as it passes from generation to generation was explained by repeat instability. Once a repeat (usually trinucleotide repeats (TNR) for example of CTG for myotonic dystrophy (De Temmerman et al, 2004)) reaches a certain threshold number, the disease of the offspring will manifest earlier. These repeats, usually from short sequences are caused by slippage during genetic replication, a phenomenon termed dynamic mutation (Richards, 2001). It has been shown that CAG repeats increase throughout litters and this phenomenon is explained by DNA integrity instability during oogenesis (Kaytor et al, 1997; McMurray, 2010). Age related TNR instability can be generated by DNA repair and recombination activities on arrested oocytes (reviewed in (Pearson et al, 2005)). Furthermore, cyclin D1 implication on the regulation of DNA stability (Casimiro et al, 2012) and DNA repair (Jirawatnotai et al, 2011) can be argued to increase the likelihood of these mutation on heterozygous mice.

Mice harbouring the phenotype of group 1 are a minority and require close maintenance to ensure that they do not die very young. Together with the evidence that consecutive breading greatly diminish the generation of group I mice, could explain why the lymphopenic phenotype of group I mice was never described.

exons 4-5 deficient mice

Our preliminary results do not yet allow us to discriminate if the exons 4-5 have a fundamental role in haematopoiesis, or if by sharing common regulatory functions with exon 1, their expression allows the development of compensatory mechanisms. The elimination of a major fraction of CLPs when mice are crossed to hCD2-CRE mice suggest an important role, but we must before demonstrate that the exons 1-3 are yet expressed in these cells. If 4-5 has a fundamental role in haematopoiesis, capable of compensatory mechanisms in D1-/-, it would be expected a more obvious phenotype from its ablation. To confirm this ablation we performed single cell sorting on the ETPs from these mice. Indeed, we found that these ETPs did express CRE, but the floxed 4-5 exons had not been deleted. These results suggest that cells not deleting these exons (since the CRE recombinase is never 100% efficient) would be strongly selected to transit to further differentiation. However, to clarify this issue we must

quantify how many of the ETPs have these characteristics. We are therefore presently studying individual ETPs to clarify this behaviour.

Future experiments

It remains to be concluded the effect of cyclin D1 exons 4 and 5 deletion in different steps of lymphopoiesis. Having CRE expressed at different steps will provide us further understanding. Pinpointing the exact moment where exons 4 and 5 might play the role in development block that they do in D1-/- mice will bring new insights as to how cyclin D1 controls lymphopoiesis.

We are currently developing a new antibody capable to recognise exons 4 and 5 both in the D1 -/- model and on the context of overexpression with our construct. A antibody specific for C terminus will also allow us to find if in the mouse, like in human cancer cell lines, there is a natural occurring mRNA of a short isoform of cyclin D1.

Having achieved so much data upon the stages of blockage and the function of cyclin D1 in lymphopoiesis, the next step will be to investigate the expression of previously described transcription factors which either control or are controlled by cyclin D1, in the context either D1 cyclin box (exons 1-3) only or of D1 exons 4 and 5 only. We aim to understand which of the D1 exons controls the expression of these TF and to determine if the role of D1 in lymphopoiesis is mediated by controlling D2 and D3 expression or through an indirect effect of TF expression.

Finally, it remains elusive as to why cyclin D1 is more expressed on new-borns and points towards the severe phenotype on group 1 mice and also the ability to escape this deficiency after compensation as the role in cyclin D1 might be of lesser importance latter on. A detailed study on the expression, localization and epigenetics of cyclin D1 both in utero and new-born might bring forward new unexpected roles for cyclin D1 that are time arbitrated.

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Manuscript

The cyclin D1 controls the cell division and differentiation of hematopoietic lineage cells.

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SUMMARY

The different D cyclin family members are believed to have redundant functions, their role in different tissues depends on their relative tissue representation. In contrast, we here show that although the hematopoietic lineage cells predominantly express the D2 cyclin, the D1 cyclin is the master cyclin controlling hematopoieses. Indeed, in the absence of D1 either in genetically deficient mice, or after acute ablation by RNA interference, the D2 and D3 cyclins are also not expressed, resulting in a major block in cell division and in hematopoietic differentiation at its earliest stage. This role was not reported in the previously described "D1 deficient" mice, because most of these mice are incomplete KOs, expressing a truncated D1 molecule. These results indicate that not all functions of individual D cyclins are redundant, and justify for the first time the prevalent association of the D1 cyclin with lymphoid cancers.

INTRODUCTION

In mammalian cells, the progression through the G1 and S phases of the cell cycle involves the expression of D type cyclins, believed to provide the link between the environmental signals and the autonomous cell cycle machinery. The most widely accepted view is that after mitogen activation D cyclins are rapidly up-regulated and positively regulate their catalytic partners the cyclin dependent kinases (CDK) 4/6. CDK4/6 bind to the D1 cyclin box, the assembled cyclin D/CDK4/6 complexes migrate to the nucleus in mid-G1, where they phosphorylate the retinoblastoma protein (Rb). Rb hyperphosphorylation disrupts its association with E2F transcription factors family members, allowing the transcription of several genes fundamental for cell cycle progression. Later in G1, the cyclin E is up-regulated and Cyclin E/CDK2 complexes phosphorylate Rb in additional and different sites, this positive feedback determines the irreversible G1/S transition (Sherr & Roberts, 1999).

The D type cyclin family comprises three members, all having a highly homologous cyclin box, but different tissue distribution. The relative role of each family member is yet subject of debate. Several data suggest that all members have largely overlapping functions. All D cyclins associate with CDK4 and CDK6 and generate complexes that are able to phosphorylate Rb *in vitro* (Sherr, 1993; Sherr, 1996). Deficiencies in the expression of individual D cyclins were reported as not preventing mouse development, such block requires the compound deficiency of all three D cyclins (Freitas & Rocha, 2000). In this perspective, the relative role of each member of the D cyclin family would be solely conditioned by its tissue distribution. The neurologic, dental and retina defects found in the previously described “D1 deficient” mice, which do not express the exons 1-3 of the cyclin D1 (1-3 D1 deficient mice) would be the consequence of the dominant expression of D1 in these tissues (Ciemerych et al, 2002). Moreover, the only function of D1 would be the induction of the cyclin E expression, as the introduction of cyclin E into the D1 locus corrected all the abnormalities found in the 1-3 D1 deficient mice (Geng et al, 1999).

Other evidence contradicts the notion of mutual redundancy. Numerous reports described that both carboxyl and amino terminal regions of the cyclin D1 have major roles in regulating gene expression, not necessarily shared by other D cyclins, and that such roles were independent of the cyclin box and the CDK4/6 kinases (Bienvenu et al, 2010; Casimiro et al,

2012; Coqueret, 2002; Musgrove et al, 2011). By genome wide Chip analysis, D1 was found to bind up to 2840 putative sites of either proximal or distal promoters (Casimiro et al, 2012). In particular, D1 bound the promoters of abundantly expressed genes, and controlled the expression of several important transcription factors (Bienvenu et al, 2010). Among these, D1 regulates Notch1 expression, whose absence is responsible for the retina degeneration found in $1\text{-}3^{-}$ D1 deficient mice (Bienvenu et al, 2010). Notch1 also controls the expression NF-kB (Rubio et al, 2012). D1 regulatory functions include the regulation of promoter accessibility by indirectly inducing histone acetylation (Bienvenu et al, 2005; Bienvenu et al, 2010; Coqueret, 2002; Fu et al, 2004), promoting genome instability (Casimiro et al, 2012) and/or contributing to an efficient DNA repair (Jirawatnotai et al, 2011). These unique features may explain the frequent association of D1 cyclin with different types of cancer. Indeed, the very frequent cyclin D anomalies associated with carcinogenesis affect almost exclusively the cyclin D1 (Sherr, 1996). Moreover, D1 is also frequently involved in T and B cell neoplasias (Musgrove et al, 2011; Rosenberg et al, 1991), although it was shown to be poorly expressed in lymphoid cells (Cheshier et al, 1999; Metcalf et al, 2010). These data suggest that the role of D1 may not always correlate with its relative abundance, but the biological significance of such differences remains largely unknown.

To address these questions we re-analysed the mice previously described as D1 deficient (Sicinski et al, 1995). These mice were produced by the insertion of a Neomycin cassette leading to the deletion of exons 1-3 (which include the N terminal regulatory region and the cyclin box) but sparing the D1 promoter, the 5’ untranslated region, and the carboxyl terminal part of the molecule coded by exons 4 and 5, which was reported to be involved in transcription regulation (Coqueret, 2002; Musgrove et al, 2011). We here show that when these mice survive to the adult age, they are incomplete KOs expressing a truncated D1 molecule. In contrast, in mice where D1 is not expressed, there is a major block in early hematopoietic differentiation, leading to fetal and peri-natal death. Fully D1 deficient mice fail to express either D2 or D3 and their hematopoietic lineage cells hardly divide. The elimination of the complete D1 cyclin molecule by RNA interference (RNAi) in adult Lineage negative, Sca1^{+} cKit^{+} (LSKs) early hematopoietic precursors induces a similar early block in their differentiation, and prevents the generation of both T and B cells. These results demonstrate that the D1 cyclin is fundamental for lymphopoiesis, explaining for the first time the frequent association of this cyclin with lymphoid cancers.

These results suggested that the function of D1 in HLC differentiation could be mediated by exons 4-5 or, alternatively, that the expression of exons 4-5 could facilitate the development of compensatory mechanisms bypassing 1-3 functions. To investigate the role of these two exons, we generated conditional deficient mice, which after crossing with mice expressing the Cre recombinase under different promoters, would continue to express D1 exons 1-3 but not exons 4-5 (hereafter referred to as 4-5 deficient mice). Two Cre lines were used for crossing. Mice expressing Cre under the hCD2 promoter, where Cre is expressed in a fraction of CLP, TN1 and TN2 cells, but fully expressed in TN4 cells and peripheral T cells, and Vav-Cre mice, which express Cre in all hematopoietic lineage cells.

RESULTS

The phenotype of 1-3 D1 deficient mice.

We found that individual mice were highly heterogeneous. In the thymus, cellularity ranged from the most severe atrophy with thymocyte yields lower than 10^6 cells, to a thymus hypertrophy when thymocyte numbers were over 400×10^6 cells (Fig.1A). Based on the thymus size we subdivided this colony in four arbitrary groups. Group I mice had severe thymus atrophy. The thymi had very few $CD4^+CD8^+$ (double positive-DP) cells (Fig.1B, upper graphs). The distribution of triple-negative (TN: $CD4^-CD8^-CD3^-$ $Lineage^-$ thymocytes) subtypes was abnormal. In contrast to wild type (WT) mice, where the more mature $CD44^-CD25^+$ (TN3) and $CD44^-CD25^-$ (TN4) populations are prevalent (Porritt et al, 2004), TN3 and TN4 thymocytes were virtually absent (Fig.1B, middle graphs). Either the TN compartment was mostly constituted by $CD44^+CD25^-$ (TN1) and $CD44^+CD25^+$ (TN2) populations (Fig.1B), or only contained TN1 thymocytes (Supplemental Fig.1). Since the TN1 thymocytes are heterogeneous, we further quantified the early thymocyte progenitors (ETP), believed to be the progenitors of all thymocytes sets and characterized by the expression of CD24 and c-kit (CD117) (Porritt et al, 2004). We found that in Group I mice ETPs were reduced up to 50 fold when compared to WT mice (Fig.1B, lower graphs). Therefore, the 1-3 D1 deficiency may block thymus differentiation at its earliest stage. The major depletion of the ETPs further suggests that differentiation blocks may precede thymus seeding, i.e, may already be present in thymocyte progenitors located in the BM. Group II mice had moderate thymus atrophy and thymocyte differentiation blocks. The thymocyte populations were enriched in TN cells and had fewer DP cells. The block on TN differentiation occurred at a later stage, i.e., in the TN3 to

TN4 transition. Analysis of the TN1 population, however, yet showed a depletion of the ETP compartment (Fig.1B). Finally, Group III and Group IV mice appeared as having a normal thymocyte population distribution. However, the comparative analysis of the TN1 compartment showed that these mice were also abnormal. In contrast to previous groups, the ETP populations were not reduced. On the contrary, these mice had more ETPs than normal mice (Fig.1B).

In WT mice B cell differentiation in the BM progresses from pre-pro B ($B220^{low}$ $CD43^{+}$ $CD24^{-}$) to pro B ($B220^{low}$ $CD43^{+}$ $CD24^{+}$) to pre-B ($B220^{low}$ $CD43^{-}$) to B cells ($B220^{high}$ IgM^{+}) (Hardy et al, 1991). In 1-3 D1 deficient mice the distribution of these subpopulations was also quite heterogeneous (Fig.2A). Thus, Group I mice had a major pre-pro B differentiation block. Most B cell lineage cells were $B220^{low}$ $CD43^{+}$ $CD24^{-}$ i.e, the 1-3 D1 deficiency also may block the B cell differentiation at its earliest stage. Group II mice yet showed a partial pre-proB block and a reduction in pre-B cells, while in Groups III, IV B cell differentiation appeared normal.

Since both T and B earliest precursors were affected in Groups I and II mice we next studied if the differentiation block may affect earlier precursors before they are committed to differentiation into T or B cell lineage cells. Indeed, the Group I mice had rare common lymphocyte progenitors (CLPs), and in the Group II mice the number of CLPs was also reduced, albeit to lesser extent (Fig.2B). In contrast, the earlier LSK precursors were not affected. Surprisingly, further analysis of the distribution of these non-committed precursors revealed that even Group III (Fig.2B) and Group IV mice (not shown) were very abnormal. Both CLP and LSKs were actually quite increased as compared to normal mice. These results show that elimination of the exons 1-3 of the D1 cyclin gene may have two opposite effects. It can block LSK differentiation even before the lymphoid lineage commitment, and thus constitute the most precocious block hematopoietic differentiation ever described. Alternatively, it can increase LSK, CLP and ETP generation and lead to lymphoid hypertrophy. It must be noted that all these studies were performed in 4 weeks old mice, since 1-3 D1 deficient mice surviving into adult age usually express “compensated” Groups III and IV phenotypes.

The peripheral mature lymphocyte pools of deficient mice.

As expected, the peripheral lymphocyte pools of these mice were also quite heterogeneous, the number of mature T and B cells recovered in each mice correlating with the blocks found in their T and B cell differentiation in primary lymphoid organs. Thus, Group I mice showed severe lymphopenia, while the number of mature lymphocytes increased progressively from Group II to Group IV (Fig.3, left graphs). Surprisingly, we also found that lymphopenia was much more severe in the lymph nodes (LN) than in the spleen, suggesting that the 1-3 deficiency could have a further impact of in LN homing. Indeed, deficient mice either failed or expressed lower levels of CCR7 and also lacked the expression CD62L both these molecules being required for LN homing (Forster et al, 2008) (Fig3. Right graphs).

Ablation of the D1 1-3 exons also affects LSK generation.

When analyzing a 684 progeny from the crosses of heterozygous 1-3 D1 deficient mice (Fig.4), we found that the yield of homozygous mice averaged 15%, i.e. was always lower than the 25% predicted by Mendelian laws, indicating pre-natal mortality. We also observed that a substantial fraction of homozygous mice died between the 1-2 two weeks of age (when they were genotyped) and four weeks of age (the time of study). The fraction of dead homozygous mice increased with the generations reaching 30% of homozygous mice by the third litter (Fig.4, supplementary Note 1). To investigate the reasons behind this high peri-natal death rate we studied litters at 1-2 weeks of age. At this age even heterozygous mice showed abnormal population distribution, resembling that of Group II mice (not shown). All 1-3 D1 deficient mice showed the severe hematopoietic differentiation block characteristic of the Group I mice and besides they also had a severe depletion of LSKs and of both erythroid and myeloid lineage cells (Fig.2C). We conclude that the 1-3 D1 deficiency already affects LSK generation. The consequent severe anemia is likely the cause of the pre-natal and early post-natal death of a fraction of homozygous mice.

It is generally believed that individual D cyclins have largely redundant functions, their different impact in organogenesis being determined by their different tissue distribution (Pagano & Jackson, 2004; Sherr & Roberts, 2004). In this context, the D1 deficiency should have a minor impact in hematopoietic cells, which were reported to express predominantly D2 (Cheshier et al, 1999). Since not all hematopoietic subpopulations were investigated, and the relative expression of D cyclins could differ in the peri-natal and adult periods, we quantified D cyclins mRNA expression in sorted individual hematopoietic subpopulations from the BM and

the thymus, in both neonatal and 4 weeks old mice. We found that cyclin D2 mRNA (*CCND2*) was the most abundantly expressed in all hematopoietic subtypes, and that cyclin D1 mRNA (*CCND1*) was poorly expressed in adult mice. However, *CCND1* was much more abundant in neonatal mice, where it could reach almost the same expression levels as the *CCND2* in some immature sub-populations (LSKs, ETPs, pro-B cells) (Fig.5). These results explain the higher impact of the 1-3 deletion during the peri-natal period, but fail to explain why this deletion yet affects differentiation in 4 weeks old mice.

Ablation of the 1-3 exons of D1 can block or promote cell division.

To investigate the behaviour of hematopoietic cells of the different groups of deficient mice, we first studied cell death and division rates in central and peripheral lymphoid organs. Cell death (evaluated by either annexin V binding, cleaved caspase 3 detection and Bcl-2 staining) was similar to that of WT mice (data not shown) but cell division was greatly modified (Fig. 6). Group I mice showed a profound block in BrdU incorporation in T cell precursors in the thymus and in B cell precursors in the BM, mature T and B cells yet showing reduced division rates. In contrast, we found an increased BrdU incorporation in other groups (Fig.6), in particular in Group II mice, which showed maximal increase in the frequency of cycling cells, suggesting that the reduced precursor frequencies were compensated by an increase in the division rates of the remaining cells (Supplementary note 1). The increased precursor frequency, together with the increased division rates of Group III-IV mice, explain why 1-3 D1 deficient mice eventually develop lymphoid hyperplasia.

Expression of D2 and D3 cyclins in 1-3 D1 deficient mice.

The phenotypes of 1-3 D1 deficient mice were perplexing. It was not clear why Group I mice had such block in hematopoietic cells division since other D cyclins prevalent in these cells should guarantee efficient division and differentiation. On the other hand, the increased division rates found in other groups suggested some compensatory mechanism, most likely due to the over-expression of other D type cyclins. If this was the case, it was also unclear why compensatory mechanisms would be engaged in some cases and not in others. To address these paradoxes, we first quantified the expression of other D cyclin family members in these mice. To prevent any influence of their different population distribution, we studied homogeneous populations of peripheral naïve CD8 T cells in all mice, before and after activation with anti-CD3 MoAb. In Group III “compensated” mice, *CCND2* and *CCND3*

expression levels, in resting cells, were equivalent to WT controls but were up-regulated to higher levels after activation. Importantly, D2 and D3 mRNAs were virtually undetectable in both resting and activated cells from Group I explaining why the vast majority of these cells do not cycle *in vivo* (Fig.7). These results implicate the D1 cyclin in the control of the expression of the other D cyclin family members in hematopoietic lineage cells.

"Compensated" mice express abnormal amounts of a truncated D1.

We next evaluated why D2 and D3 was expressed in some but not in other mice. Since D1 is located in a different chromosome, the absence of D2 and D3 transcription cannot be explained by perturbations in the locus, known to be induced by insertion of the neomycin cassettes (Pham et al, 1996). In contrast, exons 4-5 of D1 cyclin could be involved, since these domains were shown to have transcription regulatory activities and are not deleted in 1-3 D1 deficient mice.

Several mechanisms could lead to the transcription of these two exons. Firstly, both the neomycin gene and exons 4 are known to have potential transcription initiating sites. In particular in the mouse exon 4 has an ATG in position 35, together with a functional kozak sequence allowing the inframe transcription of exons 4-5 (Supplementary Fig.2). In human, (in which D1 has 85% homology with mouse D1) both exons 4 and 5 have potential transcription initiating sites and human tumor cell lines were shown to express stable transcripts coding for two types (exons 4+5 and exon 5 alone) truncated D1 molecules. Transcription rates of truncated *CCND1* were 10% of those from the regular *CCND1* but mRNAs were correctly polyadenylated *CCND1* (Terrinoni et al, 2001). Secondly in several but not all mice, we found an identical epigenetic modification leading to the generation of alternative mRNAs where the neomycin cassette was spliced out, the initial *CCND1* 5' non-translated region was followed by a fraction of the pPNT (used in the construction of the KO mice) which was spliced into exon 4. This modification allowed the inframe transcription of both exons 4-5 (Supplementary Fig. 3). These later results suggested a strong selective pressure for cells transcribing truncated *CCND1.* We thus evaluated if exons 4-5 were expressed in these mice. In Group I mice *exon-4-5 CCND1* transcription was virtually absent, and this was not substantially modified by T cell activation (Fig.7B). In contrast, resting cells from compensated mice transcribed exons 4-5 at normal levels, and responded to exogenous stimuli by up-regulating exon 4-5 to higher levels than those found in activated WT cells (Fig. 7A). If, as found in humans (Terrinoni et al, 2001), the truncated *CCND1* is expressed at much lower levels than the full *CCND1* in WT cells, these

results indicate that compensated mice transcribe truncated *CCND1* at much higher levels than WT cells.

These results provide a possible explanation for the different phenotypes of 1-3 D1 deficient mice. In Group I mice the regulatory 4-5 D1 domains are not transcribed, what could lead to the absence of other D cyclins and thus block the capacity of hematopoietic cells to respond to the environmental signals, explaining both the major blocks in hematopoietic cells division and cell differentiation. In contrast, in response to environmental signals, the cells from Group III-IV "compensated" mice express higher levels of the D1 truncated mRNA, and much higher levels of other D type cyclins mRNAs, what could explain their higher division rates, and their progression to lymphoid hyperplasia.

Elimination of the full D1 protein mimics the Group I phenotypes.

To test this hypothesis we eliminated the full *CCND1* by RNA interference. For that purpose, we generated several lentiviral constructs expressing EGFP and small hairpin RNAs (control or complementary to *CCND1*). In the Jurkat tumour cell line, we tested their capacity to inhibit specifically the expression of *CCND1*. We selected one of these constructs, which induced the down-regulation of 90% of the *CCND1* expression without affecting *CCND2* and *CCND3* expression levels (Supplemental Fig.4). Transduced adult LSKs were followed *in vivo*, or cultured *in vitro* in appropriate differentiation conditions. The deletion of the *CCND1* indeed reproduced Group I phenotypes: *In vivo*, the thymus DP compartment was reduced, and TN differentiation blocked at the TN1-2 differentiation stages (Fig.8A), while in the BM, we found the same pre-pro-B differentiation block, pro-B and pre-B cells virtually disappearing. *In vitro*, (Fig.8B) when LSKs were differentiated into T cell lineage cells in the presence of the OP-9 delta 4 stromal cell line the TN differentiation block was even more marked, since we failed to generate TN2 cells or DP thymocytes. When LSKs were cultured in B cell lineage differentiation conditions with the OP-9 cell line, the proB-pre-B differentiation block found in Group I mice was confirmed. We found a major block in the generation of $B220^{high}$ cells. The vast majority of $B220^{low}$ cells did not express CD24. These results formally demonstrate that the expression of the D1 cyclin is fundamental for hematopoietic differentiation.

The characteristics of D1 exons 4-5 deficient mice.

Our above results suggested that while the expression of exons 1-3 could be redundant, (their role being substituted by either other D cyclins or by cyclin E), the role of the carboxyl terminal part of the molecule could be fundamental for hematopoietic differentiation. Alternatively, the expression of exons 4-5 could only facilitate the establishment of compensatory mechanisms circumventing the functions of exons 1-3. To discriminate between these two hypothesis we aimed to generate mice which expressed D1 exons 1-3 but lacked exons 4-5. Since the D1 deficiency may be lethal, we generated conditional deficient mice by introducing two LoxP sites flanking the 4th exon. In the strategy selected, after Cre recombination Exon 4 should be excised completely, while after the further splicing of exon 3 to the splice acceptor site of exon 5 introduces a stop codon, preventing the expression of the 5th exon. This strategy was designed to allow the stable expression of exons 1-3, since the both splice acceptor site and the poly adenylation sites of exon 5 are not modified what should guarantee mRNA stability. We crossed these mice with two mouse strains expressing Cre respectively under the VAV promoter, which induces Cre expression already in LSKs, and under the hCD2 promoter, in which Cre is first expressed in a fraction of CLPs, TN1 and TN2 thymocytes but in all TN4, DP and mature T cells.

DISCUSSION

The present results reveal a fundamental role of the D1 cyclin in hematopoiesis. Indeed, we found that 1-3 D1 deficient mice could exhibit a very severe block in early hematopoietic differentiation, already affecting the number of LSKs and their differentiation into the common lymphocyte progenitors and into the earliest precursors of the T and B lymphocytes. Moreover, the 1-3 deletion blocked the expression of the other cyclin D family members and abrogated the division of hematopoietic precursors. The impact of the 1-3 deletion was particularly severe in the perinatal period, correlating to expression of the D1 cyclin in hematopoietic precursors, which was much higher in neonates than in adult mice. However, the D1 cyclin was yet fundamental for hematopoiesis in adults, since the elimination of its expression by RNA interference in adult LSKs blocks T and B cell differentiation *in vivo* and *in vitro*. Since D1 is poorly expressed in adult hematopoietic precursors, the fundamental role of D1 in adult hematopoiesis contradicts the notion that individual D cyclins family members have fully redundant functions, their role depending solely in their relative tissue representation.

Overall these results reveal that D1 is a master cyclin regulating hematopoiesis, what explains for the first time the prevalent association of this cyclin to lymphoid neoplasias.

One of the roles of D1 in hematopoiesis could be indirect, through the regulation of Notch 1 expression. D1 was shown to regulate Notch1 expression, the retinal degeneration induced by the 1-3 deficiency can be explained by a defect in the expression of Notch1 (Bienvenu et al, 2010). The deficiencies in Notch1 also have a major impact in lymphopoiesis (Naito et al, 2011; Tanigaki & Honjo, 2007; Weber et al, 2011) and lymphoid neoplasias are frequently associated to modifications of Notch1 activity (Buchler et al, 2005; Ji et al, 2012; Lin et al, 2010; Wang, 2011; Wang et al, 2006). D1 may also have a direct impact in the expression of the other cyclin D family members, by inducing the accessibility of their promoters. Cyclin D1 interacts with several co-activators and complexes that remodel the chromatin and allow the recruitment of the transcriptional initiation complex (P/CAF, members of the p160 co-activators, NcoA/SRC1a, AIB-1, TAFII250 and GRIP-1) (Adnane et al, 1999; Avivar et al, 2006; Coqueret, 2002; Lazaro et al, 2002; Musgrove et al, 2011; Shao & Robbins, 1995; Zwijsen et al, 1998). It is possible that once other D cyclins are expressed, D1 is no longer required to maintain their expression. We found that in the Jurkat tumor cell line already expressing *CCND2* and *CCND3*, the expression of these two mRNAs was not affected by ablation of *CCND1* by RNA*i*, indicating that once expressed, other D cyclins may become D1 independent. However, it is also possible that this relative “D1 independence” may only occur in transformed cells.

As frequently found when mice are deprived of fundamental molecules (Sherr & Roberts, 2004), 1-3 D1 deficient mice engage compensatory mechanisms that have previously hindered the identification of the role of D1 cyclin in hematopoiesis. Indeed, although mice with lymphopenia and perinatal death were previously reported in 1-3 D1 deficient colonies, these mice were disregarded as outliers and not investigated further (Sicinski et al, 1995). The results of our breeding reveal the likely cause, since the frequency of both perinatal mortality and compensated mice increased with the generations. This phenomena, which was previously referred to as genetic anticipation, was described in several human genetic disorders associated to DNA instability (reviewed in (Pearson et al, 2005)), where the severity of the disease changes from one generation to the other probably due to errors/corrections of DNA replication or/and repair, either in the germ-line cells or in individual tissues. It is tempting to speculate that similar phenomena occur in the 1-3 D1 deficiency, since this cyclin was

implicated in regulating both DNA stability (Casimiro et al, 2012) and DNA repair (Jirawatnotai et al, 2011). Because of these changes in the severity of the disease we were forced to constantly renew breeding pairs to obtain lymphopenic mice. However, colonies are usually maintained by using the same breeding pair to produce several litters. If this common strategy was followed, lymphopenic mice in other 1-3 D1 deficient colonies could be rare.

Finally, we also described “compensated” Group III/IV 1-3 mice, which had an opposing phenotype to lymphopenic mice. Indeed, in these mice, LSKs and early T and B cell precursors were increased, hematopoietic cells division rates much increased and these mice eventually developed lymphoid hyperplasia. We found that these mice were incomplete D1 deficient, since all of them yet expressed a truncated *CCND1* coded by exons 4-5, which have transcription regulatory functions.

It is well known that the introduction of neomycin cassette as a strategy to generate deficient mice induces perturbations at and around the insertion locus in such way that current gene ablation strategies always delete neomycin inserts, what was not done in 1-3 deficient D1 mice. Both the neomycin cassette (Pham et al, 1996), exons 4 and 5 have potential transcription initiating sites and stable transcripts either from exons 4-5 or exon 5 were found “ex vivo” in tumor cell lines (Terrinoni et al, 2001). Besides, we found that several mice had developed/selected cells with the same epigenetic modification ensuring the in-frame transcription of exons 4-5. Since Abs specifically recognizing products generated by these two exons are not available, we could not evaluate if this truncated protein migrated to the nucleus in the absence of the NLS provided by the cyclin box/CDK4/6/p27$^{cip/kip}$ complexes (Sherr & Roberts, 1999). However, the existence of a CDK4/6 independent, D1 nuclear import is well known (Coqueret, 2002; Musgrove et al, 2011). Several members of the nuclear protein family were described to bind the carboxyl terminal fraction of D1 in a CDK4 independent fashion (Coqueret, 2002; Musgrove et al, 2011), and mutations of the D1 protein affecting CDK4/6 binding do not prevent nuclear import, but rather result in the retention of D1 in the nucleus (Alt et al, 2000). Thus, the truncated carboxyl terminal part of D1 has all the potential to migrate to the nucleus and regulate gene expression during hematopoiesis. It is possible that the role of D1 cyclins in hematopoieses is mediated by the exons 4-5 and not by exons 1-3. Alternatively, genetic compensation may occur, if the carboxyl and amino-terminal parts of D1 share some of the fundamental regulatory roles. In this case, the expression of the exons 4-

5 will just favor the development of compensatory mechanisms, bypassing the requirement for D1 exons 1-3. Indeed, only cyclin D1 (but not other D cyclins) is able to activate several members of the nuclear protein family as the ER (Estrogen Receptor) (Neuman et al, 1997; Zwijsen et al, 1998; Zwijsen et al, 1997). Binding of D1 to ER is CDK-independent and mediated by exon 5 (Coqueret, 2002). ER enhances cyclin D2 promoter activity through the cAMP response element (CRE) (Kanda et al, 2004). In this perspective the expression of the exons 4-5 would provide an alternative pathway to activate the D2 promoter in the absence of D1 exons 1-3, allowing hematopoietic division and differentiation. In this context, the notion that the sole role of the D1 cyclin is the induction of cyclin E may require revision (Geng et al, 1999). This notion was based on data showing that the insertion of the human cyclin E into the D1 locus rescued the neurological, retinal and dental abnormalities found in 1-3 D1 deficient mice. However, the D1 locus of these mice still has the PGK-Neo cassette, and the D1 5th exon. In these circumstances, it will be necessary to exclude that any epigenetic modification or/and the use of any of the neomycin transcription initiating sites yet allows the inframe transcription of the D1 5th exon.

To summarize, a remarkable review has previously highlighted the limitations of using genetically modified mice to study the role of fundamental genes, as the engagement of strong and abnormal compensatory mechanisms may fail to reveal the physiological role of such molecules in important processes (Sherr & Roberts, 2004). Our data strongly supports this notion, and also suggests that the acute removal of such genes by RNA interference will likely be more informative on the physiological roles of important molecules, since it reduces the time period when compensatory mechanisms may be engaged. Our finding that 1-3 D1 deficient mice are incomplete KO also highlights the dangers of not removing the complete gene when generating deficient mice. Finally, our data also tunes-down the notion that all D cyclin family members are fully redundant, their role being exclusively determined by their relative representation in different tissues. Tissue representation is indeed important, as shown by the major impact of the D1 deficiency in hematopoietic cells during the perinatal period, when this cyclin is abundantly expressed. However, adult LSK differentiation is strongly affected by the removal of D1, although this cyclin is poorly expressed by adult LSKs. The fundamental role of the D1 cyclin in heamotopoiesis explains for the first time its strong association with lymphoid transformation.

MATERIALS AND METHODS

Mice.

C57Bl6 (Ly5.2) and Rag-2 deficient mice with the C57Bl6 background (Ly5.1) were bred at the Center for Development of Advanced Experimentation Techniques, Orleans, France. Mice deficient in the exons 1-3 of the D1 cyclin were kindly provided by Dr. Sicinsky.

The breeding of 1-3 D1 deficient mice.

Since these homozygous females are incapable of nursing due to an impaired mammary gland development, and the homozygous males have low sperm counts, (ref Sicinski Cell 1995, Fantl Genes & Dev 1995), 1-3 D1 Deficient mice were obtained by crossing heterozygous mice, in conditions we adapted for the D1 deficiency. Briefly, the breeding was done in isolators and in continuous direct supervision. Since mothers frequently kill fragile pups, or/and WT littermates outcompete them for feeding, at 1 week of age pups were selected by the presence of neurological/retinal abnormalities. WT or heterozygous mice were transferred to foster mothers, in such way that the D1$^{+/-}$ mother only nursed the homozygous offspring. We found that the neurological abnormality of 1-3 D1 deficient mice prevent them to climb to reach food and water, while the teeth abnormality prevented the ingestion of solid food . Thus, when pups reached three weeks of age, wet soft food was directly provided in the litter and teeth were cut regularly. Finally, couples were maintained only for two litters. When using these conditions D1 deficient mice reached normal body weight by 6 weeks of age. It must be noted that these breeding conditions are fundamental to obtain Groups I and II mice. Otherwise, most homozygous mice are of smaller size, frequently die before weaning and the few survivals have compensated phenotypes. All experiments were approved by the Ministère de l'Agriculture, de la Pèche et de l'Alimentation (France).

Antibodies and flow cytometry.

The following mAbs used for flow cytometry and/or cell sorting were obtained from BD Pharmingen: anti-CD4 (GK1.5), CD3 (145-2C11), CD8 (53.6.7), CD44 (IM7), CD25 (PC61), CD24 (J11D), CD117(3C1), B220 (RA3-6B2), CD43 (S7), anti-erytroid (Ter119), GR1 (RB6-8C5), Sca-1 (CT-6A.2), IL-7R (A7R34), Ly5.1(A20-1.7) and Ly5.2 (104-2.1). Polyclonal goat anti-mouse IgM and monoclonal rat anti-mouse IgD (11-26) Abs was obtained from Southern Biotechnologies.

These antibodies were either directly conjugated to FITC, PE, APC, PerCP or biotin, these later being revealed by streptavidin-APC or PerCP5.5. To study cells in S phase in central lymphoid organs, mice received two i.p. injections of 1mg of BrdU (Sigma) at a 4 hours interval. To analyse proliferation in peripheral organs, they were injected with 1mg of BrdU every 12h during 3 days. BrdU incorporation was detected as previously described (Lucas et al, 1993) using a FITC-conjugated anti-BrdU antibody (BD Pharmingen).

shRNA and primers.

Oligonucleotides were purchased from Invitrogen Life Technologies (Cergy Pontoise, France). Forward sequences of small hairpins used are the following:

D1 (646) 5' cgcgtccccctgggcagccccaacaacttcaagagagttgttggggctgcccaggtttttggaaaT 3'

Control 5' cgcgtccccacgggccgcccttaatacattcaagagatgtattaagggcggcccgtttttggaaat 3'

Forward and reverse oligonucleotides were annealed in 500 mM potassium acetate, 150 mM Hepes pH 7.4, 10 mM magnesium acetate. shRNA sequences were designed in a first instance by using ambion free-software (no longer available) and selected according to the rules reported to predict efficient sequences (Reynolds, Nat Biotech, 2004,326; Kim, Nature Biotech, 2005, 222; Siolas Nature Biotech, 2005, 227; Grimson, Mol Cell, 2007,91; Ui-Tei, NAR, 2004, 936; Yuan, NAR, 2004, W130; Jagla, RNA, 2005, 864 and Shabalina, BMN, 2006, 65)

Primers used for quantitative real time PCR were selected in order to include at least one intron in the amplicon, no match with other murine genes by standard blast, no genomic amplification and for identical amplification efficiency. Sequences (5' to 3') and amplicon sizes between brackets were the followings:

muRNA polymerase II: forward: catctcagaatgctagcacca; reverse: tgaatccaaaggtcgtttctg (92 pb)

muCyclin D1: forward: tgctgcaaatggaactgctt ; reverse: ccacaaaggtctgtgcatgct (150 pb)

muCyclin D2: forward: caccgacaactctgtgaagc; reverse: atgaagtcgtgaggggtgac (102pb)

muCyclin D3: forward: ctacttccagtgcgtgcaaa; reverse: agccagagggaagacatcct (115 pb)

muCyclin D1(exon4-5): forward: cccaacaacttcctctcctg; reverse tcagatgtccacatctcgca (231pb)

Quantitative RT-PCR was performed as previously described (Peixoto et al, 2004). Expression levels for all genes were calculated in arbitrary units relative to the housekeeping gene. Standard deviations were calculated from triplicates.

Production Lentivirus vectors.

We used the pLv-HTM plasmid (kindly provided by Dr. Trono Didier, Geneva University) which is a self-inactivation third generation HIV1-derived vector (Wiznerowicz & Trono, 2003). The annealed oligonucleotides coding for shRNA were ligated into *ClaI* and *MluI* double-restricted plasmid by standard cloning procedures, using restriction enzymes and T4 DNA ligase (New England BioLabs Inc). In these conditions, the production of siRNA is under the control of H1 (RNA polymerase type III) promoter, and the reporter gene, the green fluorescent protein (GFP) is expressed under the control of eukaryotic EF-1a promoter. All plasmids were verified by sequence analysis. Lentivirus particles were produced by transient transfection of 293T cells according standard protocols. Briefly, subconfluent 293T cells were co-transfected with 20 µg of plasmid vector, 15 µg of pCMVR8.74 and 5 µg of pMD.G envelope (VSVG) by calcium phosphate method. After overnight incubation, media was changed by fresh one and supernatants harvested at 48 and 72 h. Supernatants were further concentrated (100X) by ultracentrifugation, titrated and stored at -80°C until use. Viral titers expressed as TU/ml were determined by assessing transduction of 293T cells with serial dilutions of virion preparations. Batches with titers $\geq 10^8$ TU/ml were used.

Purification, transduction and differentiation of hematopoietic progenitor cells.

C57BL6 Ly5.2 mice were treated intravenously with 100mg/kg 5FU (TEVA Pharma), bone-marrow cells recovered at day 5 and depleted of Lineage (Lin) positive cells with a cocktail of rat mAbs CD11b (M1/70), CD8 (Lyt2), CD5 (Lyt1), CD45R/B220 (RA3-6B2), Gr-1/Ly6G (RB6-8C5), Ter119 (Ly76). and anti-rat IgG conjugated dynabeads (Dynal). Hematopoietic progenitors were further enriched by using Spin Sep Purification kit from Stem Cell Technologies following manufacturer instructions. Routinely, using this double step procedure, $Lin^-ckit^+Sca\text{-}1^+$progenitors were enriched 200 times from ($0.17 \pm 0.13\%$) to ($34 \pm 11\%$) (n=12) of total bone marrow cells with a recovery of 80% of the initial progenitor cells after the procedure.

Transduction of enriched progenitors was performed in the presence of 5µg/ml of protamine sulphate (Sigma) and 10µg/cm2 coated RetroNectin® (Takara). Briefly, 10E6 enriched progenitors were plated in 96 well plate in 200 µl of complete RPMI media (Gibco) in the presence of 10ng/ml mu-SCF, 50ng/ml TPO, 100U/ml muIL-6, 10ng/ml muIL-11 and 5ng/ml muFlt3-L, all cytokines from R&D Systems. Virus supernatants were added at a multiplicity of infection MOI=10. Plates were centrifuged at 1000g during 1h at 20°C and further cultured at

37°C for other 4 hours. At the end of this 5 hours transduction protocol, cells were extensively washed and injected into lethally irradiated Rag^{-} mice or culture “in vitro”. Myeloid and B cells development was performed by co-culture of these progenitors on OP9 stroma cell line whereas T cell development was induced by co-culturing progenitors on OP9-DL4 stroma as previously described by Schmitt and Zuniga-Pflucker (Schmitt & Zuniga-Pflucker, 2002). Briefly, OP9 or OP9-DL4 cells were seeded into 6-well tissue culture plates the day before the co-culture with progenitors. All cultures were performed in presence of 1ng/ml rmuIL-7 and 5ng/ml Flt3-L (both cytokines from R&D Systems) and fed every 4 days. At the indicated time points, cells were harvested, counted and their phenotype evaluated by cytometry.

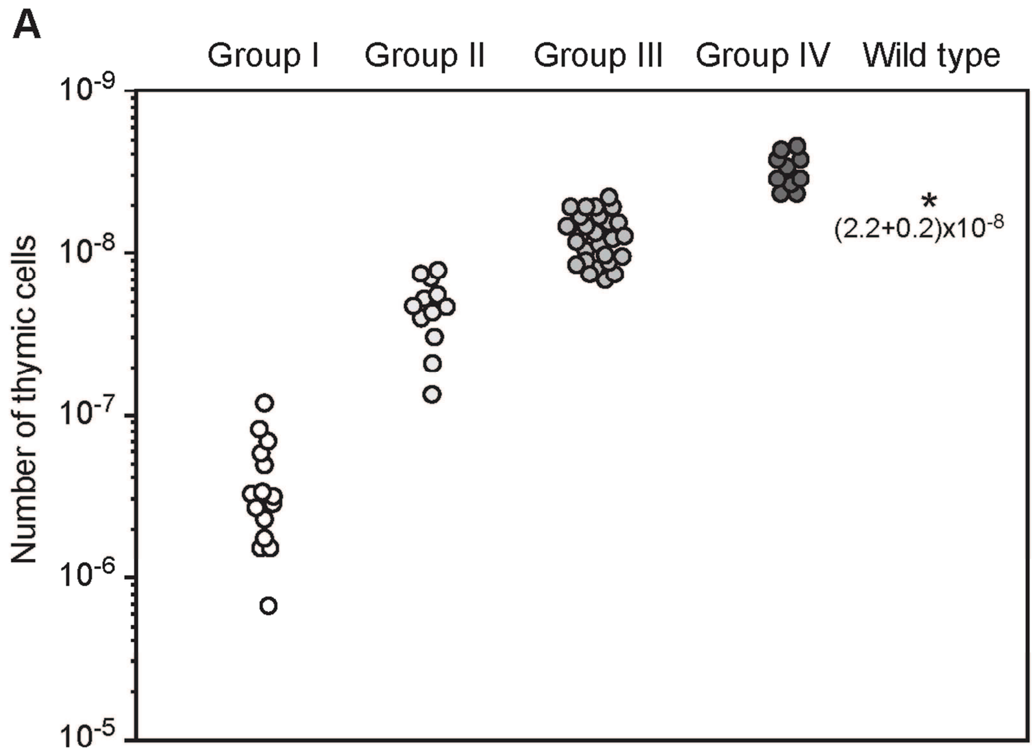
Statistic analysis.

Paired student’s t-test was performed to calculate statistic significance. Differences were considered significant when $p<0.05$.

ACKNOWLEDGMENTS AND AUTHORS CONTRIBUTIONS

We thank the advices of: Claude-Agnès Regnaud in the design of our constructs; Daniel Compagno in the selecting sequences for RNA interference: Sophie Ezine in lymphoid differentiation cultures and Jacinta Bustamante in sequencing. This work was supported by a grant from the Agence National de la Recherche, France. M. Ferreira, G. Krenn and F. Vasseur performed experiments, F. Vasseur also supervised the breeding of 1-3-D1 deficient mice and performed all the sortings. D. Laderach performed experiments, oriented the study and supervised directly the other authors. B. Rocha supervised the study and wrote the MS.

FIGURES



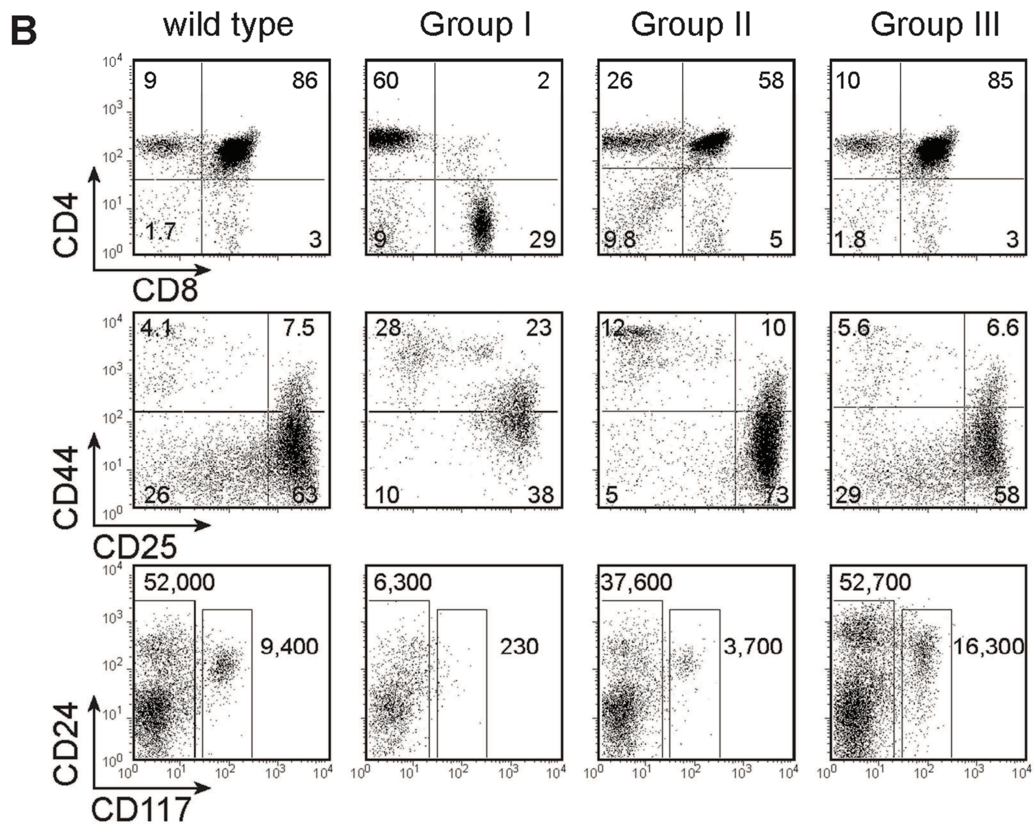


Figure 1

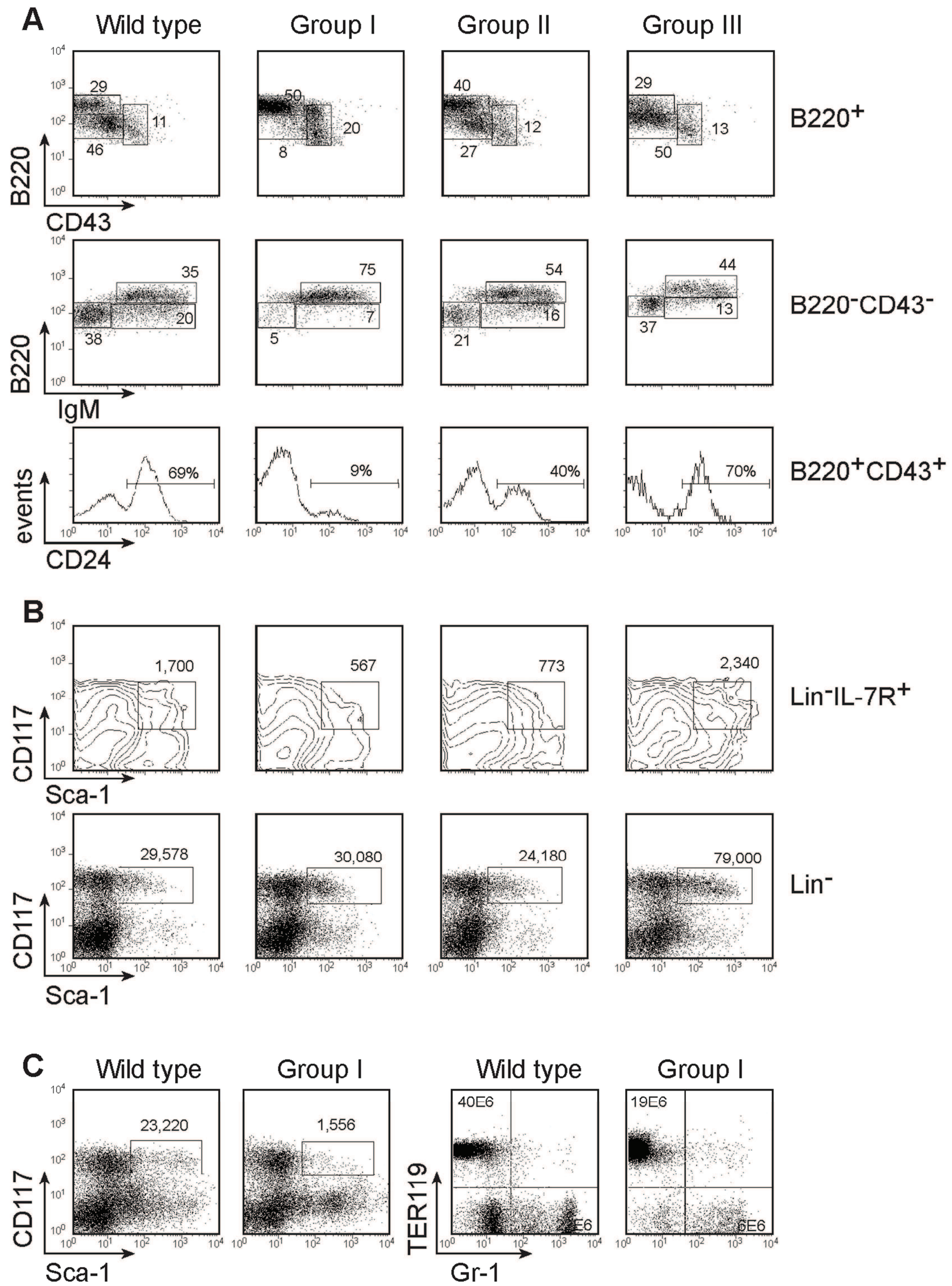


Figure 2

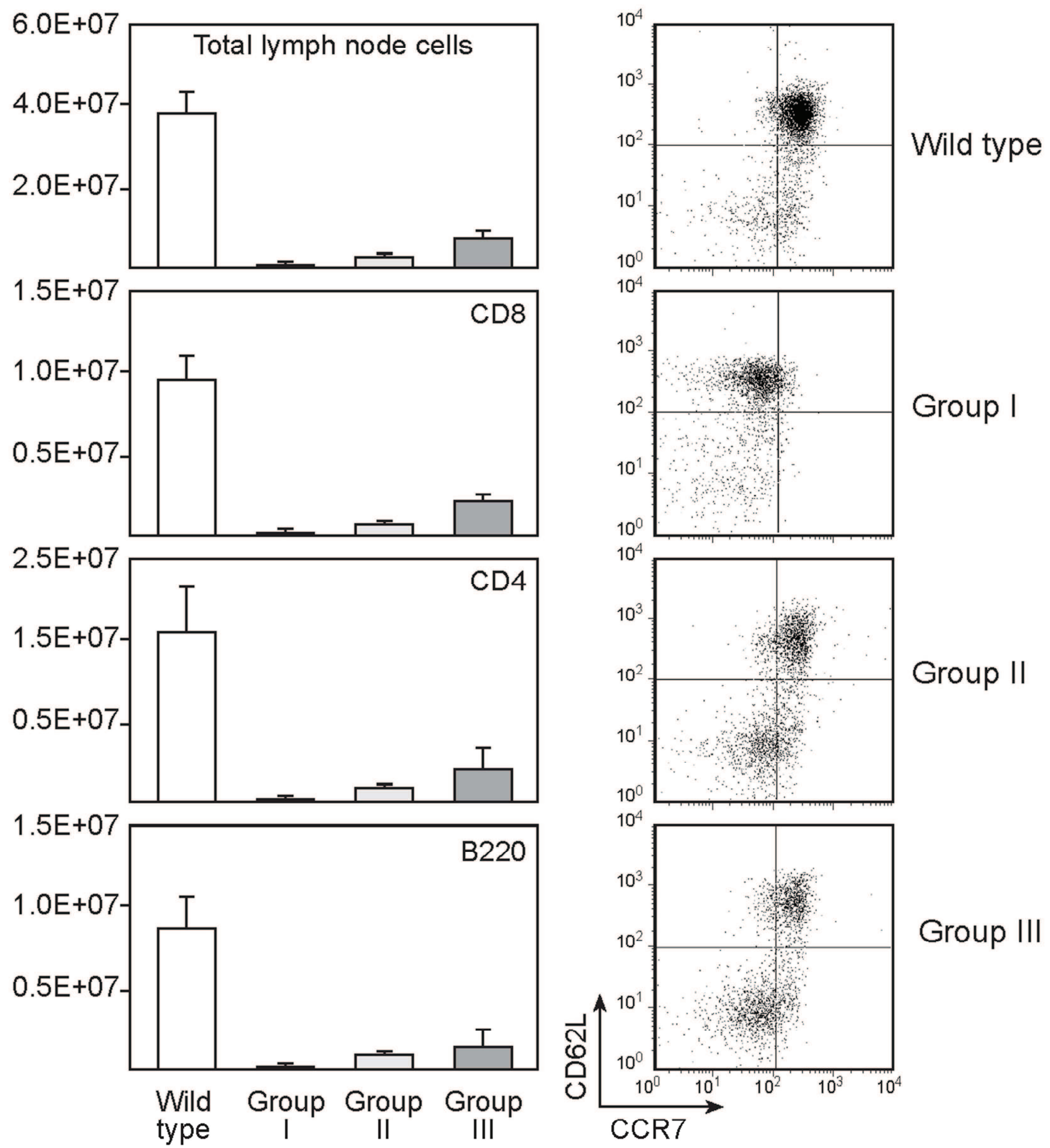


Figure 3

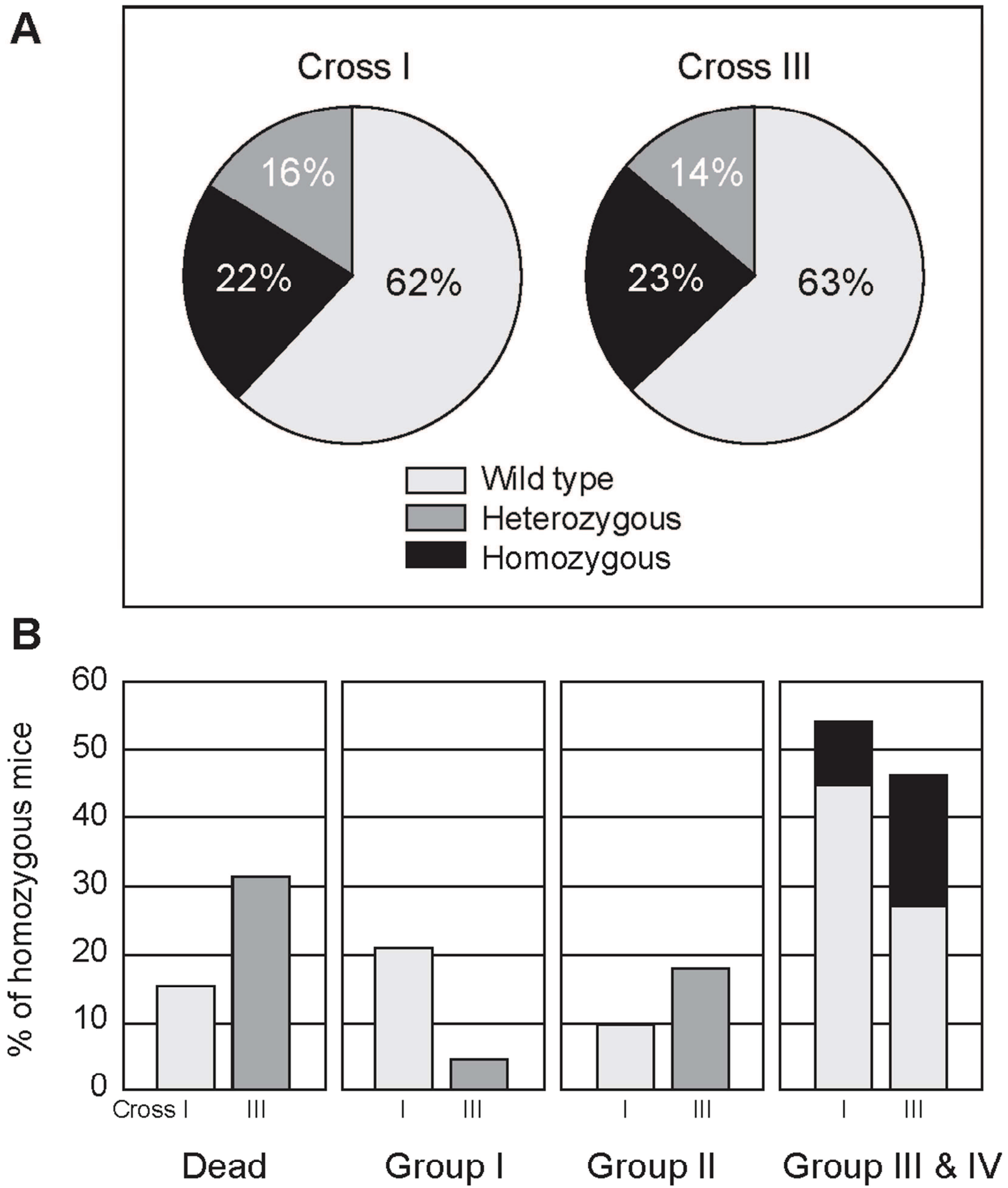


Figure 4

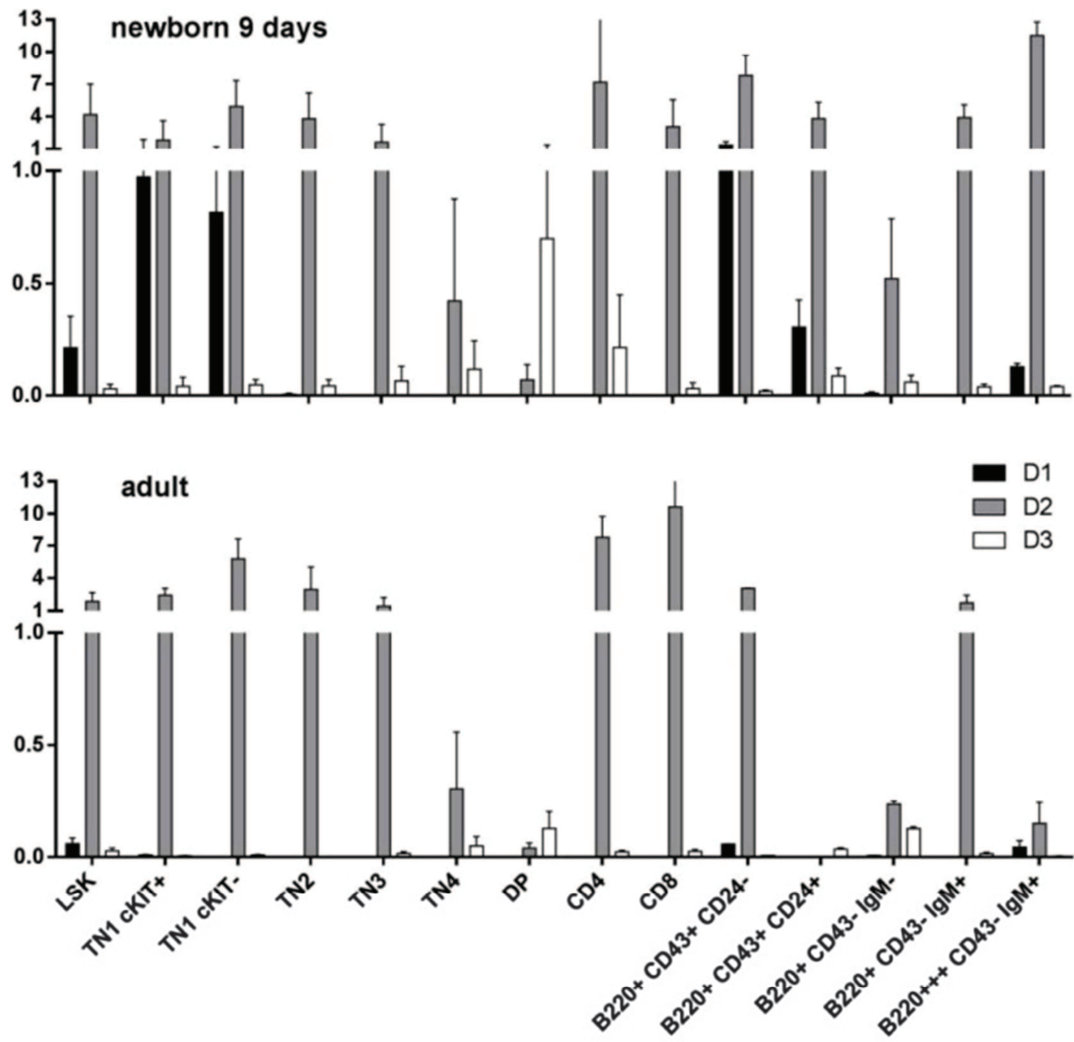


Figure 5

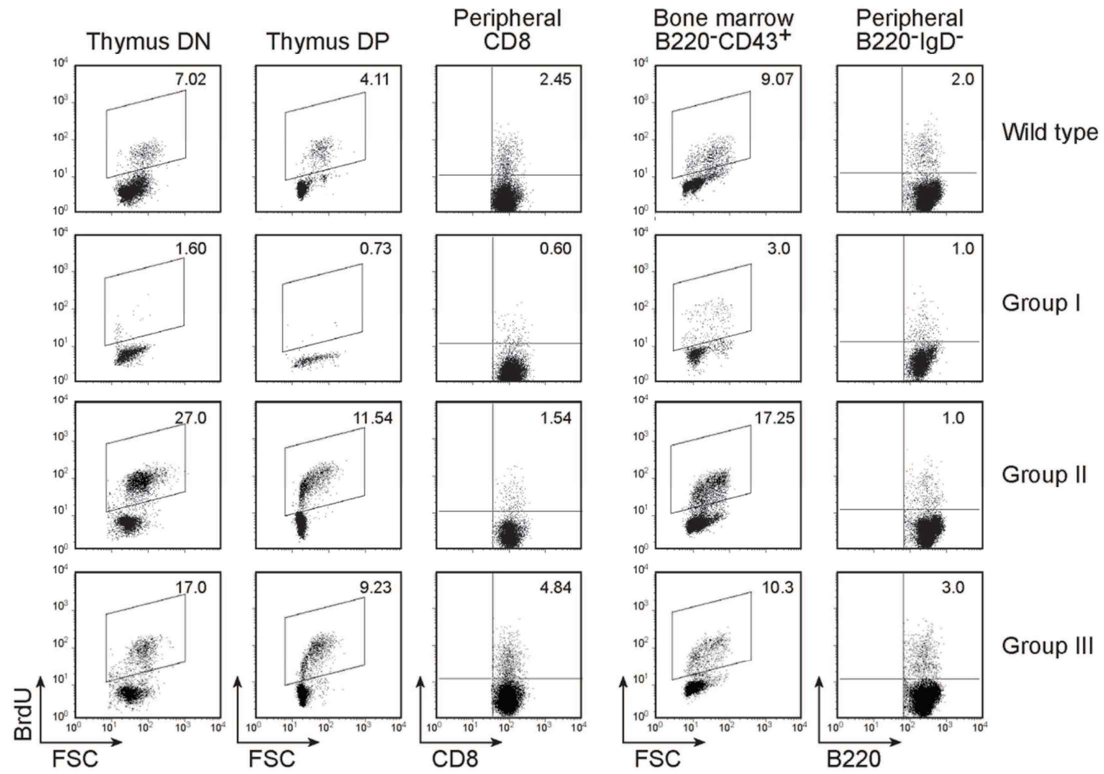


Figure 6

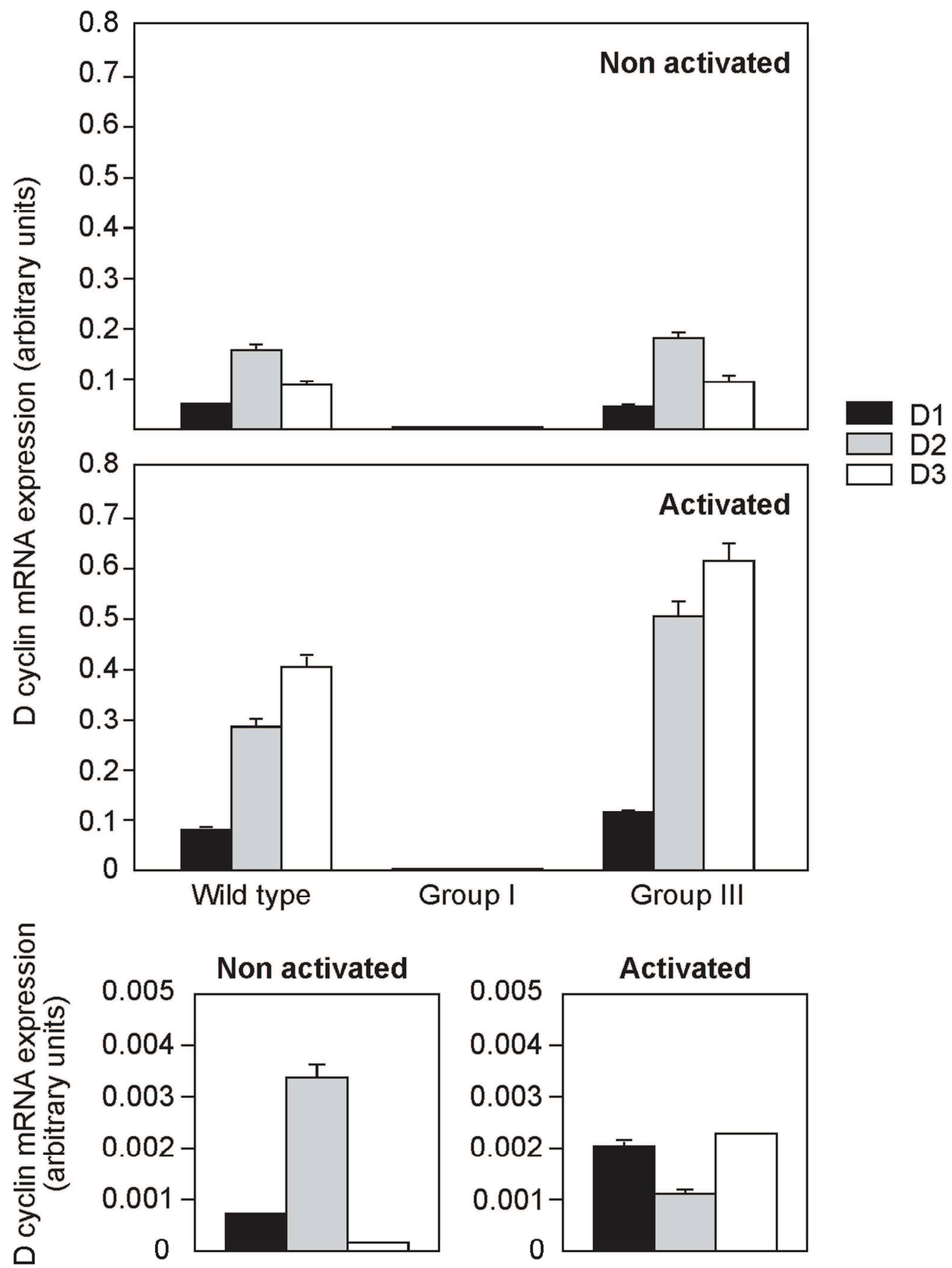


Figure 7

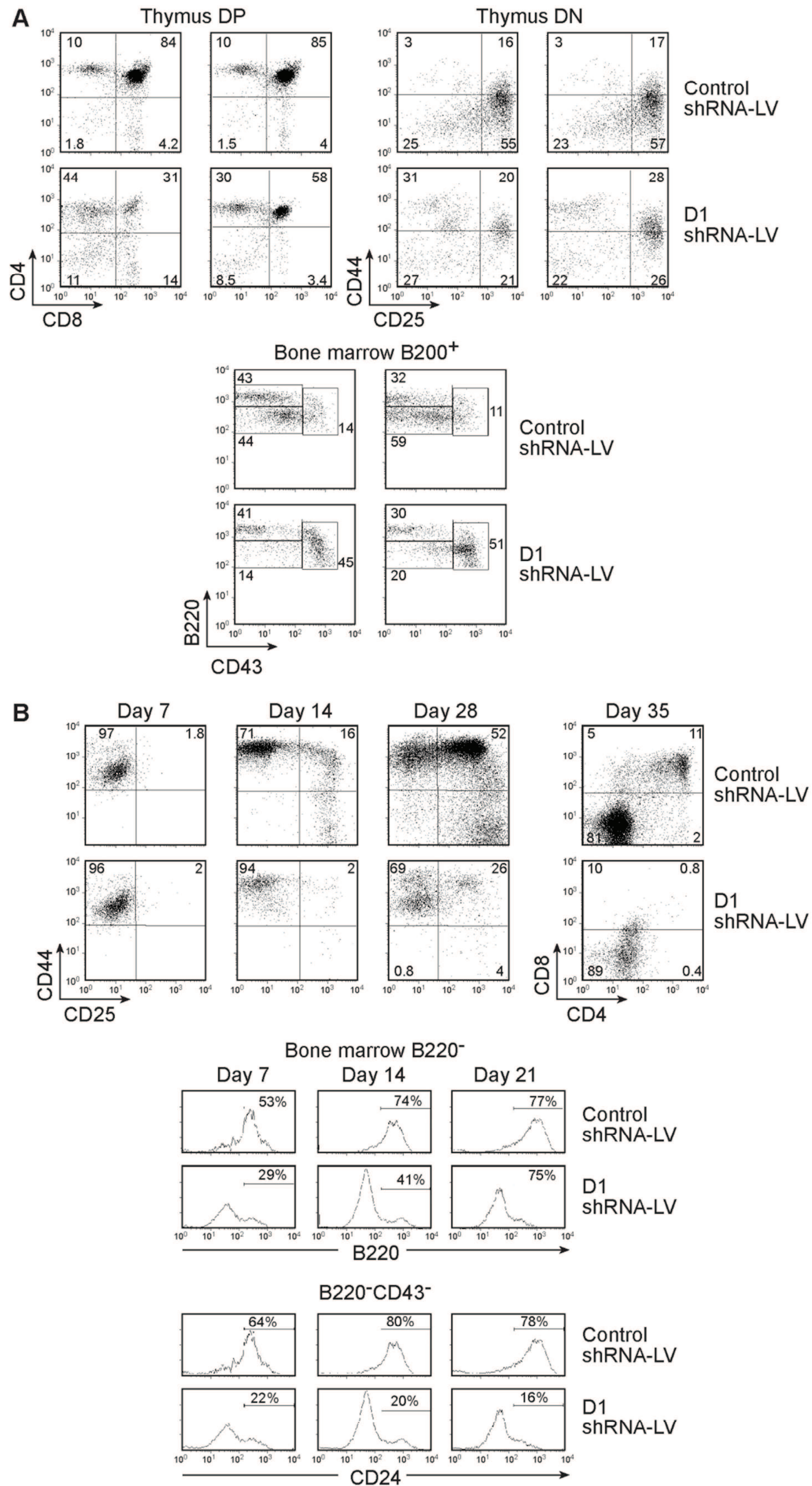
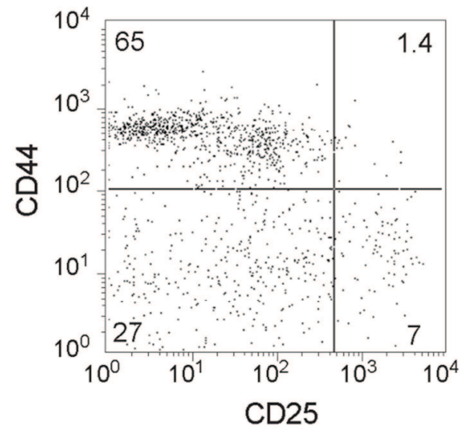


Figure 8



Supplemental figure 1

Exon 4

ATGTGAAGTTCATTTCCAACCCACCCT CC **ATG** GTA GCT GCT GGG AGC GTG GTG GCT GCG
M V A A G S V V A A

ATG CAA GGC CTG AAC CTG GGC AGC CCC AAC AAC TTC CTC TCC TGC TAC CGC ACA ACG
M Q ....

CAC TTT CTT TCC AGA GTC ATC AAG TGT GAC CCG
H F L S R V I K C D P

Exon 5

GAC TGC CTC CGT GCC TGC CAG GAA CAG ATT GAA GCC CTT CTG GAG TCA AGC CTG
D ....

CGC CAG GCC CAG CAG AAC GTC GAC CCC AAG GCC ACT GAG GAG GAG GGG GAA GTG
R ....

GAG GAA GAG GCT GGT CTG GCC TGC ACG CCC ACC GAC GTG CGA GAT GTG GAC ATC
E ....

TGA
*

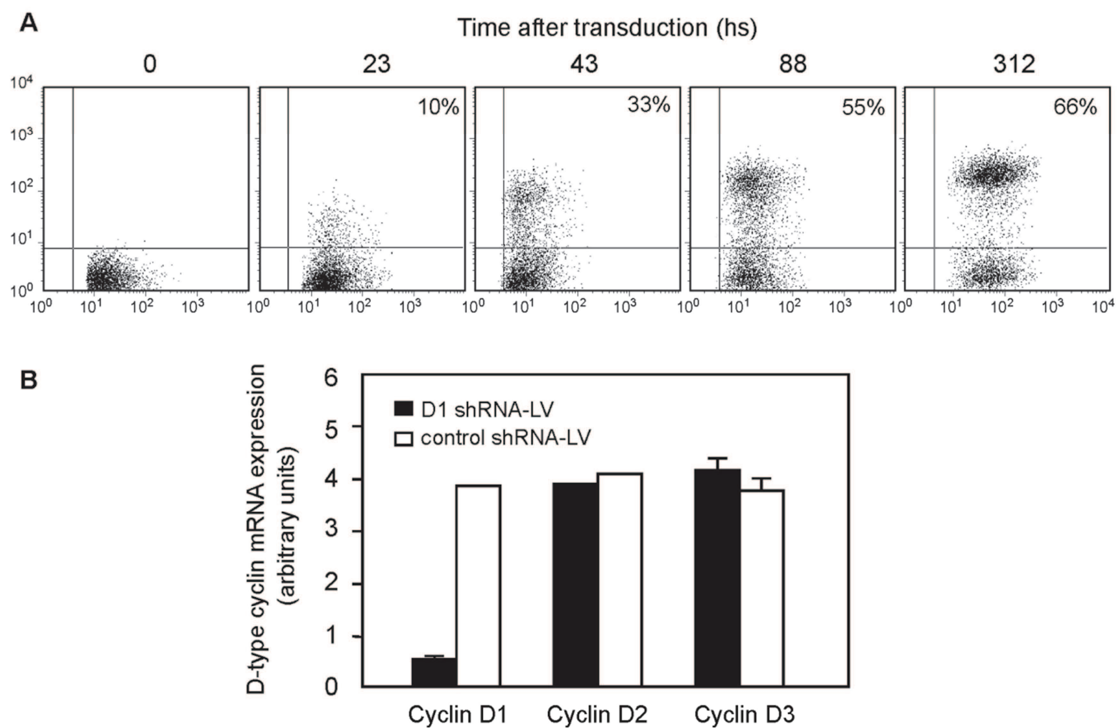
Supplemental figure 2

alternative splicing exon1-exon4

GCTCCGGAGACCGGCAGTACAGCGCGAGGCAGCGCGCGTCAGCAGCCGCCACC
GGAGCCCAACCGAGACCACAGCCCTCCCCAGACGCGCTCGAGGGCCCCTGCAG
GTCAATTCTACCGGATGTGAAGTTCATTTCCAACCCACCCTCCATGGTAGCTGCT
GGGAGCGTGGTGGCTG

Blue: cyclin D1 exon 1
Red: vector sequence
Black: cyclin D1 exon 4

Supplemental figure 3



Supplemental figure 4

FIGURE LEGENDS AND SUPPLEMENTARY NOTES

Figure 1. The thymocytes from 1-3 D1 deficient mice. Mice were four weeks old. Results show: A. Total yields, each point representing an individual mouse. B. The distribution of thymocyte populations. Results are from one mouse from each group, representative of 15 (Group I) 12 (Group II) and 22 (Group III) mice. Upper graphs: CD4/CD8 profiles within $CD19^{-}$ $TER119^{-}Gr\text{-}1^{-}$ lineage negative populations. Middle graphs: CD44/CD25 profiles in triple negative (TN: $CD3^{-}CD8^{-}CD4^{-}CD19^{-}TER119^{-}Gr\text{-}1^{-}$) cells. Lower graphs: CD44+ $CD25^{-}$ TN1 populations were subdivided by their expression of CD24 and CD117. The numbers show $CD24^{+}$ $CD117^{+}$ (ETPs) /thymus.

Figure 2. Bone marrow cells from 1-3 D1 deficient mice. A, B. Mice were four weeks old. Results are from one mouse from each group, representative of 15 (Group I) 12 (Group II) and 18 (Group III) mice. A. $B220^{+}$ B lineage cells. The percentage of cells of each sub-population is shown. B. Lineage negative (Lin^{-}: $CD3^{-}CD4^{-}CD8^{-}CD19^{-}TER119^{-}Gr1^{-}Mac\text{-}1^{-}NK1.1^{-}$) progenitors analysed according to their expression of IL-7R, Sca-1 and CD117. Upper graphs: CLPs Lower graphs: LSK/HSCs. The numbers correspond are yields/femur. C. Mice were 9 days old. Left graphs show LSK/HSC, right graphs erythroid and myeloid cells. Numbers represent yields/femur.

Figure 3. The peripheral lymph nodes of 1-3 D1 deficient mice. Results are from individual mice studied in the same experiment and correspond to a pool of the same LNs in each mice group. They show: A. Cell yields. (Please note: the scale in Y axis varies in different groups) B. CCR7 and CD62L expression levels in gated T lymphocytes. The same results were obtained in 5 other experiments.

Figure 4. The offsprings of the crosses between heterozygous $1\text{-}3^{-}$D1 mice. A. Results are from 684 mice and show the relative proportion of homozygous, heterozygous and wild type mice at birth. **B**. Group distribution and prenatal mortality in the homozygous cohort. Results are from 200 homozygous mice studied until 4 weeks of age.

Figure 5: Quantification of the expression of the D cyclins in different subpopulations of hematopoietic cells. The individual subpopulations of hematopoietic lineage cells were sorted from 9 days and 4 weeks old mice. Results show the mRNA expression of the different members of the D cyclin family, determined qRT-PCR. Please note: since the different PCR amplifications had the same efficiency, expression levels of individual D cyclins can be directly compared.

Figure 6: Division rates of hematopoietic cells from 1-3 D1 deficient mice, as evaluated by BrdU incorporation. In the thymus and bone marrow results show the % of cells in S phase after a 2h BrdU pulse. TN: ($CD3^-CD8^-CD4^-CD19^-TER119^-Gr\text{-}1^-$) DP: $CD4^+CD8^+$ thymocytes. In peripheral T and B cells, BrdU incorporation was studied in mice receiving BrdU 2 injections/day spaced by twelve hours during three days. Barriers for positive cells were established in cells from mice that were not injected with BrdU labelled simultaneously. Results are from one experiment out of 4 experiments giving the same results.

Figure 7*:* **The expression D-type cyclins in 1-3 D1 deficient mice.** Results are from sorted naïve CD8 T cells before (non activated) or after stimulation overnight with anti-CD3 MoAbs (activated). A. They show the expression levels of the transcripts from D2 and D3, and from exons 4-5 of the D1 cyclin relative to the *RPII* housekeeping gene. *RPII* housekeeping gene expression levels were similar in all mouse groups. B. Since in Group I mice expression levels were too low to fit in the same scale as those from other mice (Fig.7A), they are shown here using a scale spanning much lower values.

Figure 8: Effect of cyclin D1 RNAi in HSC differentiation. Bone marrow HSC (Lin^-Sca1^+ $ckit^{high}$) progenitors were transduced with cyclin D1 or control shRNA-LV-EGFP. **A.** They were directly injected into lethally irradiated Rag^- mice. Results showing the profiles of $EGFP^+$ cells in two different individual mice two months after HSC transfer; Upper graphs: $EGFP^+$ thymocytes; lower graphs: B cell lineage profiles in the BM, analysed as described in Fig.1. **B**. They were cultured in “in vitro” conditions promoting T cell (upper graphs) or B/myeloid cell differentiation (lower graphs). Results show the phenotype of $EGFP^+$ at different time points in culture.

Supplemental Figure 1: CD44/CD25 profile of triple negative thymocytes ($CD3^-CD8^-CD4^-CD3^-CD19^-TER119^-Gr\text{-}1^-$) in TN1 blocked Group I mice.

Supplemental Figure 2: Sequence of the exon-4-5 of D1 showing the putative start codon in exon 4 compatible with in frame transcription of C-terminal portion of the cyclin D1. The Kozak sequence is underlined.

Supplemental Figure 3: Epigenetic modifications of the D1 locus, detected in several of 1-3 D1 deficient mice.

Supplemental Figure 4: Efficiency and specificity of D1 RNA interference. Jurkat cells were transduced with control or cyclin D1 shRNA-LV-EGFP. (A). Kinetics of EGFP expression on Jurkat cells after transduction. (B) Quantification of *D type cyclin* mRNA expression levels. $EGFP^+$ cells were sorted two weeks after infection. *D type cyclin* mRNA levels were evaluated by qRT-PCR.

Supplementary Note 1: The peripheral pools of D1 deficient mice reflected the precursor compartment phenotypes. In the spleen, Group I mice were severely lymphopenic, Group II mice showed moderate T and B reduction, while Group III mice had normal lymphocyte counts and T/B population distribution (not shown). In the same way, peripheral lymph nodes were virtually absent in all groups even in Group III-IV mice that had normal yields and T and B cell numbers in the spleen (Supplemental Fig.5).

Supplementary Note 3: Examination of Exon 4-5 demonstrated an ATG initiation of transcription codon (in position 30-32 of exon 4). Analysis by using DNA Srider software demonstrated compatibility for an in frame transcript coding for C-terminal cyclin D1 peptide. This ATG sequence has a G in position +4 and has two C in position -2 and -1. Even if it lacks a purine in position -3, these patterns configure an adequate kozak sequence.

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Resumé

Les cyclines D jouent un rôle essentiel dans les mécanismes du cycle cellulaire. Cette famille de protéines est composée de trois membres (D1,D2,D3) qui partagent un domaine très homologue de la « cyclin box » (codée par les exons 1-3). Ce domaine est responsable de leur activité redondante dans la phosphorylation de la protéine du rétinoblastome lors de l'association avec les kinases cycline-dépendantes CDK4/6. Parmi les trois cyclines, la cycline D1, bien que faiblement exprimée dans les lymphocytes, est la cycline la plus impliquée dans les cancers lymphoïdes ou elle aurait une fonction de facteur de transcription indépendante de Cdk. Etant donné qu'après stimulation antigénique, les lymphocytes T et B ont une capacité remarquable de division, essentielle à la génération d'une réponse immunitaire efficace, nous avons porté un intérêt particulier au rôle des cyclines D dans la lymphopoïèse.
Pour étudier le rôle de la cycline D1 dans la différenciation des lymphocytes, nous avons utilisé des souris déficientes pour les exons 1,2,3 de la « cycline box » D1 mais conservé les exons 4 et 5. Étonnamment, ces souris présentaient des phénotypes très différents que nous avons subdivisés en quatre groupes. Dans le groupe I, les souris avaient un thymus réduit car la différenciation de la lignée lymphoïde est bloquée à un stade très précoce, avec un faible nombre de cellules progénitrices (CLP) dans la moelle osseuse. Dans le thymus, les progéniteurs des thymocytes (ETP) étaient pratiquement absents et les précurseurs $CD4^-CD8^-CD3$ (TN) immatures essentiellement constitués par des cellules $CD44^+CD25^-$ (TN1) et $CD44^+CD25^+$ (TN2) les plus immatures. De plus, les $CD4^+CD8^+$ (DP) qui donnent naissance aux thymocytes matures CD4+ et CD8+ étaient présents en très faible quantité. Dans la moelle osseuse, on observe un blocage majeur dans la différenciation de la lignée B au stade pré-proB. Dans les ganglions, la forte réduction du nombre de lymphocytes T observée était liée au faible nombre d'ETP et à l'absence du récepteur aux chimiokines CCR7. Dans le groupe II, les souris présentaient une diminution moins sévère des ETP et une atrophie modérée du thymus. La différenciation était bloquée à un stade ultérieur, soit dans la transition des étapes TN3 à TN4. Dans la moelle osseuse, les lymphocytes B ont subi un blocage partiel au stade pré-proB et une réduction des cellules pré-B. Le nombre de CLP est également réduit, mais dans une moindre mesure que dans les souris du groupe I. dans les groupes III et IV, les souris ont une répartition normale des thymocytes mais présentaient une augmentation du compartiment ETP. Alors que les souris du groupe III contenaient un nombre normal de thymocytes, les souris du group IV présentaient une hyperplasie thymique. Par ailleurs, en comparaison avec des souris normales, bien que la différenciation des lymphocytes B soit normale, on observe dans les deux groupes une augmentation des CLP et des progéniteurs hématopoïétique (LSK).
L'implication de la cycline D1 dans la transition de G1 à S nous a conduit à analyser les divisions cellulaires *in vivo*. De manière surprenante, les souris du groupe I étaient fortement dépourvues de cellules en cycle dans tous les compartiments lymphoïdes, ce qui peut expliquer les blocages de la différenciation lymphoïde. Par contre, dans les trois autres groupes, on observe une augmentation du nombre de divisions cellulaires. Ces résultats différents peuvent être dû à l'expression ou l'absence d'une protéine D1 tronquée qui contient cependant les exons 4-5. Alors que ces ARNm tronqués ne sont pas détectables dans les souris de groupe I, on observe des niveaux élevés d'expression dans les autres groupes. De plus, nous avons observé une corrélation entre l'absence d'expression des exons 4-5 et la très faible expression des gènes *CCND2* et *CCND3*, ce qui attribue à cette protéine tronquée un rôle prépondérant dans la régulation des cyclines D et permet d'expliquer l'aplasie profonde et l'arrêt de la division cellulaire de la lignée lymphoïde. Bien que le domaine de la « cyclin box » soit commun aux trois cyclines, ces résultats suggèrent, le domaine régulateur codé par les exons 4-5 est fondamental dans la lymphopoïèse. Pour corroborer ces résultats, des expériences d'inhibition dans l'expression de la protéine D1 complète par ARN Interférence *in vitro* et *in vivo* ont reproduit le phénotype des souris du groupe I.
Pour étudier le rôle des exons 4-5 du gène *CCND1* en tant que facteur de transcription impliqué dans la régulation de la lymphopoïèse, nous avons développé un vecteur lentiviral qui contient le gène tronqué et des souris knockout conditionnelles de ces exons. Ces outils permettront d'attribuer une fonction aux exons 4-5 afin d'étudier leur rôle dans la régulation des différents facteurs de transcription et comprendre le rôle majeur de la cycline D1 dans la cancérogénèse.

Abstract

D Cyclins play an essential role connecting exogenous stimulation to the intrinsic cell cycle machinery. This family of proteins is composed of three members sharing a highly homologous domain, the cyclin box (coded by exons 1-3), which is responsible for their redundant role in the phosphorylation of the retinoblastoma protein upon association with cyclin-dependent kinases Cdk4/6. Both mature T and B-cells have a remarkable division capability after antigen stimulation, essential to the generation of efficient immune responses, raising the interest of D Cyclins in lymphopoiesis. Cyclin D1, although weakly expressed by lymphocytes, is the D Cyclin most commonly implicated in lymphoid cancers and as having a Cdk-independent transcriptional role.
To study the role of Cyclin D1, we used mice deficient for the D1 cyclin box but sparing exons 4-5. Surprisingly, individual mice have very different phenotypes that we subdivided into four arbitrary groups. Group I mice show the most precocious block in lymphoid lineage differentiation, illustrated by a low cellularity of common lymphoid progenitor cells (CLP). The thymi showed very few $CD4^+CD8^+$, double positive (DP) cells, while the $CD4^-CD8^-TCR^-$, triple negative (TN) populations were found to be mostly constituted by the early $CD44^+CD25^-$ (TN1) and few $CD44^+CD25^+$ (TN2). TN1's early thymocyte progenitors (ETP) were virtually absent. At the B-cell lineage level in the bone marrow (BM) there was a major block in pre-proB differentiation. The number of peripheral T-cells was severely reduced, mainly in LN, since group I T-cells lack CCR7 expression. Group II mice presented moderate thymus atrophy. The block on TN differentiation occurs at a later stage, i.e., in the TN3 to TN4 transition, and the TN1 population was characterized by a less severe depletion of the ETP. Group II mice showed a partial pre-proB block and a reduction in pre-B-cells. CLPs were also reduced but to a lesser extent than in group I mice. Group III and group IV mice appear to have a normal thymocyte population distribution but showed an increase on ETP compartment. Group IV mice displayed thymic hyperplasia while group III mice possessed normal thymus cellularity. B-cell differentiation on both groups appeared to be normal but BM precursors had an increase in both CLP and early haematopoietic progenitor's (LSK) levels as compared with wild type mice.
Cyclin D1 involvement in G1 to S transition led us to analyse *in vivo* division rates. Strikingly, group I mice were virtually devoid of cycling cells in all lymphoid compartments, explaining why lymphoid lineage cells do not differentiate in these mice. In contrast, in all other groups we observed an increased BrdU incorporation. These contradicting phenotypes correlated with the expression or absence of a truncated D1 protein coded by exons 4-5. The presence of the *cyclin D1* truncated mRNA was not found in group I mice but high levels of expression are consistently observed in the remaining groups. In the absence of the D1 truncated protein only trace values of Cyclins D2 and D3 were found, highlighting the role of this protein as a master D cyclin regulator, which further supports the profound aplasia and arrest in lymphoid lineage division on cells that predominantly express Cyclin D2. These results suggest that, while the function of the D1 cyclin box is redundant, the regulatory domain coded by exons 4-5 is fundamental for lymphopoiesis. Full D1 protein was also eliminated by RNA interference both *in vitro* and *in vivo*. These experiments reproduced the phenotype of group I mice.
We have developed a lentiviral vector with a truncated D1 (exons 4-5) and conditional knockout (KO) mice by floxing exons 4-5 of *cyclin D1*. These tools will allow us to show Cyclin D1 Cdk-independent role as a transcription regulator in lymphopoiesis and to attribute this function to exons 4-5. Understanding how exons 4-5 regulate different transcription factors might be a key in understanding D1's unique role in carcinogenesis.