Ras mediated pathways antagonise Notch signalling, promoting B lymphogenesis in a T cell environment.

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SUMMARY

Within the immune system an important lineage commitment decision faced by the haemopoietic progenitor cells (HPCs) is whether to adopt a T or B lymphocyte fate. Recent studies have indicated that Notch, a transmembrane receptor, plays a crucial role in inducing HPCs to adopt a T cell fate. Similarly transcription factors E2A, early B cell factor and Pax5 have been shown to be important in the generation of B lymphocytes from HPCs. Many of the upstream signalling pathways that regulate the expression and activation of these transcription factors remain largely undefined.

To generate T cells from HPCs *in vitro*, we utilised foetal thymic organ culture (FTOC), high oxygen submersion FTOC techniques and the novel system OP9-DL1 a bone marrow stromal cell line expressing the Notch ligand Delta-like-1. HPCs, retrovirally infected with constitutively active Ras mutants, were cultured in each of the afore mentioned *in vitro* culture systems. Results indicate that constitutive activation of the Ras-mediated signalling pathways, by retrovirally infected cells with constitutively active Ras^{V12}, results in HPCs adopting a B cell fate within a T cell microenvironment. Furthermore, constitutive Ras-extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) signalling may be key in promoting B cell fate within these systems as $Ras^{V12}S35$, which leads to a constitutive activation of Raf binding in the absence of PI3K binding, results in the adoption of a B cell fate. By contrast $Ras^{V12}C40$, which binds PI3K but not Raf, led to normal T cell fate adoption. Importantly, we have also shown *in vivo* that retrovirally infected HPCs adoptively transferred into neonatal recombination activating gene-1 deficient mice resulted in Ras/ERK-MAPK dependent B cell commitment within the thymus. These results suggest that constitutive Ras signals antagonise Notch-1 to promote B cell fate at the expense of T cell development.

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DECLARATION

This work represents original work carried out by the author and has not been submitted in any form to any other University.

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ABBREVIATIONS

Ag	antigen
Akt	protein kinase B (PKB)
AMV RT	avian myeloblastis virus reverse transcriptase
APC	allophycocyanin
BCR	B cell antigen receptor
BM	bone marrow
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
CLP	common lymphoid progenitor
CMP	common myeloid progenitors
CREB	cAMP response element binding protein
DC	dendritic cell
DNA	deoxyribonucleic acid
DN	double negative
DP	double positive
EBF	early B cell factor
ERK	extracellular signal regulated kinase
ETP	early T-lineage progenitor
FL	foetal liver
FTOC	foetal thymic organ culture
GAP	GTPases-activating proteins
GEF	guanine exchange factor
GFP	green fluorescence protein
HOS	high oxygen saturation
HRP	horse radish peroxidase
HPC	haemopoietic progenitor cells
HSC	haemopoietic stem cell
Ig	immunoglobulin
IL	interleukin

ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LAT	linker for activation of T cells
LTR	long terminal repeat
MAPK	mitogen-activated protein kinase
M-CSFR	macrophage colony-stimulating factor receptor
MHC	major histocompatibility complex
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MPP	multipotent progenitor cells
NK	natural killer cell
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
PE	R-phycoerthrin
pErk	phospho-Erk
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A (cAMP-dependent protein kinase)
PKB	protein kinase B (Akt)
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
pMEK	phospho-MEK
PTEN	phosphatase and tensin homologue
RBP-J	recombination binding protein-J
SH2	Src homology 2
SLP-76	SH2 domain-containing leukocyte protein 76kD
SP	single positive
TBS	Tris buffered saline

TCR	T cell antigen receptor
TEC	types of epithelial cell
TNF	tumor necrosis factor
WASP	Wiscott-Aldrich syndrome protein
ZAP-70	zeta associated protein 70kD

CHAPTER 1 – Introduction

1.1 Immune System

The main function of the immune system is to protect the host from infectious microbes in the environment. Environmental pathogens threaten the host with various pathogenic mechanisms, therefore the immune response uses a complex array of protective mechanisms to control and usually eliminate these organisms. All of these mechanisms rely on detecting structural features of the pathogens that mark them as distinct from the host, so as to eliminate the pathogen without excessive damage to its own tissue.

There are two main classes of host recognition:

- Innate immunity which serves as a first line defence and includes all defence mechanisms that are encoded in the germline genes of the host, i.e., epithelial barriers and mucocoliary barriers.
- Adaptive immunity is based on clonal selection of antigen-specific receptors expressed on the surface of T and B lymphocytes enabling the host immune system to recognise any foreign antigen. The antigen-specific receptors of the adaptive response are assembled by means of somatic rearrangement of germline gene elements to form intact T cell receptor (TCR) and immunoglobulin (Ig) genes (1). The assembly of antigen receptors from a collection of a few hundred germline-encoded gene elements permits the formation of millions of different antigen receptors, each with the potentially unique specificity for a different antigen (2).

Although the innate and adaptive immune responses are fundamentally different in their mechanisms of action, synergy between them is essential for an intact and fully effective immune response.

1.2 Lymphocyte lineage Commitment

1.2.1 Haemopoiesis

The development of the various haemopoietic cell lineages are compartmentalised during foetal development and throughout adult life. Approximately by day 9 of embryonic development the foetal liver (FL) is seeded by definitive haemopoietic progenitor cells (HPCs), the FL continues as the primary site of haemopoietic development until birth, when the bone marrow (BM) takes over as the primary site in adults. Stem cell differentiation is regulated by soluble factors and cell contact dependent signals within specialised microenvironments, each of which supports the development of specific cell lineages.

Pluripotent HPCs in the FL and BM, have an extensive self-renewal potential regenerating all blood cell types throughout life by differentiating to multipotent progenitor cells (MPP), which have gradually restricted developmental potential. MPPs commit to either the lymphoid or erythro-myeloid lineages, giving rise to common lymphoid (CLP) or common myeloid (CMP) progenitors (3-5). The loss of long term self-renewal capacities in MPPs is accompanied by the expression of the tyrosine kinase receptor Flt3 (6). The early HPC cell is thought to give rise to the recently identified early T-lineage progenitor (ETP) in the thymus and to the HPC in the bone marrow, which is able to develop into four cell types: B, T, natural killer cells (NK) and dendritic cells (DC) (7).

The development of B and T lymphocytes, from HPCs is a multistep process that involves the ordered expression of a large number of genes. It is clear that the antigen receptors play essential roles not only in transducing signals for B and T cell activation and regulation of the immune response, but also in transducing signals required for development and continued survival.

HPCs with T cell potential leave the FL or BM and migrate to the thymus where they undergo a tightly regulated developmental programme involving proliferation,

differentiation and selection to produce a functionally diverse population of T cells. Although, many of the phenotypic changes associated with specification and commitment to the T cell lineage are well characterised, the signals that drive these events are less clear. On the other hand, B cell development takes place within the BM microenvironment. Commitment of HPCs to B cell lineage is dependent on the expression of the paired box transcription factor Pax5, which acts to promote the expression of B cell lineage genes while suppressing genes responsible for T cell, erythroid or myeloid cell development (8). Once committed, B cell precursors pass through a number of developmental stages marked by a series of changes in location and in the expression of genes, intracellular signalling proteins and cell surface markers.

1.2.2 Lineage choice of HPCs

The primary lineage choice that developing lymphocytes undergo is commitment to either the B or T cell lineage. Notch receptors and their corresponding ligands comprise of a family of evolutionary conserved trans-membrane proteins initially described in *Drosophila*, and found in organisms as diverse as worms and humans. Thus far, mammals possess four Notch receptors (Notch 1-4) and five ligands, Jagged 1 and 2, and Delta1, 3 and 4. Notch receptors are synthesised, as precursor proteins that are cleaved during transport to the cell surface and are expressed as heterodimers. Ligand binding to the extracellular domain initiates a cascade of proteolytic cleavages ultimately releasing the cytoplasmic domain of the Notch receptor, which translocates to the nucleus and binds to the transcription factor recombination binding protein-J (RBP-J). The binding of Notch cytoplasmic domain to RBP-J converts it from a transcriptional repressor into a transcriptional activator that leads to the activation of Notch target genes. Modulators, acting at the extracellular, cytoplasmic and nuclear levels, can regulate notch signalling.

During haemopoiesis, the T versus B lineage decision depends on Notch1 for the development of both thymus-dependent and thymus-independent T cells (9-11) (Figure 1.1). It has been shown by Pui *et. al.*, (12) that B cell development is

abolished in mice that are reconstituted with BM-HPCs expressing a constitutively active form of Notch, and that $CD4^{+}CD8^{+}$ double positive (DP) T cells develop in the BM of these mice. In a complementary experiment Notch1 deficient mice displayed a severe block in T cell development, with the simultaneous development of B cells in the thymus; thus implying that Notch signalling is crucial for the earliest stages of T cell commitment, and that Notch1 signalling must be either absent or negatively regulated in BM progenitor cells to allow B cell development, and that the Notch1 signal is sufficient for T cell commitment (13, 14).

The question that then arises is 'how do B cells still develop in the BM compartment despite the fact that Notch receptors and ligands are expressed on BM progenitors and stroma?' Among the family of Notch receptors it is predominantly Notch2 that is expressed in B cells, the level of Notch2 expression increases with B cell maturation and is prominent in splenic B cells, indicating a role in mature B cells (15). It has been shown by Souabni *et. al.* (16) that the B lineage commitment factor Pax5 represses the transcription of the Notch1 gene. B cell commitment depends on the transcription factors E2A, early B cell factor (EBF) and Pax5. E2A and EBF activate the expression of B cell specific genes, which are essential for the generation of the earliest B cell progenitors. However, the activation of the B cell specific transcription programme is not sufficient to commit early progenitors in the absence of the Pax5 expression. The absence of Pax5 arrests B cell development at an early pro-B cell stage, even in the presence of other specific transcription programmes. Subsequently the restoration of Pax5 in $Pax5^{-/-}$ pro-B cells can promote development to mature B cells (17). Whether Notch1 plays a similar role in T cell progenitors by repressing Pax5 expression remains to be seen. Therefore in an effort to understand the signalling mechanisms that control this lineage commitment decision, we will manipulate HPCs with signalling mutants, and utilising *in vitro* T cell generation systems (FTOC, HOS-FTOC and OP9-DL1) to investigate how these molecules impact B and T lineage cell fate decisions.

1.3 T Cell Development

T cell development occurs in the thymus where the unique processes of T cell differentiation and repertoire selection are induced by the specialised cellular microenvironments provided by the thymic stroma (18). This comprises an ordered three dimensional network of epithelial cells interspersed with non-epithelial stromal cells. It is broadly divided into two compartments, the cortex and the medulla, each of which contain several epithelial cell types (Figure 1.2) (19). However, lineage relationships between the thymic epithelial subpopulations and the molecular mechanisms behind their functional differences are poorly understood. Early thymus organogenesis occurs between day 9.5 and 11.5 of murine embryonic development, and results in the development from the third pharyngeal pouch of a common primordium containing prospective thymus and parathyroid domains (20, 21). The thymus and parathyroid primordia separate by day 12.5, by which time the thymic epithelial rudiment is encapsulated by the mesenchyme. Mesenchyme derived signals are required to support thymic development. HPCs colonise the thymus from day 11.5, and during organogenesis interactions between thymocytes and thymic epithelial cells are required to establish proper organisation and function of the cortical and medullary compartments (19, 20). The *nu/nu* phenotype (*nude*) of congenital athymia is caused by mutation of the transcription factor *Foxn1* (*whn*), while not involved in the initiation of thymic organogenesis, it has been shown to be required for the development of all major thymic epithelial subpopulations (22). Deficiency in Whn results in a cystic thymic rudiment in adults and consequently causes a marked reduction of T cells, with some cells still being generated extrathymically (20).

Early T cell progenitors undergo a number of differentiation steps that are typically defined by the cell surface expression of CD4 and CD8. HPCs entering the thymus at day 11.5, from the FL or BM, lack expression of CD4 or CD8 and are hence termed double negative (DN) thymocytes (23). They then become $CD4^+CD8^+$ double positive (DP) thymocytes, and lastly mature into single positive (SP) $CD4^+$ or $CD8^+$ T cells (20) (Figure 1.3). DN thymocytes can be further

characterised by the ordered expression of two cell surface molecules CD117 (stem cell factor receptor) and CD25 (IL-2 receptor alpha): $CD117^{+}CD25^{-}$ (DNI); $CD117^{+}CD25^{+}$ (DNII); $CD117^{-}CD25^{+}$ (DNIII); $CD117^{-}CD25^{-}$ (DNIV) (24) (Figure 1.3). DNIs still have multi-lineage potential (including T/B/NK/dendritic cell (DC)), DNIIs have lost their ability to give rise to B cells but can give rise to T and NK cells, whereas DNIIIs are fully committed to T cell lineage with no B cell potential (13).

The specificity of the adaptive immune response is partly dependent on the clonal expression of the mature TCR on lymphocytes. The expression of a newly rearranged TCRβ chain with a pre-Tα and CD3 complex on DNIII cells, to form the pre-TCR complex, represents a critical checkpoint in T cell differentiation known as β selection (25). The pre-TCR complex triggers a maturation programme within developing thymocytes that includes: rescue from apoptosis; inhibition of further DNA recombination at the TCRβ gene locus (allelic exclusion) (26); proliferation; down-regulation of CD25; up-regulation of CD4/CD8 and inhibition of TCRα gene rearrangement. The second checkpoint occurs when DP thymocytes undergo positive and negative selection, which enables the differentiation of MHC-restrictive and self-tolerant $CD4^{+}$ or $CD8^{+}$ SP αβ T cells (23).

1.3.1 Pre-TCR complex

The TCRβ chain couples to the pre-Tα chain, which together with the CD3 molecules (ε, γ, δ, and ζ) form the pre-TCR complex. The pre-TCR complex drives the generation of DP cells by signal transduction through the CD3 complex. The activation of the pre-TCR does not appear to depend on ligand engagement (unlike most lymphocyte surface receptor complexes), in fact ligand engagement of the pre-TCR *in vivo* arrests maturation of the thymocyte prior to the DP stage (23). The potential ligand binding exodomains of pTα and TCRβ have been shown to be dispensable for pre-TCR function (27), suggesting that engagement by a specific ligand is not responsible for initiation of pre-TCR signalling *in vivo*. Therefore, it is

thought that the pre-TCR needs to be transported to the cell surface of immature thymocytes to be *in situ* to meet crucial signalling components. It would therefore appear that the pre-TCR has a unique ability to transduce β-selection signals even when expressed at low levels and when not engaged by a surface receptor. There are several hypotheses to explain this phenomenon, all of which suggest that the pre-TCR complex signals constitutively (27). The lack of specific ligand recognition could be explained by the recent findings which show that the pre-TCR complex (directed by the presence of the palmitoyl moiety on the pTα chain) has the ability to spontaneously target the distinct lipid microenvironments (lipid rafts) in the plasma membrane of pre-T cells. The lipid rafts provide a framework for signalling protein complexes and therefore provide a platform for the initiation of downstream signalling cascades, which compensates for the requirement of ligand binding. This is in direct contrast to the TCRαβ complex, where ligand binding by the antigen receptor is absolutely essential for the further differentiation of DP thymocytes and activation of mature T cells.

Another finding that has helped to explain how the pre-TCR complex has such a high signal efficiency in the absence of ligand recognition, is the activation threshold of pre-T cells (23). Pre-T cells have a high expression of activation marker CD25, possibly implicating that they are in a heightened activation state, therefore it is thought that pre-T cells may have a lower threshold of activation (28). Therefore the pre-T cell may provide a unique environment that is poised to respond to differentiation signals upon generation of a functionally rearranged TCRβ chain.

1.3.2 Downstream signalling events of the pre-TCR complex

Due to the structural similarity between the pre-TCR and the mature TCR complex, and the reliance of both receptors on the immunoreceptor tyrosine-based activation motifs (ITAMs) containing CD3 moieties to transmit signals from the cell surface to the nucleus, it is thought that the signalling properties of both receptors may be similar (29). Upon pre-TCR formation, Lck phosphorylates tyrosines within the ITAMs of the TCRζ resulting in the recruitment of ZAP-70 (zeta associated protein, 70 kD) to the pre-TCR complex. In return ZAP-70 phosphorylates the adaptor molecules LAT (linker for activation of T cells) and SLP-76 (SH2 domain-containing leukocyte protein, 76 kD). SLP-76 is responsible for the coupling of TCRαβ with phospoholipase C-γ1 (PLCγ1), and Ras mediated signalling pathways which result in downstream signalling events such as: Ca^{2+} mobilization; protein kinase C (PKC) activation; and extracellular signal regulated kinase-mitogen-activated protein kinases (ERK-MAPK) activation (30-32). Such signalling events are essential for the activation of T cell effector function. The notion that the active Ras/Raf signalling cascade plays a central role during β selection is supported by data obtained from mice bearing targeted genetic deficiencies or mutations of key signalling molecules such as Lck, LAT or SLP-76, also showing the importance of these molecules in pre-TCR signalling (33-35). These mice display a block in T cell development at the DN stage, implying that these molecules are essential for differentiation to the DP stage of development. The importance of PKC activation during β selection has been shown by Michie *et al* (36), SLP-76 is responsible for binding to and activating PLCγ which in turn activated PKC. Michie *et al* introduced a retroviral construct encoding a dominant negative PKCα into developing thymocytes, which resulted in a block in the generation of DP thymocytes. The molecules involved during the initial activation steps that mediate β selection are outlined in Figure 1.4.

Further insights into which signalling molecules are important for the β selection checkpoint have been gained by the introduction of transgenes encoding activated signalling components into recombination activating gene-1 or-2-deficient ($RAG^{-/-}$)

mice (37). These mice are halted at the DNIII stage of T cell development due to an inability to initiate antigen receptor rearrangement. When productively rearranged TCRβ transgenes were introduced into $RAG^{-/-}$ mice they allowed the further differentiation of DN cells, indicating that a functional TCRβ chain is sufficient for progression to the DP stage. Moreover, $RAG^{-/-}$ mice containing a transgene encoding constitutively active GTPase Ras (Ras^{V12}) display cellular proliferation and generation of DP cells by bypassing the pre-TCR dependent β selection processes (38). This suggests that Ras is involved in promoting β selection, by activating the ERK-MAPK cascade. Although it can be shown that ERK-MAPK can mediate proliferation and differentiation of DN thymocytes, Crompton *et al* has shown by the introduction of constitutively active MAPK kinase-1 (MEK1) into developing thymocytes from $RAG^{-/-}$ mice only resulted in the generation of immature single positive thymocytes (39). Therefore suggesting that additional signals upstream of MEK1 may be responsible for full differentiation of DN thymocytes to the DP stage. Michie *et al* (40) have developed a novel experimental system which allows the introduction of ELK-1-reporter-plasmids into thymic lobes, using gene gun-mediated transfection. This system facilitated the study of specific biochemical signalling events that ensure upon the formation of the pre-TCR complex. Using this system they have been able to show that the ERK signalling cascade was activated in developing thymocytes as a result of pre-TCR complex formation *in vivo* (41).

1.4 B Cell Development

The processes controlling B cell development are tightly regulated to ensure a constant supply of B cells expressing Ag receptors of distinct specificity, which enables identification of any already encountered Ag, yet simultaneously avoiding the generation of auto-reactive B cells that recognise 'self' Ags. This complex process involves the integration of numerous signals at a number of stages, including Ag, soluble mediators and accessory cells. Once committed, the pre B cells pass through a number of development stages marked by a series of changes in location and in the expression of genes, intracellular signalling proteins and cell

surface markers. B cell development can be broadly divided into two quite distinct phases; Ag-independent and Ag-dependent (42). The Ag-independent phase occurs in the BM and involves the production of a repertoire of immature B cells bearing functional Ag receptors. During this process of receptor editing an encounter with Ag leads to death by apoptosis, or anergy, a process by which the B cell becomes unresponsive to future encounters with its particular Ag. B cells that emerge into the periphery are termed immature B cells, and these migrate to the secondary lymphoid organs, such as the spleen and lymph nodes (LNs). At the periphery, B cell recognition of Ag (in association with specialised Ag-presenting cells and stromal cells) can lead to one of several developmental pathways in the production of mature B cells from transitional B cells: anergy and/or apoptosis; activation, proliferation and differentiation into high rate Ab secreting plasma cells; or differentiation into memory B cells (43).

The earliest marker that defines cells committed to the B cell lineage is the CD45R isoform B220, a membrane tyrosine phosphatase (Figure 1.5). B220 and CD19 are expressed through out B cell development, with levels increasing during the transition from the immature to the mature stage, which exhibit an increased BCR expression. At the late pro-B cell stage CD117 expression is lost, while CD24 (HSA) is upregulated. Immature cells are characterised by the expression of membrane IgM (mIgM), which is required for the continual survival of mature B cells as they migrate to the periphery where they become resting mature B cells. An additional hallmark of B cell development is the ordered expression of surrogate, and ultimately mature, BCRs (44).

Competent BCR complexes promote positive selection and migration of B cells from the BM to the peripheral lymphoid organs, including spleen, and gut-associated lymphoid tissues. It is at these secondary sites, that B cells stimulated by antigens generate memory B cells and plasma cells which secret antibodies at the terminal stages of B cell development. The early stages of B cell development are characterised by the expression of transcriptional regulatory proteins, which

can both activate or repress genes (45). Transcription factors including PU.1, E2A and EBF are essential for the earliest B lymphocyte developmental stages (46).

1.4.1 PU.1 and B cell lineage commitment

PU.1 transcription factor is an important regulator of HPC cell commitment and of B cell transcription factors. It has been reported that independently generated mouse lines with targeted deletions of PU.1 genes completely lack B cells, T cells and myeloid cells. Some studies reported $B220^+$ cells present that lack Ig heavy chain rearrangements (47-49). As a result of these findings it has been hypothesised that PU.1 may control the commencement of B cell development, DeKoter *et al* have shown that PU.1-deficient progenitors drives lineage determination in a dose-dependent manner, with low expression favouring the generation of B cells (50). The generation of B cells is indicated by the activation of B lineage specific genes, such as mb-1 (Ig-α); B29 (Ig-β); VpreB and λ5 surrogate light chain components of the pre-BCR; RAG-1 and RAG-2; ebf1 (which encodes EBF) and interleukin-7 receptor α (IL-7Rα) (51). This data suggests that although PU.1 is essential for the generation of B cells, it does so only at low concentrations. In support of these findings Zou *et al* have shown that the knockdown of PU.1 by small interfering (si) RNA's in $CD34^+$ haemopoietic progenitors promoted B lineage development at the expense of another lineage (52). The knockdown of PU.1 up-regulated the expression of IL-7Rα, EBF and Pax5 essential for B cell lineage commitment.

1.4.2 E2A, EBF and B cell commitment

B cell fate is characterised by the expression of genes encoding components of the B cell antigen receptor and rearrangement of the Ig heavy chain locus (53, 54). The transcription factors E2A and EBF are essential for the determination of the B cell fate. The E2A gene encodes two basic helix-loop-helix proteins (E12 and E47) generated by differential splicing, whose activities are induced during B cell development. EBF is an atypical helix-loop-helix zinc finger protein that expressed exclusively in the B lineage within the haemopoietic system (55, 56). It has been

shown that the targeted interference of E2A or EBF gene results in a block in the B cell development prior to the onset of early B lineage gene expression and the initiation of D-J rearrangements (56-59). Supporting this hypothesis Romanow *et al* have demonstrated that either E2A or EBF can induce specific D-J_H rearrangements in non-lymphoid cells when ectopically expressed with the RAG-1 and RAG-2 genes.

1.4.3 Lineage Commitment by Pax5

The activation of B cell specific genes and V(D)J recombination by E2A and EBF alone is not sufficient to commit HPCs to the B cell lymphoid lineage in the absence of Pax5 (60). It has been shown that Pax5 is the critical B lineage commitment factor that restricts the developmental options of early progenitors to the B cell pathway. Nutt *et al* have demonstrated that $Pax5^{-/-}$ pro-B cells still retain a broad lympho-myeloid potential, characteristic of uncommitted progenitors, due to their ability to differentiate *in vitro* into functional NK cells, DCs, macrophages, osteoclasts, and granulocytes, upon IL-7 withdrawal and appropriate cytokine stimulation (17, 61, 62). However, this multilineage potential is suppressed by retroviral restoration of Pax5 expression, which promoted development to the mature B cell stage (58).

Pax5 plays a dual role during lineage commitment, by repressing inappropriate lineage genes and also simultaneously activating B cell specific genes, leading to the initiation of the B-lymphoid gene expression programme (17). Pax5 represses macrophage colony stimulating factor receptor (M-CSFR) and Notch1 genes in committed B lymphocytes ensuring that these cells no longer respond to the myeloid cytokine M-CSF or to T cell inducing Notch1 ligands (16, 17). Activated Pax5 target genes also code for essential components of the pre-BCR signalling pathway, including the receptor signalling chain Igα (mb-1), the stimulatory co-receptor CD19 and the central adaptor protein BLNK (61, 63-66).

1.4.4 Pre-BCR complex

The pre-BCR is composed of the IgH chain and the surrogate light chain encoded by VpreB and lambda 5. The pre-BCR has been implicated in precursor B cell proliferation, differentiation and IgH chain allelic exclusion (67). As with the BCR complex of immature and mature B cells, the Ig of the pre-BCR complex couples to the signal transduction molecules Igα and Igβ. These are first expressed on the surface of late pro-B cells in association with the integral membrane protein and molecular chaperone calnexin, which are required for pre-BCR signal transduction (44). Unlike the immature and mature B cell receptor there has been no antigen identified for the pre-BCR. However, it has been proposed that ligand independent basal signals generated through the ITAMs of Igα and Igβ are sufficient to promote the development of pro-B cells into pre-B cells (68, 69), or that a BM stromal factor such as galectin-1 may bind to and activate the pre-BCR (70). Alternatively, it has been suggested by Ohnishi *et al* that the induction of signals be the pre-BCR involves the ligand independent aggregation of components of the pre-BCR (71).

Successful rearrangement of IgH gene locus, and subsequent expression of this chain as a functional pre-BCR complex, is fundamental for the development of pre-B cells. The pre-BCR initiates critical signals essential for the survival and proliferation of the developing B cell, such as the Erk-MAPK cascade (72). It is thought that the pre-BCR complex uses similar intracellular signalling molecules as the BCR complex, such as the protein tyrosine kinase Btk (Brunton's tyrosine kinase) which is essential for optimal pre-BCR signalling and if deficient results in impaired B cell development at the pre-B cell stage (73). Furthermore, it has been shown that mice deficient in the tyrosine kinase Syk have impaired pre-BCR signalling leading to a disruption in the B cell development at the pro-B to pre-B cell transition. IL-7 is a survival factor for the pro-B cells due to its ability to activate Erk (74, 75). Thus, signals activated downstream of the pre-BCR promote survival and proliferation of the pre-B cells, therefore cells that are unable to rearrange genes encoding Ig heavy chain, and express a functional pre-BCR, do not undergo proliferation and are blocked at this stage of development.

1.5 Involvement of Ras signalling in lymphocyte development

The G protein Ras (small GTPase) is a regulatory GTP hydrolase that cycles between two conformations: activated (Ras-GTP) or inactivated (Ras-GDP) (76, 77). It is activated by guanine exchange factors (GEFs), which are themselves activated by mitogenic signals through feedback from the Ras itself. It is inactivated by GTPases-activating proteins (GAPs), which increase the rate of GTP hydrolysis, returning Ras to its GDP-bound form, while simultaneously releasing an inorganic phosphate. Ras is attached to the cell membrane by prenylation, is a key component in many pathways which couple growth factor receptors to downstream mitogenic effectors involved in cell proliferation or differentiation (78). Ras activates a number of pathways (Figure 1.6) but an especially important one seems to be the MAPKs, which themselves transmit signals downstream to other protein kinases and gene regulatory proteins (79).

1.5.1 The Ras-MAPK pathway

MAPKs are activated by a wide range of extracellular stimuli mediating an array of cellular functions, from proliferation and activation to growth arrest and cell death. The MAPK family is subdivided into three groups; the classical signal-regulated kinases (ERK-MAPK), the c-Jun N-terminal kinases, also known as the stress activated protein kinases (JNK/SAPK) and the p38 MAPKs (80). Activation of each group is determined by distinct upstream MAPK kinases (MEKs) and MAPKK kinases (MEKK). MAPKs are activated by dual phosphorylation on tyrosine and threonine residues, located in a T-X-Y motif, where the X is individual to each sub-class of kinase. Following MAPK activation a number of downstream transcription factors are activated such as ELK-1 and c-myc (ERKMAPK); c-Jun, ATF-2 and p38 (JNK); and ATF-2 and MAX (p38 MAPK). Therefore, phosphorylation and activation of these transcriptional regulators enables the MAPK families to regulate gene expression and hence, cellular responses.

Activated Ras functions as an adaptor that binds with high affinity to Raf kinases and causes their translocation to the cell membrane, where full activation occurs. The exact mechanism behind this is not known, however it requires Ras binding and multiple phosphorylations including autophosphorylation at threonine 372 in the conserved region 2 domain (81). Activated Raf then binds to and phosphorylates the dual specificity kinases MEK1/2, which in turn phosphorylates ERK 1/2 on the conserved TEY motif in their activation loops. Ras is extremely efficient in its activation capacity, as evidenced by the fact that 5% Ras activation leads to complete ERK 1/2 activation (82). ERK 1/2 targets cytoplasmic proteins, membrane proteins, cytoskeletal proteins and nuclear proteins (83). Rsk 1-3, Mnk1 and Mnk2 are subset targets of ERK 1/2 and have been implicated in the promotion of protein synthesis (84-86). In addition to enhancing gene expression by intermediary kinases, ERK 1/2 can also directly phosphorylate transcription factors such as Elk-1, cFos and c-Jun, therefore increasing transcription of pro-proliferative genes (87-89). ERK 1/2 can also phosphorylate membrane protein substrates phospholipase A_2 (PLA_2) and epidermal growth factor (90, 91). Differential utilisation of these pathways by the ERK module is implicated in many diverse downstream cellular responses including proliferation, survival and apoptosis.

1.5.2 PI3 Kinase pathway

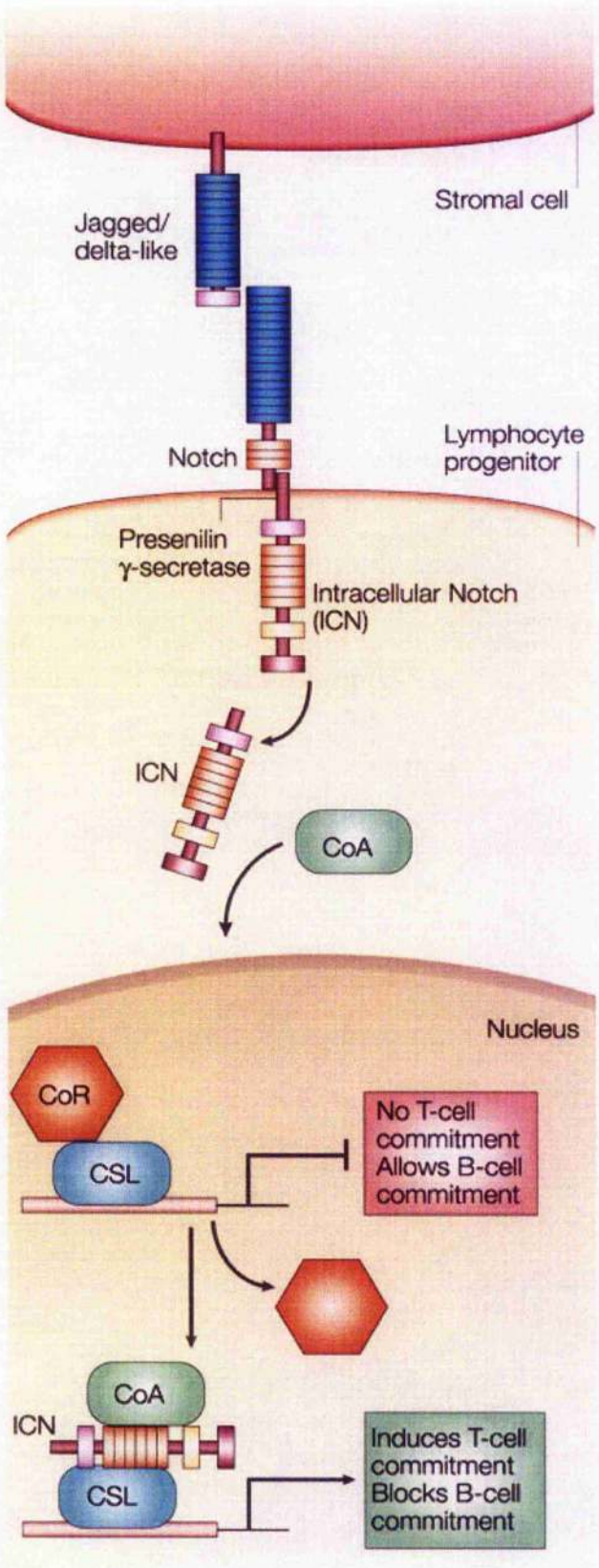
PI3K phosphorylates inositol phospholipids on the 3 position of the inositol ring, resulting in the production of phospholipids that are present at very low levels prior to receptor engagement (92-94). PI-(4,5)-P2, the major substrate of PI3K, is phosphorylated to produce PI-(3,4,5)-P3, both of these molecules can act as second messengers by acting as ligands for pleckstrin homology (PH) domains found in a large number of cytosolic proteins. This enables the co-localisation of the PH domain containing proteins as well as recruitment to the plasma membrane. PI3K also produces the phosphoinsitides PI-(3)-P and PI-(3,4)-P2 which along with PI(3,4,5)-P3 are known to govern events such as cell survival, cell growth, cytoskeletal remodelling and the trafficking of intracellular organelles (95).

AKT (PKB) is a major downstream target of the PI3K pathway that can modulate cell fate decisions such as survival and proliferation. The AKT protein family consists of three isoforms AKT1, AKT2 and AKT3 that are all regulated by similar PI3K and phosphorylation dependent mechanisms. AKT contains a PH domain that act to recruit AKT to the plasma membrane where it undergoes dimerisation and consequently a conformational change, allowing AKT to be phosphorylated by serine/threonine kinases such as PDK1 (96). Once AKT has been phosphorylated, it translocates to the specific cellular locations where it has multiple pro-survival actions targeting both mitochondrial and caspase-dependent events (97).

1.6 Aims and objectives

The initial aim of this study was to investigate the role of Ras and how its activity impacted the biological outcome of β selection, utilising a number of T cell generating culture systems. Upon emergence of sustained $B220^+$ cells within these T cell environments we then went on to investigate the mechanisms that may account for this phenomenon. We are interested in expanding the knowledge gained in these studies and addressing how modulators of Ras activity can impact the biological outcomes of β selection and B/T cell lineage decisions.

Figure 1.1 **Schematic diagram of Notch signalling.** Notch ligands (Jagged/DL) are expressed by stromal cells and bind to Notch receptors on the HPCs. This induces the presenilin-dependent γ-secretase-mediated cleavage of intracellular Notch (ICN) (98). ICN translocates to the nucleus, where it interacts with a transcriptional co-activator (CoA), which in turn displaces a co-receptor complex (CoR) containing silencing mediator of retinoid and thyroid hormone receptors (SMR) and histone deacetylase (HDAC1) from its association with the DNA-binding protein CSL (CBF1/RBF-J), to induce the transcription activation of T-cell-lineage-specific genes. Diagram taken from Nature Reviews: Immunology 2004, 'T- cell development made simple' by J.C. Zuniga-Pflucker (99).



Nature Reviews | Immunology

Figure 1.2 **Schematic diagram of intrathymic T-cell development.** The thymic architecture is organised into discrete cortical and medullary areas, each of which is characterised by the presence of particular stromal cell types, as well as thymocytes precursors at defined maturation stages (100). Interactions between Notch receptor-expressing thymocytes and thymus stromal cells that express Notch ligands induce a complex programme of T-cell maturation in the thymus, which ultimately results in the generation of self-tolerant $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. Diagram taken from Nature Reviews: Immunology 2001, 'Lymphostromal interactions in thymic development and function' by Anderson and Jenkinson (20).

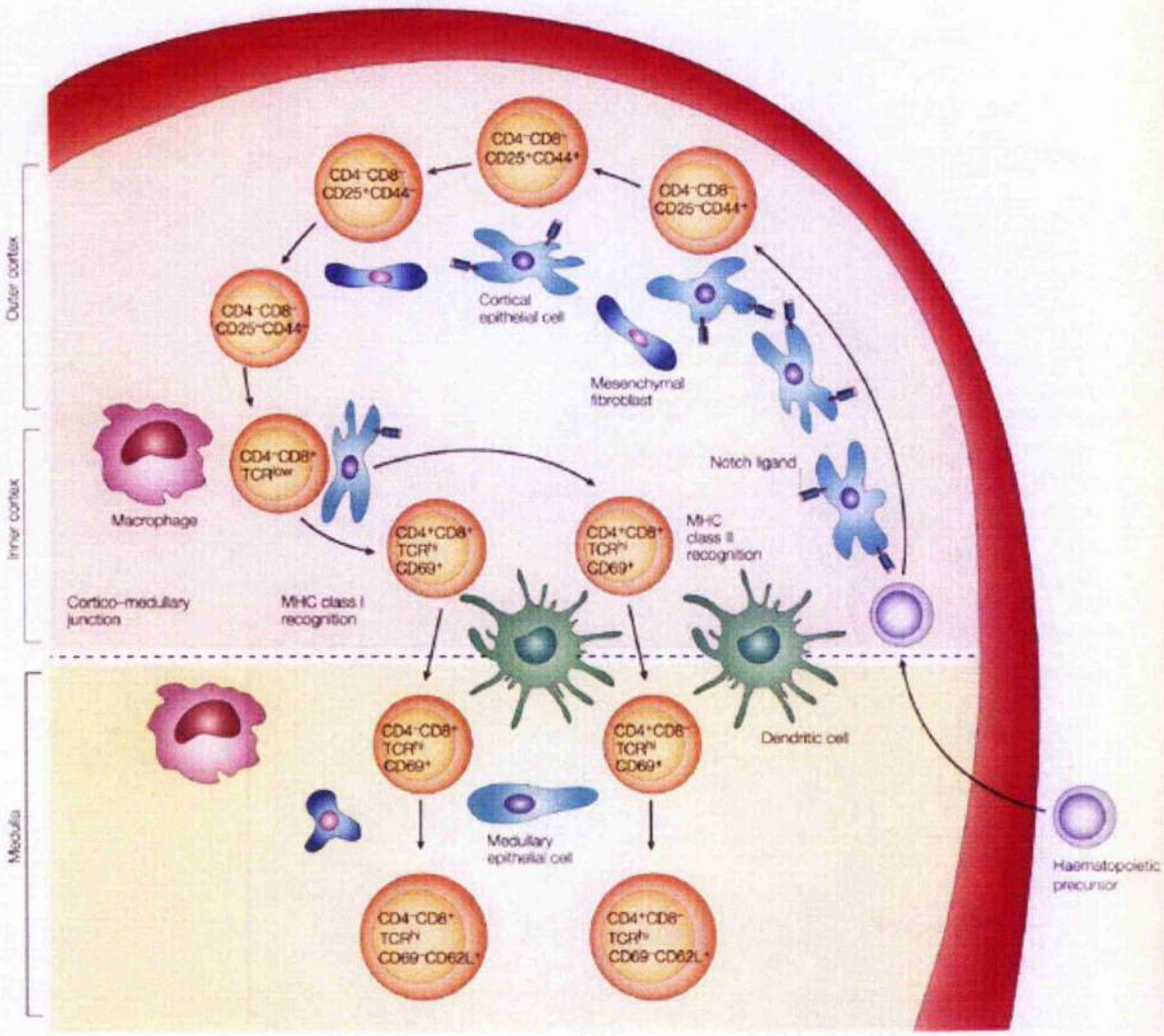

Outer cortex
Inner cortex
Medulla
CD4⁻CD8⁻ CD25⁺CD44⁺
CD4⁻CD8⁻ CD25⁺CD44⁻
CD4⁻CD8⁻ CD25⁻CD44⁺
CD4⁻CD8⁻ CD25⁻CD44⁺
Cortical epithelial cell
Mesenchymal fibroblast
Notch ligand
CD4⁻CD8⁺ TCR^low
Macrophage
CD4⁺CD8⁺ TCR^hi CD69⁺
CD4⁺CD8⁺ TCR^hi CD69⁺
MHC class II recognition
Cortico-medullary junction
MHC class I recognition
Dendritic cell
CD4⁻CD8⁺ TCR^hi CD69⁺
CD4⁺CD8⁻ TCR^hi CD69⁺
Medullary epithelial cell
Haematopoietic precursor
CD4⁻CD8⁺ TCR^hi CD69⁻CD62L⁺
CD4⁺CD8⁻ TCR^hi CD69⁻CD62L⁺

Figure 1.3 **Primary checkpoints in T cell development.** Thymocyte development is characterised by the cell surface expression of CD4 and CD8. DN HPCs entering the thymus from the FL or BM have to pass through the primary checkpoint (β selection), ensuring that only thymocytes that have generated a functionally rearranged TCRβ chain can be selected to differential to the DP stage. The second checkpoint occurs when DP thymocytes undergo positive and negative selection, allowing for the differentiation of MHC-restricted and self-tolerant $CD4^+$ or $CD8^+$ αβ-T cells (101).

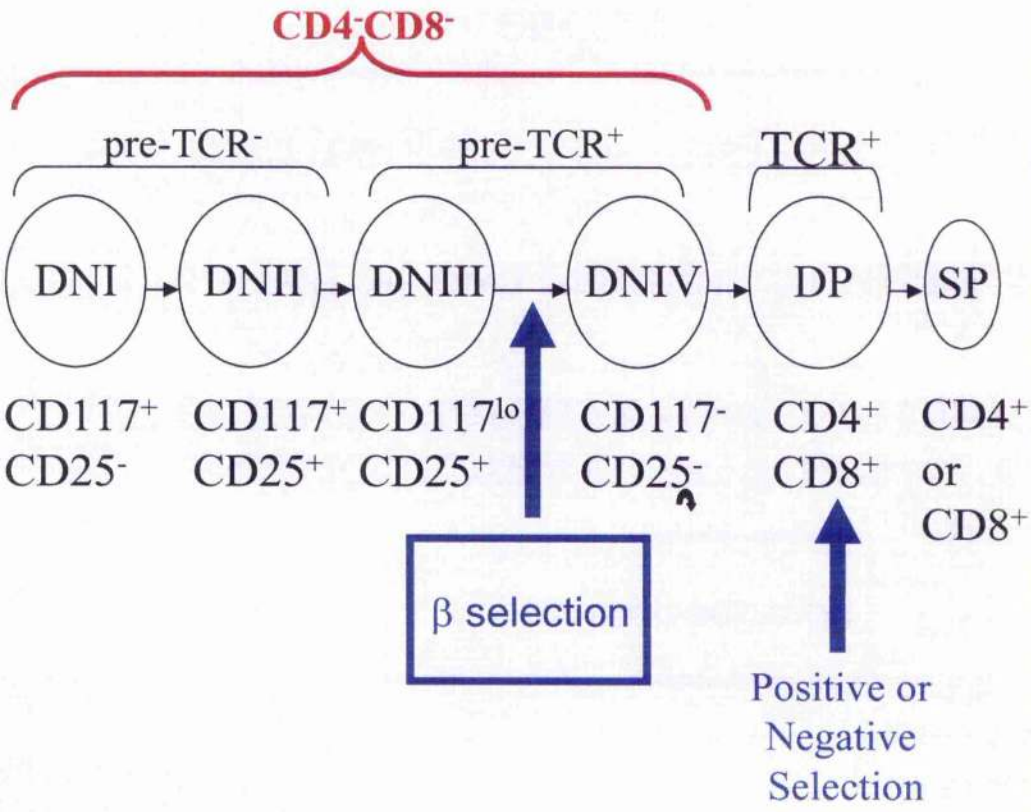

CD4-CD8-
pre-TCR-
pre-TCR+
TCR+
DNI
DNII
DNIII
DNIV
DP
SP
CD117+ CD25-
CD117+ CD25+
CD117lo CD25+
CD117- CD25-
CD4+ CD8+
CD4+ or CD8+
β selection
Positive or Negative Selection

Figure 1.4 **Signalling downstream of the pre-TCR.** The CD3 components that form part of the pre-TCR contain several immunoreceptor tyrosine-based activation motifs (ITAMs) within their cytoplasmic domain. Phosphorylation of the ITAMs creates docking sites for the SH2-domain containing Syk-family PTKs (protein tyrosine kinases), ZAP-70 and Syk. Adaptors, exchange factors and GTPases, regulate more distal events in pre-TCR signalling.

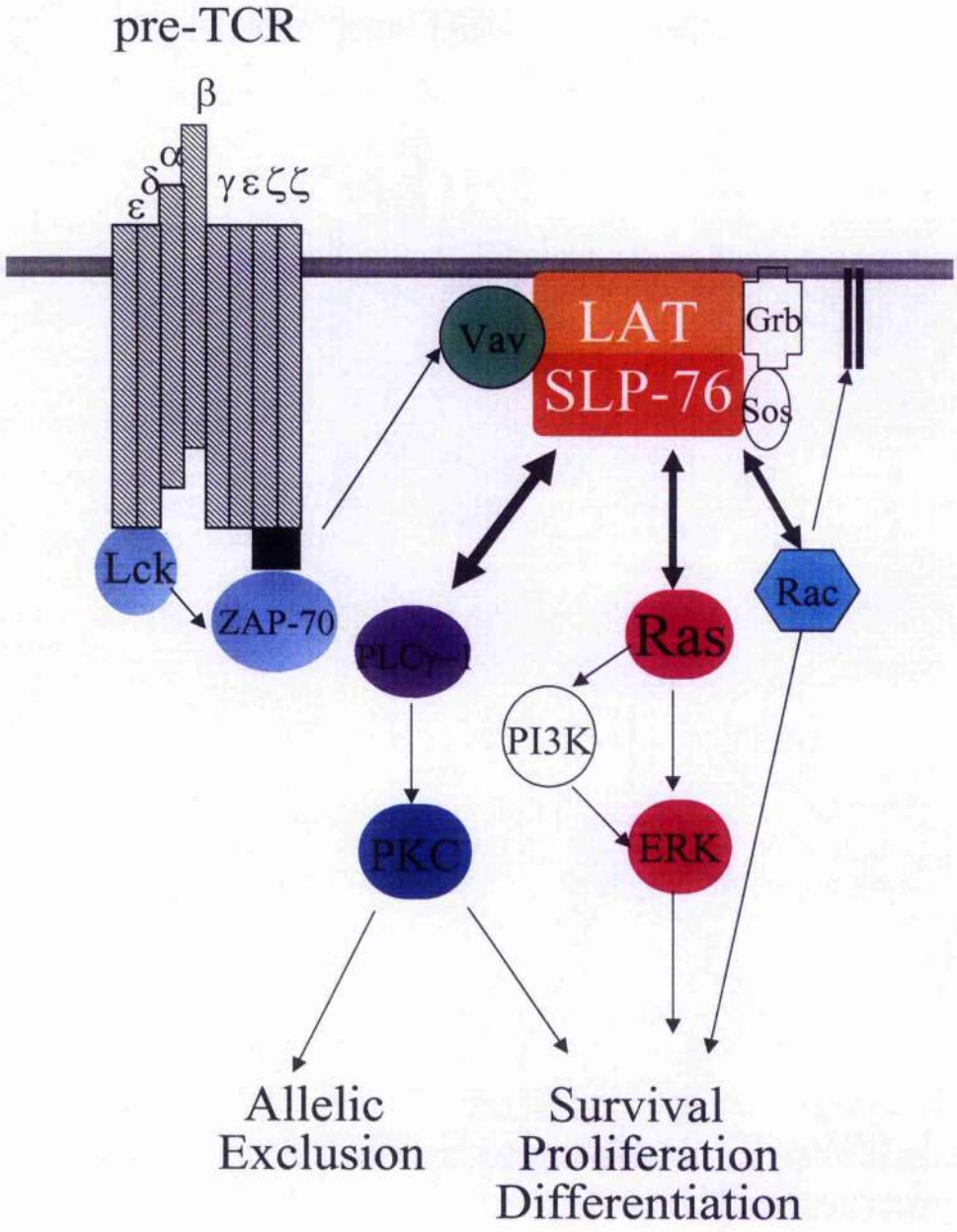
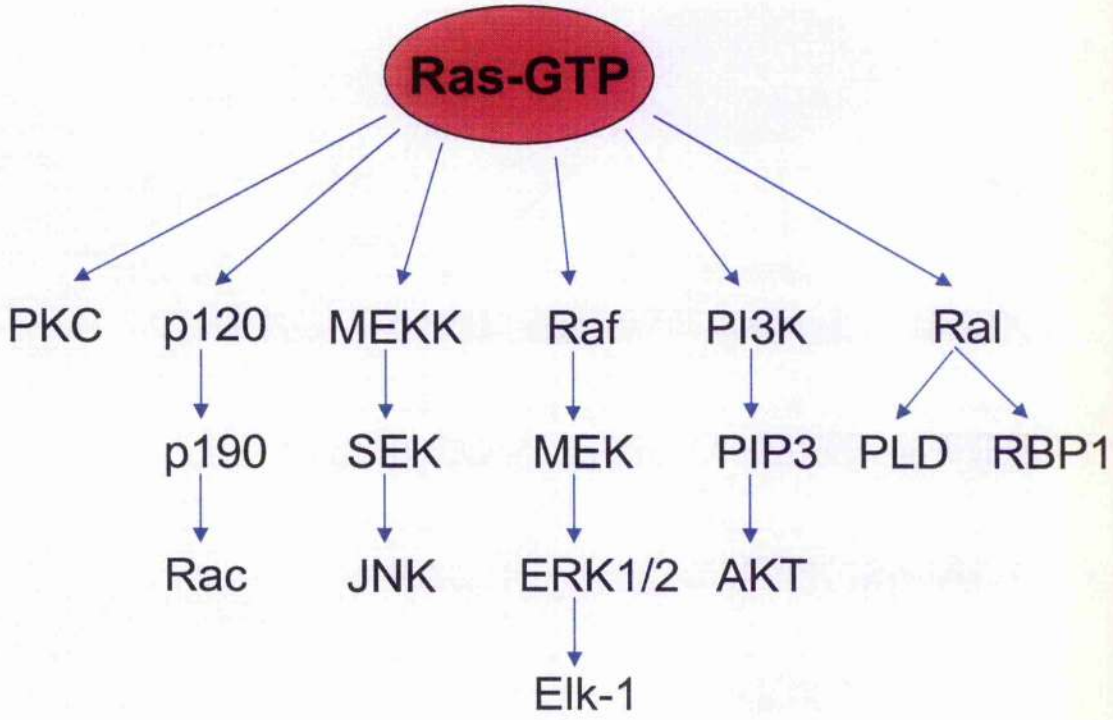

pre-TCR
β
α
δ
ε
γεζζ
Vav
LAT
SLP-76
Grb
Sos
Lck
ZAP-70
PLCγ-1
Ras
Rac
PI3K
PKC
ERK
Allelic
Exclusion
Survival
Proliferation
Differentiation

Figure 1.5 **Phenotypic characterisation of B cell development.** Definition of developmental stages of the various B cell types, present throughout B cell development. The classification into various stages of B cell development can be defined by the absence or presence of specific surface markers. The earliest marker that defines B cells committed to the B cell lineage is the CD45R isoform B220. B220 is expressed at all stages of B cell development, and levels increase during transition from the immature to the mature stage.

Cell type:	Pro-B	Late pro-B	Large pre-B	Small pre-B	Immature B	Mature B
Surface Markers:	$B220^{+}$ $CD19^{+}$ $CD43^{+}$ $CD24^{-}$ $CD25^{-}$ $CD117^{+}$ IgD^{-} $CD23^{-}$	$B220^{+}$ $CD19^{+}$ $CD43^{+}$ $CD24^{+}$ $CD25^{-}$ $CD117^{+}$ IgD^{-} $CD23^{-}$	$B220^{+}$ $CD19^{+}$ $CD43^{-}$ $CD24^{++}$ $CD25^{+}$ $CD117^{-}$ IgD^{-} $CD23^{-}$	$B220^{+}$ $CD19^{+}$ $CD43^{-}$ $CD24^{++}$ $CD25^{+}$ $CD117^{-}$ IgD^{-} $CD23^{-}$	$B220^{+}$ $CD19^{+}$ $CD43^{-}$ $CD24^{++}$ $CD25^{-}$ IgM^{++} $CD117^{-}$ IgD^{-} $CD23^{-}$	$B220^{++}$ $CD19^{++}$ $CD43^{-}$ $CD24^{+}$ $CD25^{-}$ IgM^{+} $CD117^{-}$ IgD^{++} $CD23^{+}$

Figure 1.6 **Signalling downstream of Ras.** Schematic diagram showing some of the many signalling pathways downstream of Ras. Ras is activated through G-protein coupled receptors, receptor tyrosine kinases, Janus kinase 1 or increases in intracellular calcium. Ras targets multiple effectors.


Ras-GTP
PKC
p120
MEKK
Raf
PI3K
Ral
p190
SEK
MEK
PIP3
PLD
RBP1
Rac
JNK
ERK1/2
AKT
Elk-1

CHAPTER 2 – Materials and Methods

2.1 Animals and Cell Lines

2.1.1 Animals and Primary Cells

Wild type ICR mice were purchased from Harlan-Olac (Oxon, UK) and maintained at the University of Glasgow Central Research Facilities. The mice were mated overnight and examined for a copulation plug the next morning (day 0 gestation). FL cells and foetal thymus (FT) were collected from ICR mice at day 14 of gestation.

RAG-1$^{-/-}$ mice (37) were bred and maintained in-house. All animals were maintained in accordance with local home office regulations. Rag-1 and Rag-2 genes are responsible for encoding the early lymphocyte-specific proteins necessary for initiation of V(D)J recombination (102). Inactivation of these genes results in a complete lack of functional lymphocytes as a result of a block in lymphocyte development at the progenitor B and T cells stages deriving from the abrogation of the initiation of V(D)J rearrangement.

2.1.2 Bone Marrow Stromal Cell Lines

OP9 cells are a BM stromal cell line commonly used to investigate the differentiation of HPCs into B cell lineage. This cell line does not express macrophage colony stimulating factor (M-CSF) which was found to inhibit the differentiation of HPCs to blood cells other than macrophages (103). The cell line was maintained in OP9 media (High Glucose-DMEM media containing 10% FBS (Bio Whittaker UK Ltd, Wokingham, UK), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 10mM HEPES, 50 μM 2-mercaptoethanol and 10 μg/ml gentamicin (Invitrogen, Paisley, UK) supplemented with 0.5 mg/ml geneticin (Invitrogen).

OP9-DL1 is a derivative of the OP9 cell line which has been retrovirally transduced to express the Notch-ligand DL1. OP9-DL1 cells have been shown to lose the ability to support B cell development, while gaining the ability to support normal T cell development (13). The cell line was maintained in OP9-DL1 media (α MEM containing 100 U/ml penicillin, 100 μg/ml streptomycin and 20% FBS (Invitrogen, Paisley, UK)). Cells were kept under a humidified atmosphere of 5% CO_2 at 37°C, and split every four days with media changes every two days.

2.1.3 SL-12β.12 Cells

SL-12β12 cell line is a pre-T cell line derived from a spontaneous SCID mouse thymoma, that stably expresses functionally-rearranged TCR-β chain at the cell surface with endogenous pTα to form the pre-TCR (Figure 2.1) (104). The cell line was maintained in OP9 media supplemented with 0.5mg/ml G418/geneticin, and kept under a humidified atmosphere of 5% CO_2 at 37°C.

2.1.4 Retroviral packaging cell lines.

Retroviral constructs were engineered by subcloning the gene of interest (Ras^{V12}, $Ras^{V12}S35$, $Ras^{V12}C40$; gifts from Prof. J. Downward) into the retroviral backbone (MIEV), 5' of the internal-ribosomal entry site, allowing the bicistronic expression of the gene of interest and green fluorescent protein (GFP). The retroviral vectors were stably expressed in the retroviral packaging line, GP+E.86 (105) and maintained in OP9 media.

2.2 Retroviral Infection

2.2.1 Mitomycin C treatment of packaging cell lines

The GP+E.86 cell lines were seeded at $2x10^4$ cells/well and cultured in OP9 media supplemented with 10 μg/ml mitomycin C (Sigma-Aldrich Co., Poole, UK) for 2-3 hours at 37°C one day prior to retroviral infection of primary cells. The GP+E.86 cells were washed three times with OP9 media prior to co-culture with HPCs.

2.2.2 Preparation of HPCs-enriched from FLs

Single cell suspension was prepared from ICR FL by crushing the tissue with a glass bottle stopper and passing through 70μm mesh to remove cell debris. HPC-enriched-FL cells were obtained by carrying out a CD24 (Heat stable antigen (HSA) (BD Biosciences, Oxford, UK)-antibody complement-mediated cell lysis on the FL single cell suspension for 30 min at 37°C, as described previously (106). The red/dead cells were removed by density configuration over Lymphocyte-Mammal (VH Bio Ltd. Newcastle, UK) at 600g for 15 min at room temperature(RT). The enriched population was removed from the interface between the media and Lymphocyte-Mammal, and washed in foetal thymic organ culture (FTOC) media (OP9 media supplemented with 5% FBS).

2.2.3 Retroviral Infection of HPC enriched population

The HPC enriched population was co-cultured with the mitomycin C-treated retroviral packaging line in FTOC media supplemented with 5 μg/ml polybrene (Sigma-Aldrich) and 10 ng/ml cytokines (rIL-6, rIL-7 and stem cell factor (SCF) Table 2.2) for 24 hr prior to co-culture on the BM stromal cell lines OP9/OP9-DL1 or in FTOC to assess their ability to give rise to B/T lineage cells.

2.3 Generation of lymphoid lineage cells from HPCs

2.3.1 FTOC

In order to analyse the T cell developmental potential, retrovirally-infected HPCs were cultured with alymphoid deoxyguanosine (dGuo)-treated FTs. DGuo-treated FTs were generated by incubating d14 FTs with 1.1 mM dGuo for 5 days in FTOC configuration (FT are placed on a nucleopore filter (Merck Ltd.) at the air-liquid interphase in a humidified incubator, 5% CO_2 at 37°C) (107) (Figure 2.2). After 5 days in culture the dGuo was removed from the medium and the cells were cultured for a maximum of 2 days prior to reconstitution with retrovirally-infected HPCs. dGuo-treated FTs were seeded with $1x10^4$ retrovirally-infected HPCs in Terlsaki plates (Fisher Scientific, Loughborough, UK) and incubated in hanging drop for 24 hr then placed in FTOC for 7-10 days (media was changed every 4-5 days) and analysed by flow cytometry.

2.3.2 High Oxygen Submersion (HOS)

In FTOC media d14 ICR FTs were placed into 96 well plates (2 lobes per well), pre-seeded with mitomycin C treated retroviral packaging cells lines. The 96 well plate was placed into a plastic bag, the air was saturated with 70% O_2/5% CO_2/25% N_2 and sealed. After 36 hr incubation the FTs were transferred onto FTOC and cultured in FTOC media for 7-11 days.

2.3.3 OP9-DL1 co-culture

Transduced HPC-enriched-FL cells were cultured on OP9-DL1 cells at a density of 2×10^4 cells/well in the presence of OP9-DL1 media containing 10 ng/ml of mouse IL-7 and Flt-3 ligand (Table 2.2). Media was replenished every two days. Cell commitment and developmental progression was assessed by flow cytometry during the course of the culture.

2.3.4 Intraperitoneal Injection of retrovirally infected HPCs

Retrovirally infected ICR HPC-enriched-FL cells were removed from the GP+E.86 co-culture by gentle pipetting and the cell concentration was adjusted to 1 x 10^6 cells/100 µl in sterile PBS for intraperitoneal (IP) transfer to neonatal recipient $RAG^{-/-}$ mice. Cells were injected in 100 µl volumes using an insulin syringe (23-gauge needle) at least 3 mice per group as indicated in figure legends. After 6-8 weeks, the mice were sacrificed and the development of lymphoid cell populations was determined by flow cytometry from the spleen and thymus.

2.4 Flow Cytometry

R-phycoerthrin (PE)-, allophycocyanin (APC)-, fluorescein isothiocyanate (FITC) and biotin-conjugated anti-mouse antibodies were used for flow cytometric analysis (BD Biosciences, Table 2.1). Cells were prepared for flow cytometric analysis as described in section 2.2.2 (106). Briefly, the retrovirally-infected HPC-enriched cells were either removed from the OP9 or OP9-DL1 cell lines by gentle pipetting, single cell suspensions were generated by crushing the FTOCs or organs and resultant cells were washed in FACS buffer (HBSS containing 1% BSA and 0.1% sodium azide (Invitrogen)). Cells were incubated with the appropriate antibodies (diluted 1:300), as indicated in the figure legends, for 30 min at 4°C. The cells were then washed twice in FACS buffer and resuspended in FACS buffer. The cells were acquired using a FACSCalibur flow cytometer using the CELLQuest software package (BD Biosciences) and analysed using FlowJo (Treestar, Stanford, CA). All data shown are live gated by size and lack of propidium iodide (PI) uptake.

2.5 Semi-Quantitative RT-PCR

Total RNA was isolated from retrovirally infected ICR FL derived HPCs co-cultured on OP9-DL1 cells at days 0 and day 6, using RNAeasy mini kit (Qiagen Ltd., Crawley, West Sussex, UK.) following the manufacturers instructions. In order to

amplify RNA, the RNA substrate needs to be converted into DNA this can be achieved through the use of the reverse transcriptase AMV RT (avian myeloblastis virus reverse transcriptase). The AMV synthesizes the new cDNA strand at sites determined by the primers used. The resulting cDNA can then be used as a template for PCR. Therefore, cDNA from each sample was prepared from 1μg RNA using the first strand cDNA synthesis kit (Roche Diagnostic Systems, Lewes, UK) following the manufacturers instructions.

Semi-quantitative PCR was performed using the same serially diluted cDNA batches shown for β-actin (13, 108). Samples were amplified by PCR, utilizing gene specific primer pairs (Table 2.3), Taq polymerase (Helena Biosciences, Fermentas, UK), dNTPs and magnesium sulphate buffer (Roche Diagnostic Systems), on an Eppendorf Personal Mastercycler (Eppendorf UK Ltd., Cambridge, UK). To separate the PCR products they were pulled down 1 % agarose gels/1 x TAE (0.04 M Tris, 0.001 M EDTA-Na_2-salt and 0.02 M Acetic acid), and visualized using ethidium bromide staining and UV imager (Gene genius Bio imaging system, Syngene (Synoptics)). All PCR products correspond to the expected molecular sizes.

2.6 Transfection of SL-12β.12 cells

All electroporations were carried out using a BioRad Gene Pulser II (BioRad, Hemel Hempstead, UK). SL-12β.12 cells were electroporated at 960μF, 260 V, attaining a time constant of 50-55 μsec. Up to $3x10^7$ cells were transfected with desired plasmid DNA (up to 40μg), as indicated in the figure legends. Each sample was transfected with PathDectect reporter plasmids (Stratagene, Amsterdam, NL); as indicated in figure legends) and 2μg plasmid encoding β-galactosidase (pCMV-β-gal). The addition of a fixed amount of β-galactosidase plasmid allowed for the control of transfection efficiency during the experiment. The β-galactosidase activity was used to index the luciferase signal detected, as we were able to assay for luciferase and β-galactosidase activity within the same sample (see below and

(41)). Cells were washed in electroporation media (RPMI 1640 containing 20% FCS) and resuspended in 250 μl electroporation media per transfection. DNA and cells were combined in 4 mm sterile cuvettes (BioRad) and incubated on ice for 10min. Transfected cells were put into fresh complete media and incubated for 24 h at 37°C, with or without the addition of exogenous stimuli or pharmacological inhibitors, as indicated in the figure legends. The cells were then lysed and analysed for luciferase and β-galactosidase activity.

2.6.1 Reporter plasmid system

This system allows us to determine which upstream kinases are affecting Elk activity downstream of the pre-TCR complex ligation within SL-12β.12 cells. The reporter system utilizes two plasmids: a fusion-activator plasmid (pFA-Elk), which encodes for the trans-activation domain of Elk, fused with the DNA-binding domain of GAL4; and a luciferase-reporter plasmid (pFR-Luc), which encodes for the luciferase gene under the control of five GAL4 binding elements. Hence, in transfected cells, phosphorylation of the Elk-fusion protein by upstream kinases can be readout in the form of luciferase activity (Figure 2.3).

2.6.2 Luciferase and β-galactosidase Assay

SL-12β.12 cells transfected with the PathDetect reporter plasmids (Stratagene) were assayed for luciferase and β-galactosidase activities utilizing substrates that generate light emission. The cells were lysed in lysis buffer (40 mM tricine, pH 7.8, 50 mM NaCl, 2 mM EDTA, 1 mM $MgSO_4$, 5 mM dithiothretol (DTT), and 1% Triton X-100). Supernatant (25 μl) was combined with an equal volume of luciferase reaction buffer (30 mM tricine, pH 7.8, 3 mM ATP, 15 mM $MgSO_4$, 1mM coenzyme A, and 10 mM DTT), and after addition of 1 mM luciferin (BD Biosciences), the samples were immediately assayed for luciferase activity with a Lumat LB 9507 Luminometer (Berthold Technologies, Bad Wildbad, Germany). To assay for β-galactoside activity, Galacton-Star (substrate for β-galactosidase; Applied

Biosystems, Bedford, USA) was diluted 1:50 with Galacton-Star Reaction Buffer Diluent (Applied Biosystems) and 100µl was added to each tube after the luciferase assay was completed. The tubes were incubated for 30-60 min at RT and then the samples were assayed for β-galactosidase activity, measured as light emission with the Lumat LB 9507 Luminometer. Samples were assayed in triplicate and the results represent the average luciferase activity indexed for β-galactosidase activity.

Table 2.1 Antibodies used

Specificity	Conjugation	Clone	Isotypes
CD117	PE,APC,FITC	2B8	Rat IgG2bκ
CD4	PE,APC	RPA,TA	Rat IgG2aκ
CD8	APC		Rat IgG2aκ
CD25	PE,FITC	PC61,7D4	Rat IgG2bκ
TCRβ	APC,Biotin	H57-597	Armenian Hamster IgG2λ
NK1.1	APC	PK135	Mouse IgG2aκ
CD11b	PE,FITC	M1/70	Rat IgG2bκ
CD19	APC	ID3	Rat IgG2aκ
CD45R	PE,APC,FITC	RA3-6B2	Rat IgG2aκ
IgM	APC	IL/41	Rat IgG2aκ
CD24	-	30-F1	

Antibodies were purchased from BD Pharmingen or Cell Signalling Technologies.

Table 2.2 Cytokines

Cytokine	Species	Supplier
rIL-7	Mouse	Caltag-MedSystems Ltd.
rIL-6	Mouse	Caltag-MedSystems Ltd.
rSCF	Mouse	Caltag-MedSystems Ltd.
rFlt-3 ligand	Mouse	R&D Systems

Table 2.3 Oligonucleotide primers for semi quantitative PCR

Primer	Species	Primer Sequence	Expected Size (bp)
bActin F bActin R	Mouse	5'- GAT GAC GAT ATC GCT GCG CTG –3' 5'- GTA CGA CCA GAG GCA TAC AGG –3'	539
Pax5 F Pax5 R	Mouse	5'- CTC GGA CCA TCA GGA CAG GAC –3' 5'- TCA TCC CTC TTG CGT TTG TTG –3'	

All Primers were purchased from MWG-Biotech.

Figure 2.1 **Schematic diagram of the SL-12β.12 cell line.** The SL-12β.12 cell line is a pre-T cell line derived from a spontaneous SCID mouse-derived thymoma, that stably expresses a functionally rearranged TCRβ chain at the cell surface with endogenous pTα to form the pre-TCR (106).

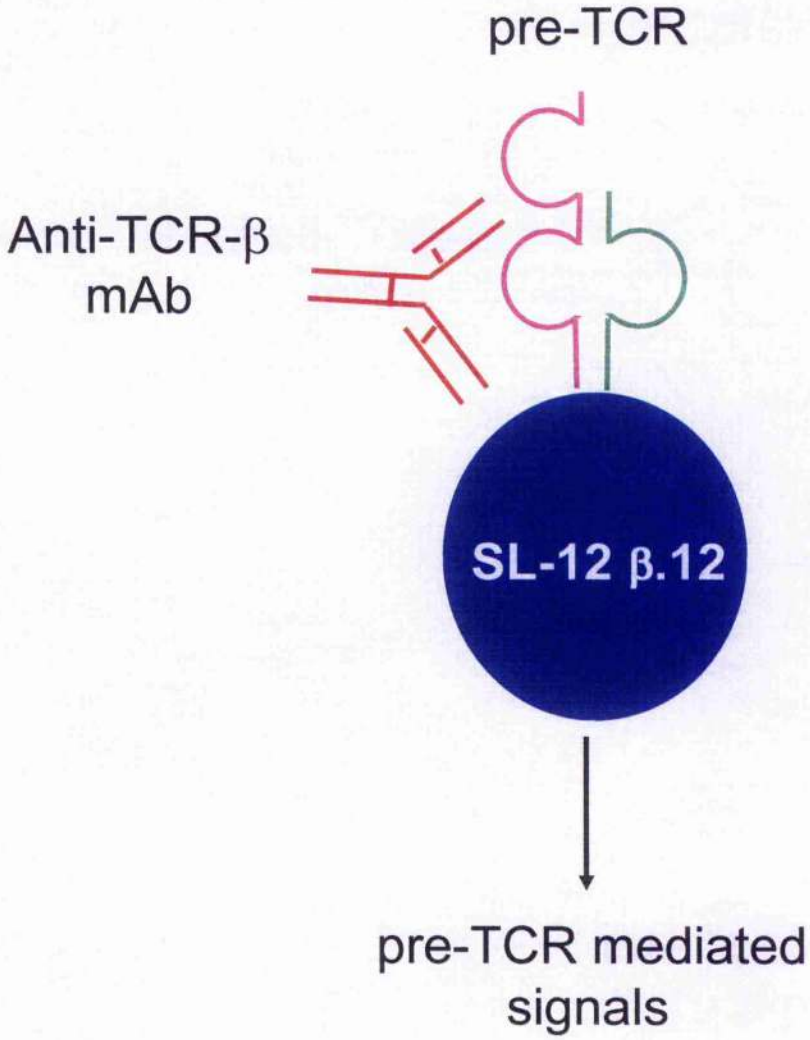

pre-TCR
Anti-TCR-β
mAb
SL-12 β.12
pre-TCR mediated
signals

Figure 2.2 ***In vitro* and *in vivo* models for studying T lymphocyte development.** The internal ribosome entry site (IRES) of the bicistronic retroviral vector MIEV permits the translation of two open reading frames from one messenger RNA, therefore the expression of GFP is directly proportional to the level of the gene of interest (Ras^{V12}, $Ras^{V12}S35$ and $Ras^{V12}C40$). HPCs are prepared from the FL of d14 RAG/wild type mice and incubated overnight on the GP+E.86 retroviral packaging cell line as described in the materials and methods (section 2.2.3). The retrovirally infected HPCs are used for FTOC, HOS-FTOC, OP9-DL1 co-culture or adoptive transfer assays.

Bicistronic Retroviral Vector (MIEV):

Retroviral Infection:

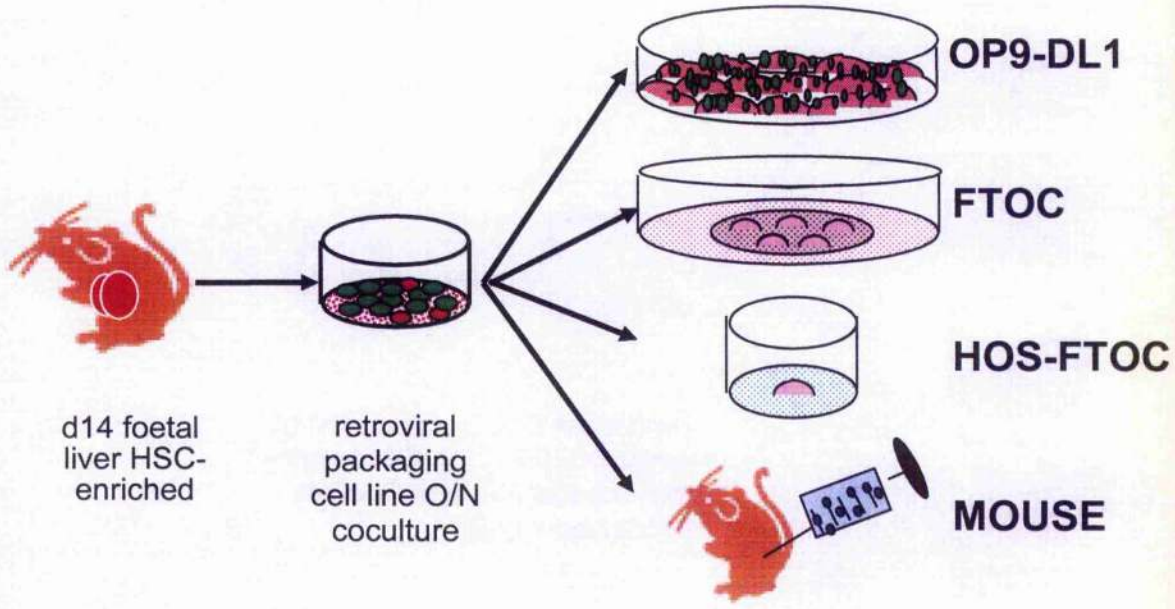
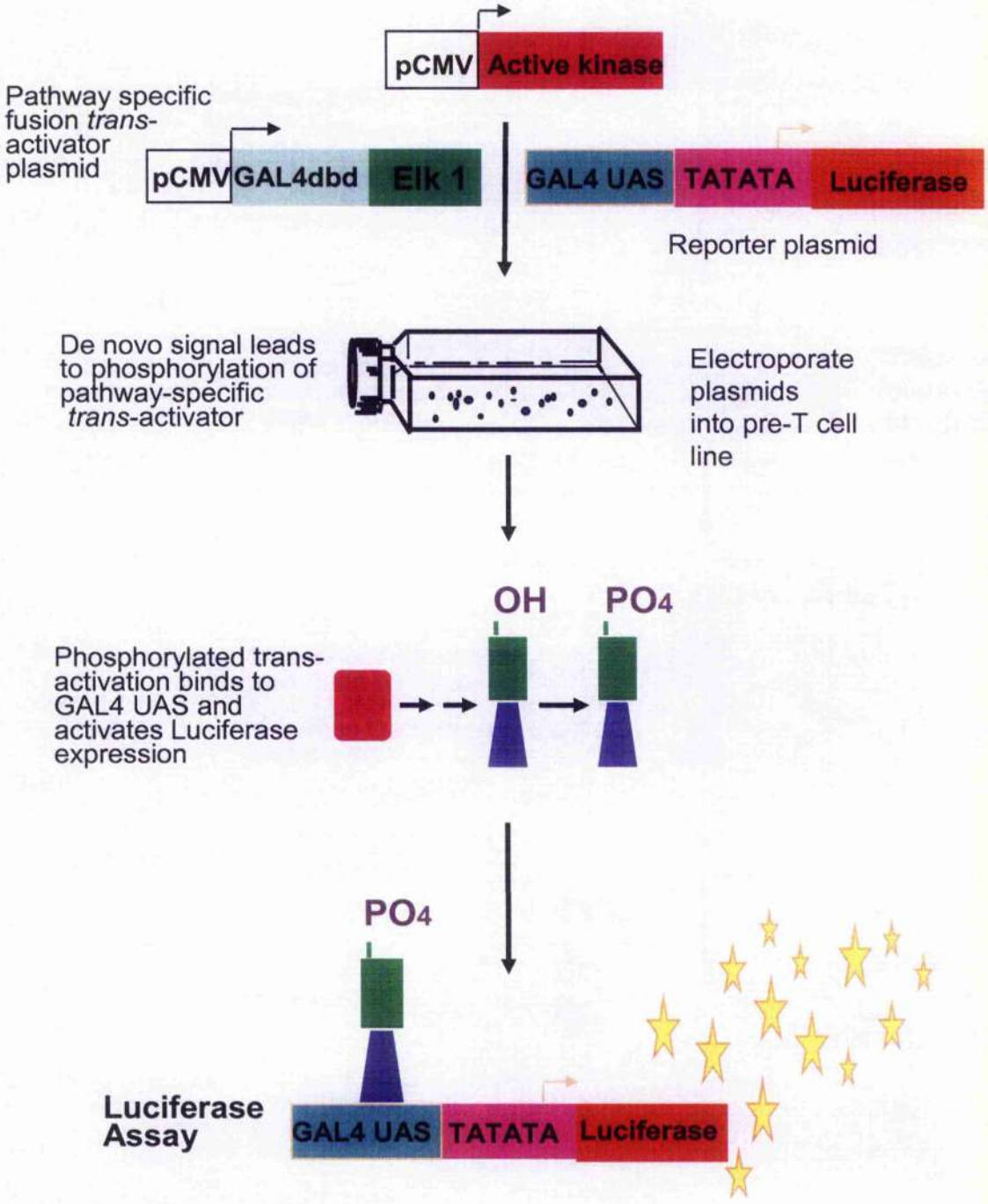


Figure 2.3 **Schematic representation of the Reporter Plasmid System.** The trans-activator plasmid system allows us to determine whether or not our protein of interest directly or indirectly activates Elk 1 and therefore Erk-MAPK. The reporter plasmid, trans-activator plasmid and expression vector along with our gene of interest are cotransfected into the pre-T cell line SL-12β.12. The expression of luciferase from the reporter plasmid (pFR-Luc) indicates that our gene of interest is directly or indirectly involved in the phosphorylation and activation of the transcriptional factor in the trans-activator plasmid, and therefore involved in the respective signal transduction pathway.


pCMV
Active kinase
Pathway specific fusion *trans*-activator plasmid
pCMV
GAL4dbd
Elk 1
GAL4 UAS
TATATA
Luciferase
Reporter plasmid
De novo signal leads to phosphorylation of pathway-specific *trans*-activator
Electroporate plasmids into pre-T cell line
OH
PO4
Phosphorylated trans-activation binds to GAL4 UAS and activates Luciferase expression
PO4
Luciferase Assay
GAL4 UAS
TATATA
Luciferase

CHAPTER 3

3.1 Introduction

3.1.1 Retroviral Infection System

A retroviral vector is an infectious virus used to introduce a non-viral gene into mitotic cells *in vivo* or *vitro* (109, 110). They originate from replication-competent viruses isolated from either rodents or chickens, the vectors are modified to aid the transduction process. Retroviral vectors are efficient in achieving stable and transient transduction of a gene or genes into cells such as primary cells that are not easily transfected by other means, such as electroporation. Retroviral infection is dependent on binding to specific cell surface receptors, many of these receptors, such as ectotropic and amphotropic receptors are expressed on many cell types including most primary cells. As a result it is possible to infect most cells with retroviruses.

Although retroviral genomes are made of RNA, they produce a DNA copy of their genome immediately after infection of the host cell by utilizing both retroviral and host factors. Mediated by the viral *int* gene product, the viral DNA integrates into the host genome where it is referred to as a provirus. Infectious retroviral stocks are made by transfecting the retroviral vector plasmid into cell lines, called packaging lines (111). Once in a cell line, transcription proceeds from the viral long terminal repeat (LTR) promoter encoded by the plasmid, generating an RNA viral genome. The viral genome is then encapsulated by viral structural proteins, and the infectious viral particles are produced by budding from the cell surface (Figure 3.1).

We utilized the ecotropic packaging cell line GP+E-86 allowing the infection of rat and mouse cells only (112). The GP+E-86 cell line has been generated to express *gag-pol* and *env* from different plasmids, therefore eliminating the generation of an intact retroviral genome. When GP+E-86 cells are co-cultured with the progenitor

cells, infectious viral particles containing our Ras mutants are emitted from the GP+E-86 cells into the media and thereby infecting our cells of interest. In order to determine the role of Ras mediated signalling in T cell lineage commitment, we developed the retroviral vector MIEV containing Ras^{V12}, $Ras^{V12}S35$ and $Ras^{V12}C40$ to manipulate HPCs. This vector allows for the transcription of the gene of interest plus green fluorescent protein (GFP), thus enabling the retrovirally infected cells to be identified by flow cytometry via the expression of GFP. The Ras^{V12} mutant $Ras^{V12}S35$ is known to constitutively bind Raf to activate the ERK-MAPK cascade, whereas the $Ras^{V12}C40$ mutant signals constitutively binds to or activates PI3K, and Ras^{V12} signals via both .

3.1.2 *In vitro* generation of T cells

The traditional method of studying T cell development *in vitro* is the FTOC system, which allows T cell development to be studied in isolation and allows for important manipulations. FTs can be depleted of endogenous thymocytes by treatment with dGuo which allows defined subsets of stem cells or progenitors to reconstitute these empty thymic lobes (99, 107, 113). Until recently, efforts to induce T cell differentiation *in vitro* have been unsuccessful in the absence of a thymic microenvironment (114). The BM stromal cell line OP9 is derived from the $OP^{-}OP^{-}$ mouse, deficient in MCSF, and supports the differentiation of HPCs into B cells (115-117). These OP9 cells were analysed to determine whether they expressed Notch ligands, which provide essential cell contact dependent signals required for T cell commitment and differentiation (13). The analysis revealed that transcripts for Delta-like-1 and Delta-like-4 were undetectable in OP9 cells, but were present in thymus stroma-enriched cell suspensions. Delta-like-1 has been shown to engage and induce Notch receptor signalling, therefore it was hypothesised by Schmitt *et al* that lack of Delta-like-1 expression by OP9 cells may be responsible for their inability to support T cell lineage commitment and differentiation. Therefore, they generated OP9 cells expressing high levels of Delta-like-1, OP9-DL-1, which proved to be successful in supporting T cell commitment and differentiation, while inhibiting the development of B cells in lymphoid progenitor

cells. HPCs that were induced to differentiate on OP9-DL-1 cells under went clonal expansion and gave rise to both $\gamma\delta TCR^+$ (10%) and the $CD8^+TCR\alpha\beta^+$ SP cells which were found to be functionally mature (99).

3.2 Results

3.2.1 ERK is activated downstream of the pre-TCR via the Ras/MAPK pathway

To address the question of how ERK is activated downstream of the pre-TCR, we chose to utilise the pre-TCR cell line, SL-12$\tilde{\beta}$12. This cell line is commonly used as a cellular model which is known to activate ERK downstream of pre-TCR ligation (41). We employed a reporter-plasmid system that would allow us to determine the involvement of Ras mediated MAPK, and PI3K cascades on ERK activation, outlined in the material and methods (section 2.6.1). The addition of a fixed amount of β-galactosidase plasmid, to every electroporation, allowed for the control of transfection efficiency during the experiment. The β-galactosidase activity was used to index the luciferase signal detected, as their activities were assayed within the same sample. Hence, in transfected cells, phosphorylation of the ELK-1 fusion protein, and therefore ERK-MAPK activity can be read in the form of luciferase activity.

Initially, we wanted to address what the optimal concentration of our constitutively active pcDNA3.Ras^{V12} plasmids would be. Therefore, SL-12$\tilde{\beta}$12 cells were electroporated with pFR-Luc, pCMV-β-gal and pFA-Elk alone, or with 3μg, 10μg or 30 μg of the pcDNA3.Ras^{V12} plasmid. Cells transfected with pFR-Luc, pCMV-β-gal and pFA-ELK alone displayed background luciferase activity (Figure 3.2 (a)). Luciferase activity increased about 38.5 fold above background levels in cells transfected with pFR-Luc/pCMV-β-gal/pFA-ELK and 3 μg pcDNA3.Ras^{V12}. This increase in luciferase activity fell to 12.6 fold above background luciferase activity in cells transfected with 10 μg pcDNA3.Ras^{V12}, and to below background luciferase

activity in cells transfected with 30 μg pcDNA3.Ras^{V12}. Therefore, the optimal concentration of the pcDNA3.Ras^{V12} plasmid is 3 μg.

Upon electroporation of SL-12β.12 cells with pFR-Luc/pCMV-β-gal/pFA-ELK and stimulation with anti-TCR-β mAb, a 10 fold increase in luciferase activity was observed (Figure 3.2 (b)). The luciferase activity noted in unstimulated cells may be indicative of low-level constitutive ERK activity within SL-12β.12 cells, as it is partially blocked by the addition of the MEK1 inhibitor PD98059 (Figure 3.2 (b)) (41). These data indicate that the engagement of the pre-TCR complex results in the activation of ERK and subsequent phosphorylation of the ERK-1/2 substrate, Elk-1 (41, 118). SL-12β.12 cell were electroporated with pFR-Luc/pCMV-β-gal/pFA-ELK/pcDNA3.Ras^{V12} and stimulated with anti-TCR-β mAb, which indicated a 20 fold increase in luciferase activity in the presence of a constitutively active Ras signal (Figure 3.2 (c)). The addition of the MEK1 inhibitor PD98059 results in a partial inhibition of luciferase activity, which may imply that other signalling molecules and pathways are involved in pElk-1 as well as the MAPK pathway.

To investigate further the involvement of the MAPK and PI3K pathways in the ERK activation downstream of the pre-TCR, we electroporated SL-12β̃12 cells with pFR-Luc/pCMV-β-gal/pFA-ELK/pcDNA3.Ras^{V12}S35 or with pFR-Luc/pCMV-β-gal/pFA-ELK/pcDNA3.Ras^{V12}C40 . The transfected cells were then stimulated with immobilised anti-TCR-β mAb (Figure 3.2 (c)). Notably, in the cells electroporated with pFR-Luc/pCMV-β-gal/pFA-ELK/pcDNA3.Ras^{V12}C40 the levels of luciferase activity were less than that of the pFR-Luc, pCMV-β-gal and pFA-ELK alone cells, indicating that the PI3K signalling pathway may not be involved in ERK activation downstream of the Ras in SL-12β̃12 cells.

To investigate the role of Ras-MAPK signalling in ERK activation downstream of the pre-TCR complex, we electroporated SL-12β.12 cell with pFR-luc/pCMV-β-gal/pFA-ELK/pcDNA3.Ras^{V12}S35, and stimulated with anti-TCR-β mAb (Figure 3.2 (c)). These electroporated cells show a 12 fold increase in luciferase activity when

unstimulated, and a 24 fold increase in luciferase activity when stimulated. Upon addition of PD98059 we observe a 3 fold decrease in luciferase activity in unstimulated cells, and a 2.5 fold decrease in luciferase activity in stimulated cells, indicating that the MAPK pathway may not be the only signalling mechanism involved downstream of the pre-TCR in SL-12β.12 cells in activating Elk-1.

Taken together, these results imply that Ras activates ERK downstream of the pre-TCR, in SL-12β.12 cells. However, this does not appear to involve signalling through the Ras-mediated PI3K pathway. The MAPK signalling pathway does show ERK activation, but only 41% of the activation levels expressed in the presence of a constitutively active Ras^{V12} signal. Suggesting, that either other signalling molecules apart from MEK may be playing a role in ERK activation downstream of the pre-TCR complex in SL12-β.12 cells (e.g. PKCs, PDEs, etc.), or that it may be the result of a complementary synergistic activation of both signalling pathways.

3.2.2 Introduction of Ras^{V12} retrovirally infected wild type FL-derived HPCs into FTOC results in the production of $CD45R^+$ B cells

Freshly prepared wild type FL cells were retrovirally infected with either vector alone (MIEV), or with Ras^{V12} encoding vector (MIEV.Ras^{V12}). Retrovirally infected HPCs ($CD117^+GFP^+$) were separated by flow cytometric cell sorting and placed into FTOC for 13 days. After this time FTOCs were analysed by flow cytometry for the ability of Ras^{V12} expressing progenitor cells to progress through T cell development (Figure 3.3). After 13 days incubation, we observed a high percentage of GFP^+ cells in both the MIEV control (78%) and Ras^{V12} (95%) reconstituted FTOCs. Upon analysis of the GFP^+ population of cells we noted that the MIEV-FTOC culture had progressed through β selection with an emergence of DP $CD4^+$ and $CD8^+$ thymocytes, with minimal B cell commitment as expected (Figure 3.3). However, analysis of the Ras^{V12}-FTOC culture showed that there was a 32% decrease in DP thymocytes, and a 70% increase in B cell commitment (Figure 3.3). Thus, indicating that B lymphocytes are being generated in a T cell

microenvironment in the presence of a constitutively active Ras signal, suggesting that there may be an inhibition of T cell lineage commitment.

To investigate this further, freshly prepared FL cells were retrovirally infected with vector alone (MIEV), MIEV.Ras^{V12}, MIEV.Ras^{V12}S35, or MIEV.Ras^{V12}C40. The retrovirally infected foetal thymic lobes were placed into HOS-FTOC for 8-12 days. Although all the samples have GFP populations, they are at lower levels than that of the FTOC, this may be due to an inability for retroviral particles to penetrate/infuse into FT three dimensional structure. Upon analysis of T cell surface markers by flow cytometry, the GFP$^+$ population of the MIEV culture showed the emergence of CD4$^+$CD8$^+$ DP cells at days 8 and 12, as expected (Figure 3.4). The Ras^{V12}C40 HOS-FTOCs also displayed an emergence of CD4CD8$^+$ DPs at days 8 and 12 (27% and 46% respectively). However, there was a marked decrease in T cell commitment and differentiation seen in the Ras^{V12} and Ras^{V12}S35 HOS-FTOCs, in comparison with MIEV control. Ras^{V12} culture produced 5% DPs at day 8, and 1% DPs at day 12. The Ras^{V12}S35 culture contained 15% DPs at day 8, and 5% DPs at day12 (Figure 3.4). From this, we concluded that the constitutive activation of the ERK-MAPK signalling cascade, via Raf, in HOS-FTOC is significantly delaying, or inhibiting T cell development.

Figure 3.5 shows the flow cytometric analysis of NK1.1, CD19 and igM lymphoid markers present at day 8. The Ras^{V12} and Ras^{V12}S35 HOS-FTOCs displayed an increase in CD19 expression (55% and 28% respectively) compared to MIEV and Ras^{V12}C40 (0% and 3% respectively). In conclusion, HOS-FTOCs reconstituted with Ras^{V12} and Ras^{V12}S35 showed a decrease in T cell commitment and an increase in B cell expression marker CD19.

3.2.3 Ras^{V12}-ERK signals compete with Notch

We were interested in using the OP9-DL1 model system to investigate the mechanisms involved in T cell development, so initially we set out to generate mature T lymphocytes by co-culturing HPCs on OP9-DL1 cells. To determine if our OP9-DL1 cells have the ability to support and maintain T cell commitment and differentiation, FL-derived HPCs were obtained from wild type mice at day 14 of gestation, and co-cultured with the OP9-DL1 cells, in the presence of IL7 and Flt3L. Flow cytometric analysis was carried out at days 4, 8, 12 and 16 of the cultures. Our results indicated that by day 8 the majority of HPCs had given rise to $CD117^{-}CD25^{+}$ DNIII cells (Figure 3.6 (a)) suggesting commitment to the T cell lineage (14, 119). Day 16 culture analysis showed that the OP9-DL1 cells give rise to, and support, DP thymocytes while down regulating CD25 (Figure 3.6 (b)), demonstrating our ability to generate T cells by OP9-DL1/HPCs co-culture.

To further investigate the role that constitutively activated Ras plays in T cell development, freshly prepared wild type FL cells were retrovirally infected with MIEV, MIEV.Ras^{V12}, MIEV.Ras^{V12}S35, or MIEV.Ras^{V12}C40. The retrovirally infected HPCs cells were placed onto OP9-DL1 cultures for 4-8 days. After this time the FL-derived cells were analysed by flow cytometry to determine the ability of constitutively active Ras expressing cells to progress through T cell development. From figures 3.8 and 3.9 it can be seen that GFP^{+} cells were evident in all cultures at days 4 and 8. Flow cytometric analysis of the cultures at day 4 (Figure 3.7) and day 8 (Figure 3.8), revealed normal T cell commitment and development in the uninfected (GFP^{-}) cell populations, as evidenced by the presence of $CD117^{+}CD25^{+}$ DNII and $CD117^{-}CD25^{+}$ DNIII thymocytes. Overall, at day 8 (Figure 3.8), the GFP^{-}, GFP^{lo} and GFP^{hi} Ras^{V12}C40 cultures showed a DNII to DNIII shift (66%, 60% and 28% respectively) when compared to the MIEV control (18%, 21% and 18% respectively), Further analysis is required to confirm whether an increase in advancement through T cell development is evident via constitutive PI3K signalling.

Upon analysis of the GFP^+ cells in Figure 3.9, we observed an increase in B cell populations in both the Ras^{V12} and $Ras^{V12}S35$ cultures, when compared to the MIEV alone or $Ras^{V12}C40$ cultures. At day 4, the GFP^{lo} and GFP^{hi} Ras^{V12} cultures showed a 9% increase in CD45R expression when compared to the respective MIEV cultures. At this time it was noted that the GFP^{hi} $Ras^{V12}S35$ culture showed a 1.5% increase in CD45R expression when compared to the MIEV control. At day 8, the Ras^{V12} cultures showed a 54% (GFP^{lo}) and 84% (GFP^{hi}) increase in CD45R expression when compared to MIEV (Figure 3.9). The ability of HPCs constitutively expressing Ras^{V12} to generate B lymphocytes corresponds to our HOS FTOC (Figure 3.5). Also at day 8, it can be observed that the $Ras^{V12}S35$ culture showed a 23% (GFP^{lo}), and a 54% (GFP^{hi}) increase in CD45R expression (Figure 3.9) when compared to MIEV. The differences between the GFP^{lo} and GFP^{hi} populations appear to be dose dependent, with the greater the expression of GFP relating to the greater the expression of the gene of interest (36, 106, 120, 121). Interestingly, from figures 3.8 and 3.9 we noted that the $Ras^{V12}C40$ culture contained no B cell commitment at day 4 or day 8.

3.2.4 Retrovirally infected $RAG^{-/-}$ FL-derived HPCs commit and differentiate when cultured on OP9-DL1 cells

To confirm that the activation of the ERK-MAPK cascade plays a central role in B cell lineage commitment, we retrovirally infected freshly prepared $RAG^{-/-}$ FL cells with either MIEV alone or containing Ras^{V12}, $Ras^{V12}S35$, or $Ras^{V12}C40$. As mentioned previously $RAG^{-/-}$ mice are blocked at the β selection stage of development and, therefore, are normally unable to develop mature B and T cells (38).

The $RAG^{-/-}$ retrovirally infected HPCs were placed onto OP9-DL1 cultures for 4-8 days. After this time the FL-derived cells were analysed by flow cytometry for the ability of Ras expressing cells to commit to the T cell lineage. Figures 3.10 and 3.11 show that GFP^+ cells were evident in all cultures at days 4 and 8. Flow cytometric analysis of the uninfected (GFP^-) cell populations at day 4 and day 8,

revealed normal T cell commitment and development by $RAG^{-/-}$ mice, as evidenced by the presence of DNII and DNIII thymocytes.

At day 8 (Figure 3.11), when we analysed for T cell surface markers we saw a decrease in T cell markers in the Ras^{V12} and $Ras^{V12}S35$ cultures, when compared to MIEV and $Ras^{V12}C40$. In the MIEV culture we see 41% (GFP^{lo}) and 36% (GFP^{hi}) DNIIIs present at day 8, however in the Ras^{V12} culture we see 5% (GFP^{lo}) and 1.7% (GFP^{hi}) DNIIIs, and in the $Ras^{V12}S35$ culture we observe 6% (GFP^{lo}) and 4% (GFP^{hi}) DNIIIs. Therefore, in comparison to MIEV, the Ras^{V12} shows a 36% (GFP^{lo}) and a 34% (GFP^{hi}) decrease in DNIII thymocytes. Also, the $Ras^{V12}S35$ culture shows a 32% (GFP^{lo}) and a 32% (GFP^{hi}) decrease in DNIII thymocytes (Figure 3.11).

Upon analysis of the GFP^{+} cells in Figure 3.12, we saw an increase in B cell populations in both the Ras^{V12} and $Ras^{V12}S35$ cultures, when compared to the MIEV alone or $Ras^{V12}C40$ cultures. In comparison to the MIEV culture, at day 4 the Ras^{V12} population showed an 8% (GFP^{lo}) and 1.5% (GFP^{hi}) increase in CD45R expression, and the $Ras^{V12}S35$ population showed a 9% (GFP^{lo}) and 15.5% (GFP^{hi}) increase in CD45R expressing cells. At day 8, in comparison to the MIEV culture the GFP^{lo} Ras^{V12} and $Ras^{V12}S35$ populations showed a 15% and 10% increase in CD45R expression respectively. Also at day 8, the GFP^{hi} Ras^{V12} and $Ras^{V12}S35$ populations showed a 29% and 11% increase in CD45R expression respectively. It was noted that the $Ras^{V12}C40$ culture contains no B cell commitment at day 4 or day 8 (Figure 3.12).

Thus far, the data obtained from the *in vitro* mouse model systems examined have concurred to show the same trend and outcome.

3.2.5 Ras/ERK-MAPK signalling in HPCs results in the development of B cells *in vivo*

As it is important, where possible, to study the development of lymphocytes in a normal physiological environment we utilised an adoptive transfer system. In order to determine the ability of our wild type derived retrovirally infected HPCs to commit to B cell development, within a T cell environment, we adoptively transferred them into neonatal $RAG^{-/-}$ mice. A mixture of both GFP^- and GFP^+ cells were injected into the recipient mice, therefore providing a useful internal control of wild type cells and allowing a comparison of lymphocyte development within the same host mouse.

The ability of the retrovirally infected wild-type donor cells to reconstitute the thymus of the host RAG $1^{-/-}$ mice was assessed 6-8 weeks post-injection. Figure 3.13 showed that the RAG $1^{-/-}$ thymus has been reconstituted with the wild type HPCs, due to the presence of $CD4^+CD8^+$ double positive cells. On comparison it was seen that the MIEV control and $Ras^{V12}C40$ mice had similar double positive populations, 86% and 82% respectively at the GFP^{lo} level. In contrast the $Ras^{V12}S35$ animal only had 19%, thus supporting the *in vitro* data shown above.

Analysis of B cell markers CD45R and IgM showed that the $Ras^{V12}S35$ mouse contained 26% $CD45R^+IgM^+$ cells in comparison with the MIEV and $Ras^{V12}C40$ animals, 0.1% and 0% respectively, at the GFP^{hi} level (Figure 3.13). This showed that constitutive activation of the Ras-ERK/MAPK pathway within the *in vivo* thymic environment resulted in the emergence of B cells, which was not seen in the control populations. However, constitutive activation of the PI3-K pathway, or lack of constitutive activation of ERK/MAPK, resulted in cell populations almost identical to the control.

3.2.6 Activation of the Ras/ERK-MAPK signalling cascade promotes the up-regulation of Pax5 expression.

The entry of lymphoid progenitors into the B cell pathway depends on the transcription factors E2A, EBF, and Pax5 (16). Pax5 is essential for the commitment of early progenitors to B lymphoid lineage, homozygous mutation of the Pax5 gene arrests B cell development at the early pro-B cell stage in the bone marrow (17). Semi-quantitative PCR analysis of retrovirally infected HPCs co-cultured on OP9-DL1 cells revealed that the constitutive activation of $Ras^{V12}S35$, induced the expression of Pax5 (Figure 3.14) (16, 17, 45, 58, 122, 123). Pax5 is detectable at low levels at d0 cultures, this may be due to small percentage of B lineage cells present in FL-HPC enriched populations (Figure 3.14). However, we observed an increase in expression of Pax5 in our day 6 cultures of $Ras^{V12}S35$ retrovirally infected HPCs. Pax5 was not detectable in the MIEV or $Ras^{V12}C40$ cultures at day 6, as these cultures contained mainly T cell lineage cells. This data concurs with the results obtained from the various *in vitro* systems that we have examined throughout the course of this research.

Figure 3.1 **Life cycle of replication competent retrovirus.** All retroviruses infect the host cells through an interaction with a specific viral receptor on the host cell surface, thereby entering the host cell and initiating reverse transcription of the RNA viral genome. The viral transcript contains the packaging sequence ψ which is recognised by the capsid proteins and allows it to be packaged into virus particles. A fully infectious viral particle containing the vector genome is thus budded form the packaging cell.

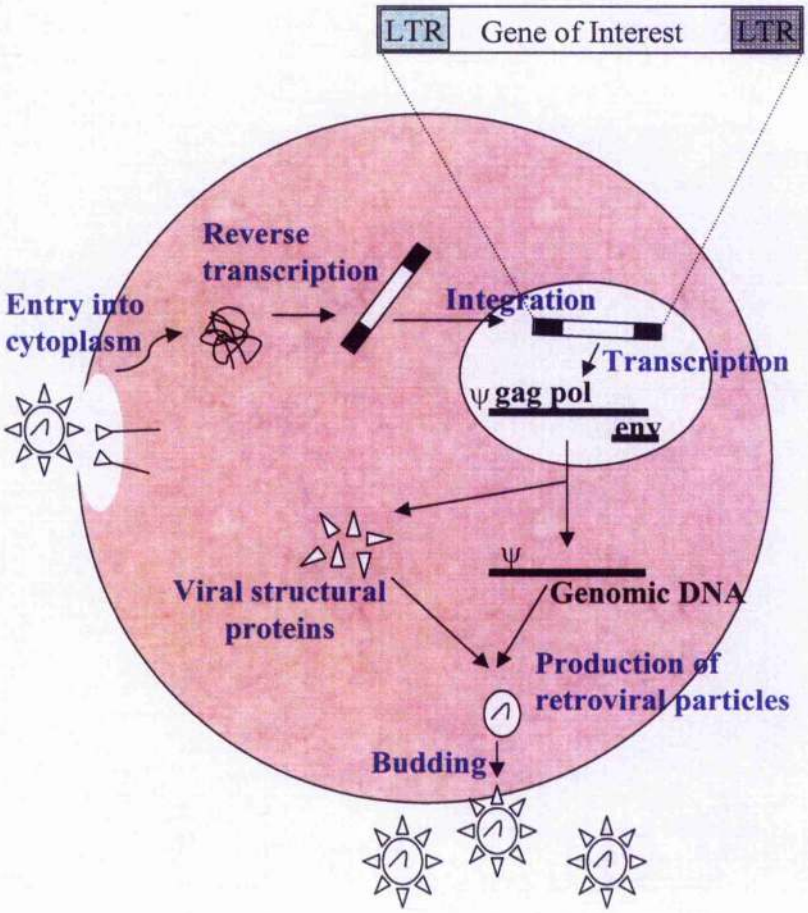
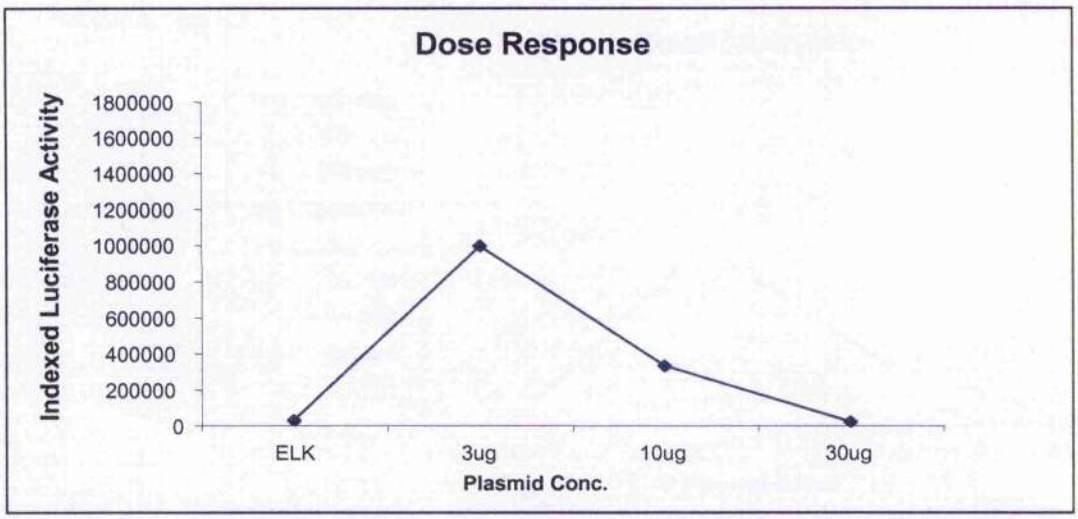
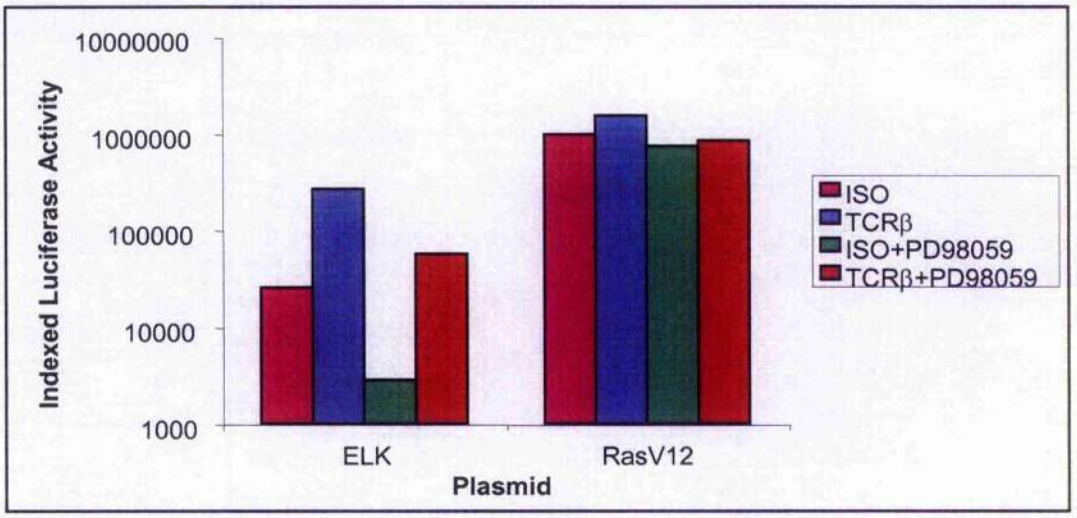

LTR
Gene of Interest
LTR
Reverse transcription
Entry into cytoplasm
Integration
Transcription
ψ gag pol
env
Viral structural proteins
ψ
Genomic DNA
Production of retroviral particles
Budding

Figure 3.2 **ERK activation downstream of the pre-TCR via the constitutively active pcDNA.Ras mutants.** (a) SL-12$\tilde{\beta}$12 cells were electroporated with pFR-Luc (5μg), CMV-β-gal (2μg), pFA-Elk (5μg) alone, or with 3μg, 10μg or 30μg of constitutively active pcDNA.Ras^{V12}. The transfected cells were then cultured for 16-20 hr, then lysed, and the lysates assayed for luciferase and β-galactosidase activity. (b) SL-12$\tilde{\beta}$12 cells were electroporated with pFR-Luc (5μg), CMV-β-gal (2μg), pFA-Elk (5μg) alone, or with constitutively active pcDNA.Ras^{V12} (3μg). These cells were then pre-treated with, or without, the MEK-1 inhibitor PD98059 (10μM) for one hour before being stimulated with, or without, anti-TCRβ Ab overnight. The transfected cells were then cultured for 16-20 hr, then lysed, and the lysates assayed for luciferase and β-galactosidase activity. (c) SL-12$\tilde{\beta}$12 cells were electroporated with pFR-Luc (5μg), CMV-β-gal (2μg), pFA-Elk (5μg) alone, and with constitutively active pcDNA.Ras^{V12}C40 or pcDNA.Ras^{V12}S35 (3μg). These cells were then pre-treated with, or without, the MEK-1 inhibitor PD98059 (10μM) for one hour before being stimulated with, or without, anti-TCRβ Ab overnight. The transfected cells were then cultured for 16-20 hr, then lysed, and the lysates assayed for luciferase and β-galactosidase activity. This data is representative of one experiment.

a


Dose Response
Indexed Luciferase Activity
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
ELK
3ug
10ug
30ug
Plasmid Conc.

b


Indexed Luciferase Activity
10000000
1000000
100000
10000
1000
ELK
RasV12
Plasmid
ISO
TCRβ
ISO+PD98059
TCRβ+PD98059

c

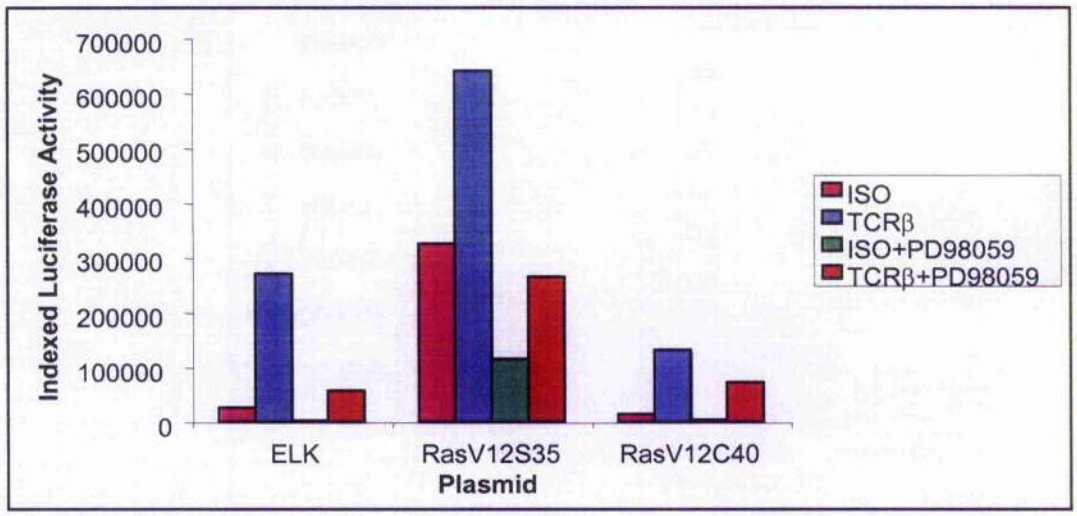
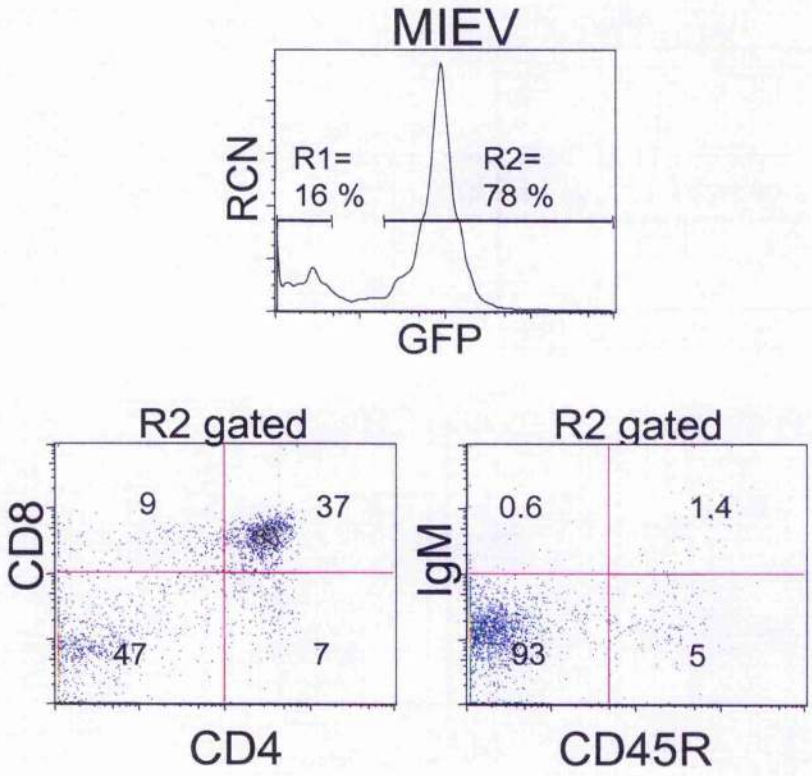

Indexed Luciferase Activity
700000
600000
500000
400000
300000
200000
100000
0
ELK
RasV12S35
RasV12C40
Plasmid
ISO
TCRβ
ISO+PD98059
TCRβ+PD98059

Figure 3.3 **Introduction of Ras^{V12} retrovirally infected wild type FL-derived HPCs into FTOC results in the production of $CD45R^{+}$ B cells.** FL cells were removed from time pregnant wild type mice (day 14 gestation) and retrovirally infected with either vector alone (MIEV) or Ras^{V12} for 24 hr. GFP^{+} $CD117^{+}$ cells were separated by flow cytometric cell sorting and placed in hanging drop with dGuo-treated FTs. After 24 hr the FT were incubated in FTOC for 13 days. Flow cytometric analysis of CD4 vs. CD8 and IgM vs. CD45R cell surface expression on thymocytes gated for GFP expression is shown (R2: GFP^{+}).


MIEV
RCN
R1=
16 %
R2=
78 %
GFP
R2 gated
CD8
9
37
47
7
CD4
R2 gated
IgM
0.6
1.4
93
5
CD45R

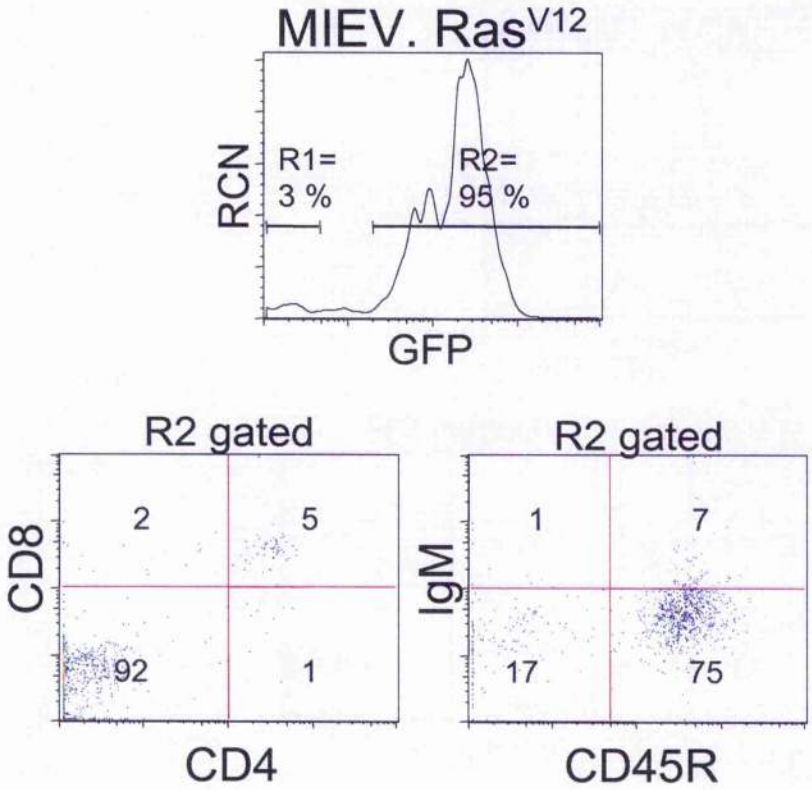
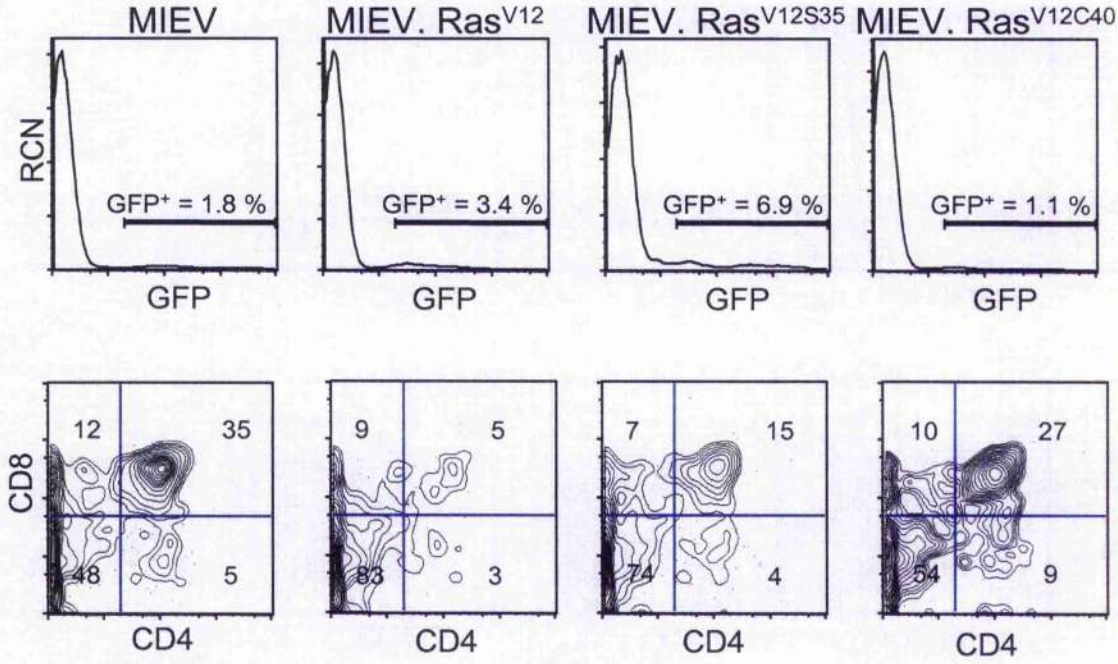

MIEV. Ras^{V12}
RCN
R1=
3 %
R2=
95 %
GFP
R2 gated
CD8
2
5
92
1
CD4
R2 gated
IgM
1
7
17
75
CD45R

Figure 3.4 **Analysis of T cell expression markers upon the introduction of Ras^{V12}S35 wild type FL-derived HPCs into HOS FTOC mimic's the effect of Ras^{V12}.** FLs were removed from time pregnant wild type mice (day 14 gestation) and retrovirally infected as described in section 2.3.2. After 24 hr the FT were transferred to FTOC and cultured for 7 to 11 days. Flow cytometric analysis of days 8 and 12 CD4 vs. CD8 cell surface expression on thymocytes gated for GFP expression is shown (R2: GFP^+). This data is representative of three separate experiments.

Day 8


MIEV
MIEV. Ras^{V12}
MIEV. Ras^{V12S35}
MIEV. Ras^{V12C40}
RCN
GFP$^+$ = 1.8 %
GFP$^+$ = 3.4 %
GFP$^+$ = 6.9 %
GFP$^+$ = 1.1 %
GFP
CD8
12
35
48
5
9
5
83
3
7
15
74
4
10
27
54
9
CD4

Day 12

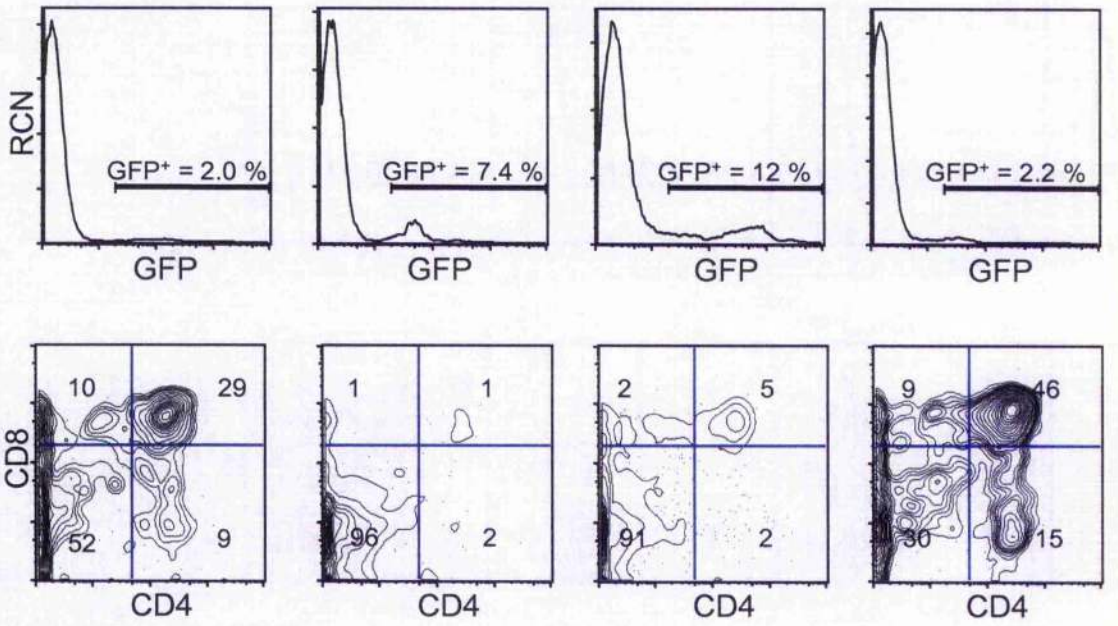

RCN
GFP$^+$ = 2.0 %
GFP$^+$ = 7.4 %
GFP$^+$ = 12 %
GFP$^+$ = 2.2 %
GFP
CD8
10
29
52
9
1
1
96
2
2
5
91
2
9
46
30
15
CD4

Figure 3.5 **Introducing $Ras^{V12}S35$ wild type FL-derived HPCs into HOS FTOC mimics the effect of Ras^{V12}.** FLs were removed from time pregnant wild type mice (day 14 gestation) and retrovirally infected as described in section 2.3.2. After 24 hr the FT were transferred to FTOC and cultured for 7 to 11 days. Flow cytometric analysis at day 8 of CD19 and IgM cell surface expression on thymocytes gated for GFP expression is shown (R2: GFP^+). This data is representative of three separate experiments.

Day 8

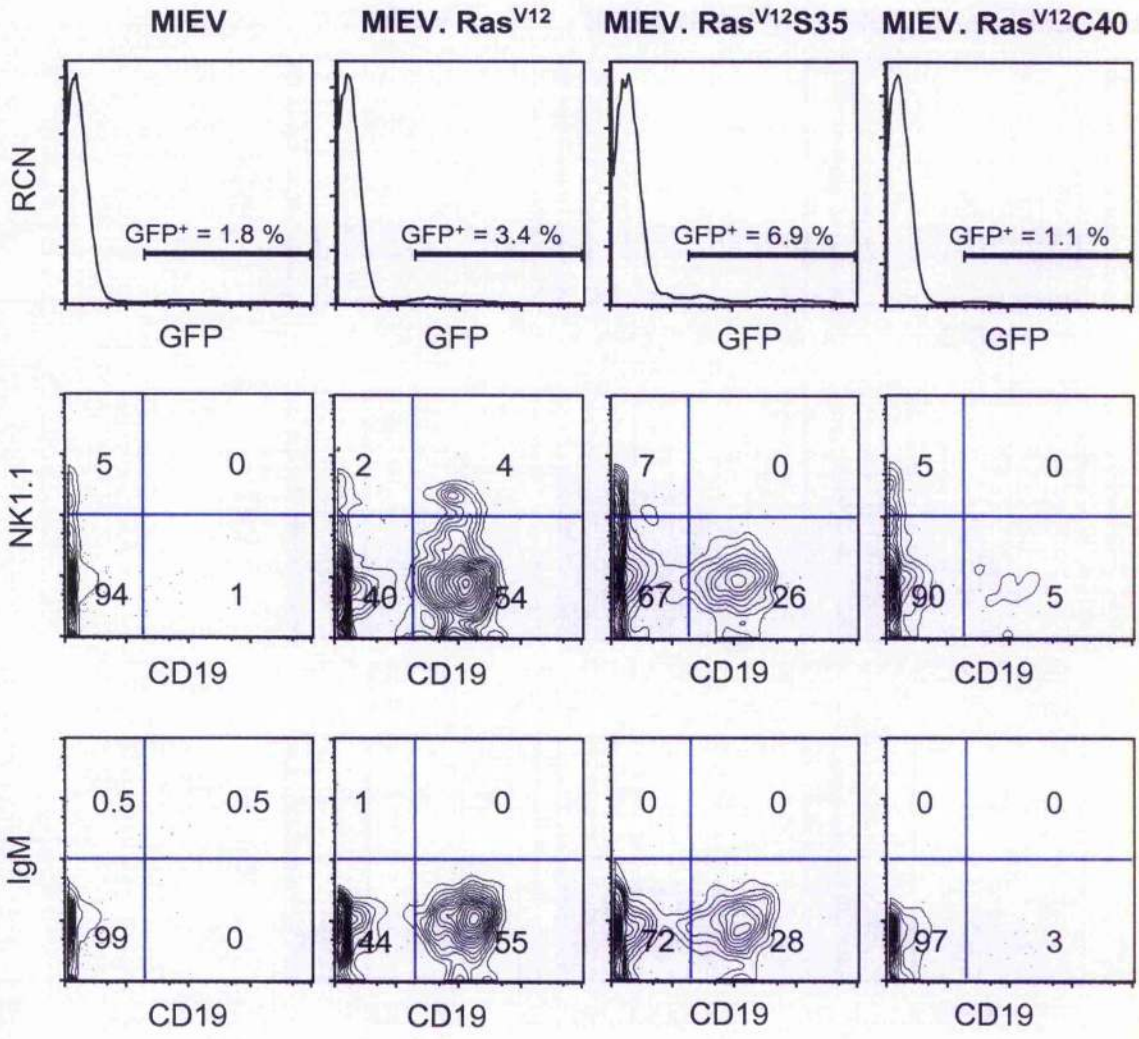

MIEV
MIEV. Ras^{V12}
MIEV. Ras^{V12}S35
MIEV. Ras^{V12}C40
RCN
NK1.1
IgM
GFP$^+$ = 1.8 %
GFP$^+$ = 3.4 %
GFP$^+$ = 6.9 %
GFP$^+$ = 1.1 %
GFP
CD19
5
0
94
1
2
4
40
54
7
0
67
26
5
0
90
5
0.5
0.5
99
0
1
0
44
55
0
0
72
28
0
0
97
3

Figure 3.6 **Generation of mature T lymphocytes on OP9-DL1 cells.** Wild type HPCs were prepared (as described in the materials and methods section 2.2.2) and placed onto co-culture for 4-16 days with OP9-DL1 cells. At these time points flow cytometric analysis for T cell markers (a): CD117, CD8 and CD25, and (b): CD8 and CD4 are shown. It has been found that the expression of the Notch ligand Delta-Like-1 on the BM stromal cell line OP9, supports the differentiation of HPCs into $CD4^{+}CD8^{+}$ immature DP T cells after 7 days of co-culture, and these DP T cells accounted for the majority of HPC-derived cells by day 12. HPCs co-cultured with OP9-DL1 cells failed to give rise to B cells, while the differentiation of NK cells was readily observed throughout (13). This data is representative of four separate experiments.

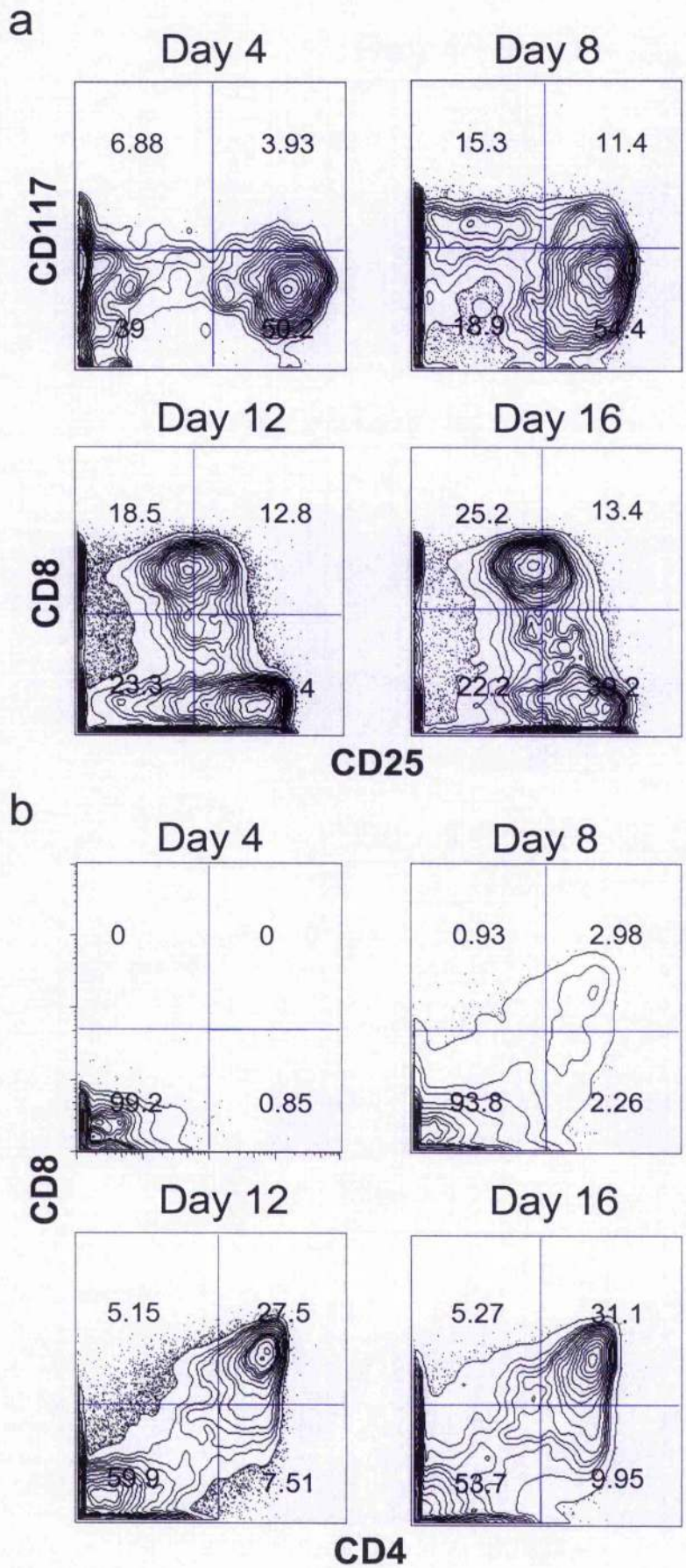

a
Day 4
Day 8
Day 12
Day 16
CD117
CD8
CD25
6.88
3.93
39
50.2
15.3
11.4
18.9
54.4
18.5
12.8
23.3
25.2
13.4
22.2
b
0
0
99.2
0.85
0.93
2.98
93.8
2.26
5.15
27.5
59.9
7.51
5.27
31.1
53.7
9.95
CD4

Figure 3.7 **Analysis of T cell expression markers on retrovirally infected wild type FL-derived HPCs cultured on OP9-DL1 cells for 4 days.** FL cells were removed from timed pregnant wild type mice at day14 of gestation and retrovirally infected with vector alone (MIEV) or Ras^{V12}, $Ras^{V12}S35$ or $Ras^{V12}C40$, for 24 hr. After 24 hr the FL cells were incubated on OP9-DL-1 cells for 4 days. Flow cytometric analysis of cell markers CD117 and CD25 is shown at day 4. This data is representative of three separate experiments.

Day 4

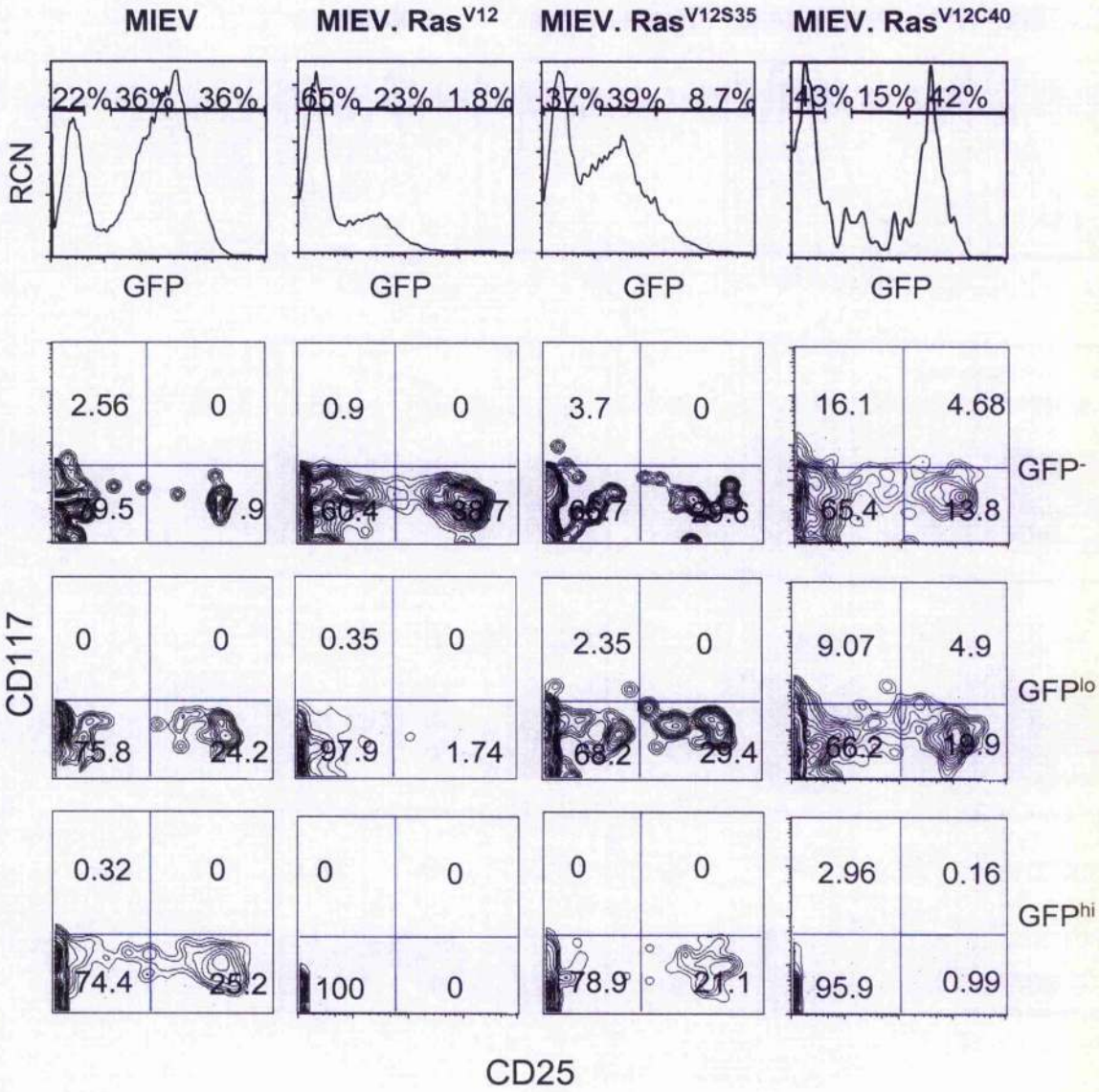

MIEV
MIEV. Ras^{V12}
MIEV. Ras^{V12S35}
MIEV. Ras^{V12C40}
RCN
22% 36% 36%
65% 23% 1.8%
37% 39% 8.7%
43% 15% 42%
GFP
GFP
GFP
GFP
CD117
2.56 0 79.5 17.9
0.9 0 60.4 38.7
3.7 0 65.7 30.6
16.1 4.68 65.4 13.8
GFP-
0 0 75.8 24.2
0.35 0 97.9 1.74
2.35 0 68.2 29.4
9.07 4.9 66.2 19.9
GFP^{lo}
0.32 0 74.4 25.2
0 0 100 0
0 0 78.9 21.1
2.96 0.16 95.9 0.99
GFP^{hi}
CD25

Figure 3.8 **Analysis of T cell expression markers on retrovirally infected wild type FL-derived HPCs cultured on OP9-DL1 cells for 8 days.** FL cells were removed from timed pregnant wild type mice at day14 of gestation and retrovirally infected with vector alone (MIEV) or Ras^{V12}, $Ras^{V12}S35$ or $Ras^{V12}C40$, for 24 hr. After 24 hr the FL cells were incubated on OP9-DL-1 cells for 8 days. Flow cytometric analysis of cell markers CD117 and CD25 is shown at day 8. This data is representative of three separate experiments.

Day 8

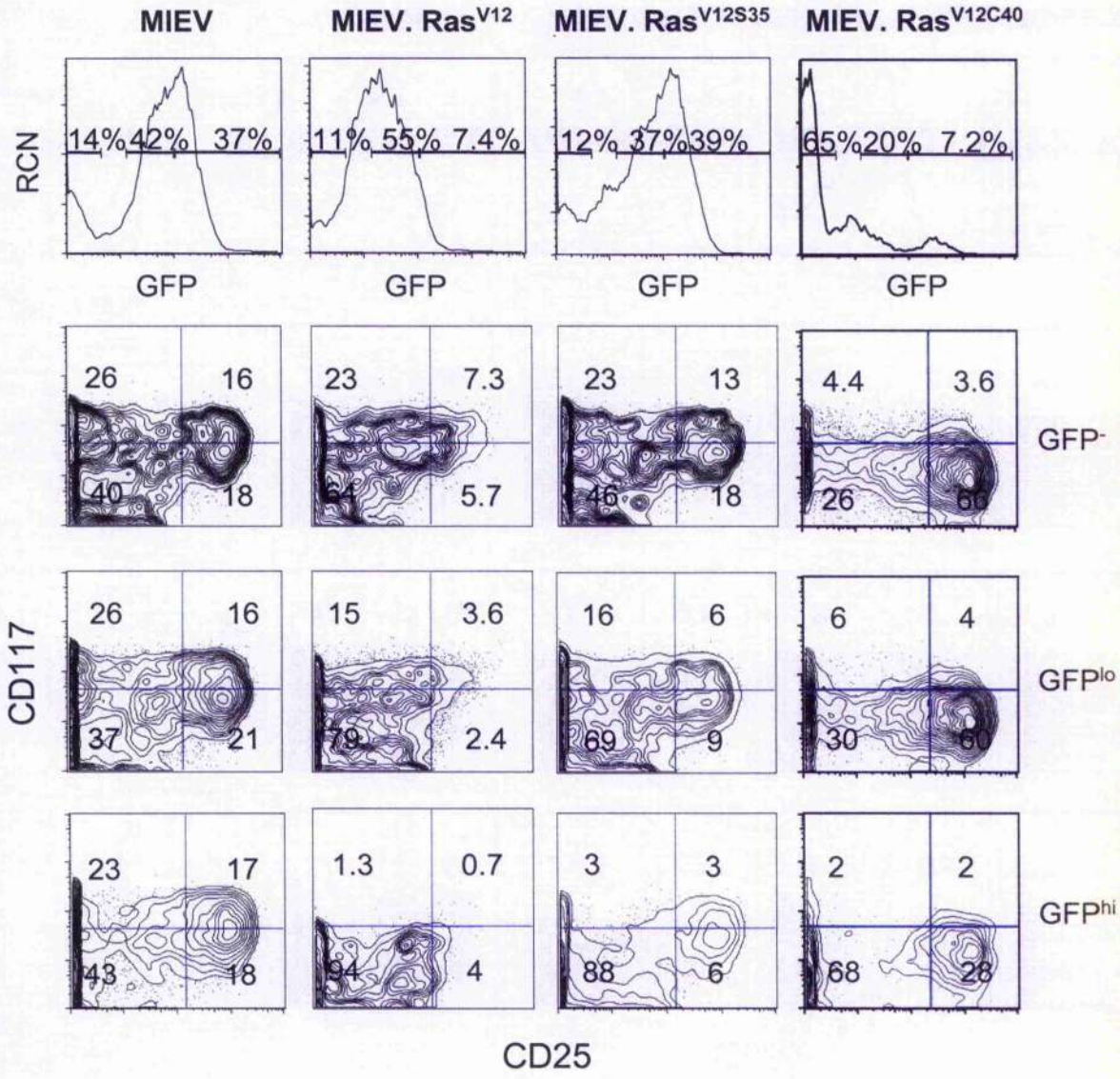
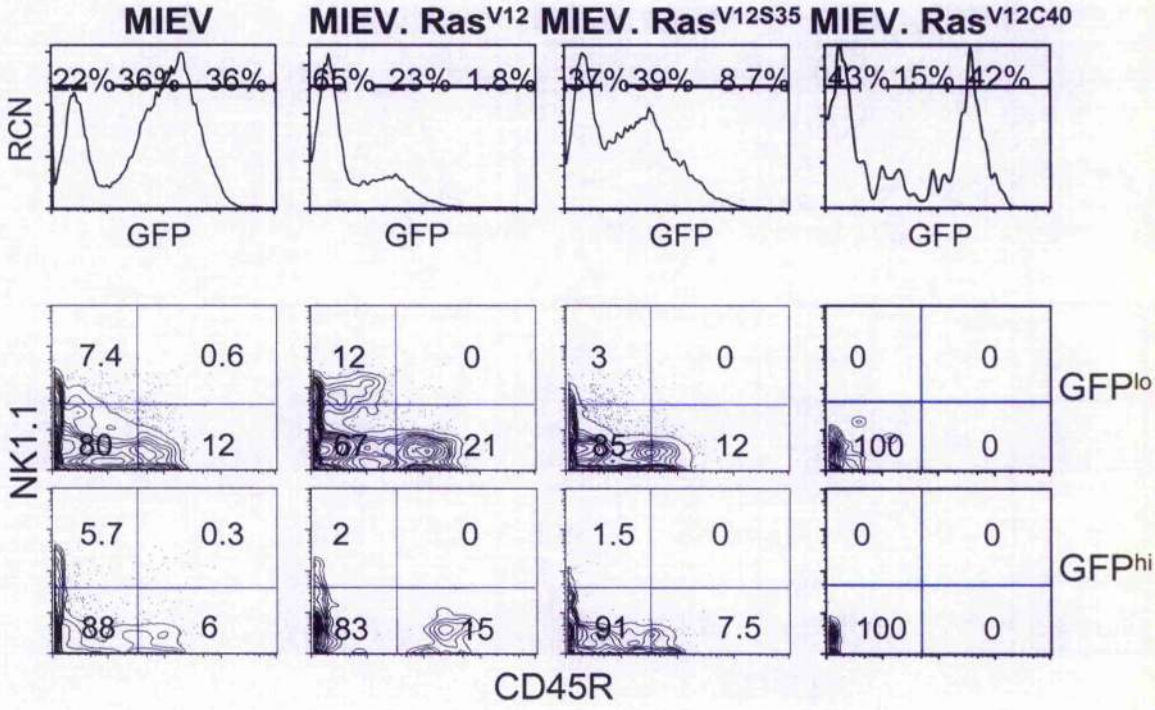

MIEV
MIEV. Ras^{V12}
MIEV. Ras^{V12S35}
MIEV. Ras^{V12C40}
RCN
14% 42% 37%
11% 55% 7.4%
12% 37% 39%
65% 20% 7.2%
GFP
GFP
GFP
GFP
26 16 40 18
23 7.3 64 5.7
23 13 46 18
4.4 3.6 26 60
GFP^{-}
CD117
26 16 37 21
15 3.6 79 2.4
16 6 69 9
6 4 30 60
GFP^{lo}
23 17 43 18
1.3 0.7 94 4
3 3 88 6
2 2 68 28
GFP^{hi}
CD25

Figure 3.9 **Analysis of lymphoid cells surface expression markers on retrovirally infected wild type FL-derived HPCs cultured on OP9-DL1 cells mimic FTOC.** FL cells were removed from timed pregnant wild type mice at day 14 of gestation and retrovirally infected with vector alone (MIEV) or Ras^{V12}, $Ras^{V12}S35$ or $Ras^{V12}C40$, for 24 hr. After 24 hr the FL cells were incubated on OP9-DL-1 cells for either 4 or 8 days. Flow cytometric analysis of cell markers NK1.1 and CD45R is shown at days 4 and 8. This data is representative of three separate experiments.

Day 4


MIEV
MIEV. Ras^{V12}
MIEV. Ras^{V12S35}
MIEV. Ras^{V12C40}
RCN
22% 36% 36%
65% 23% 1.8%
37% 39% 8.7%
43% 15% 42%
GFP
NK1.1
7.4 0.6 80 12
12 0 57 21
3 0 85 12
0 0 100 0
GFP^{lo}
5.7 0.3 88 6
2 0 83 15
1.5 0 91 7.5
0 0 100 0
GFP^{hi}
CD45R

Day 8

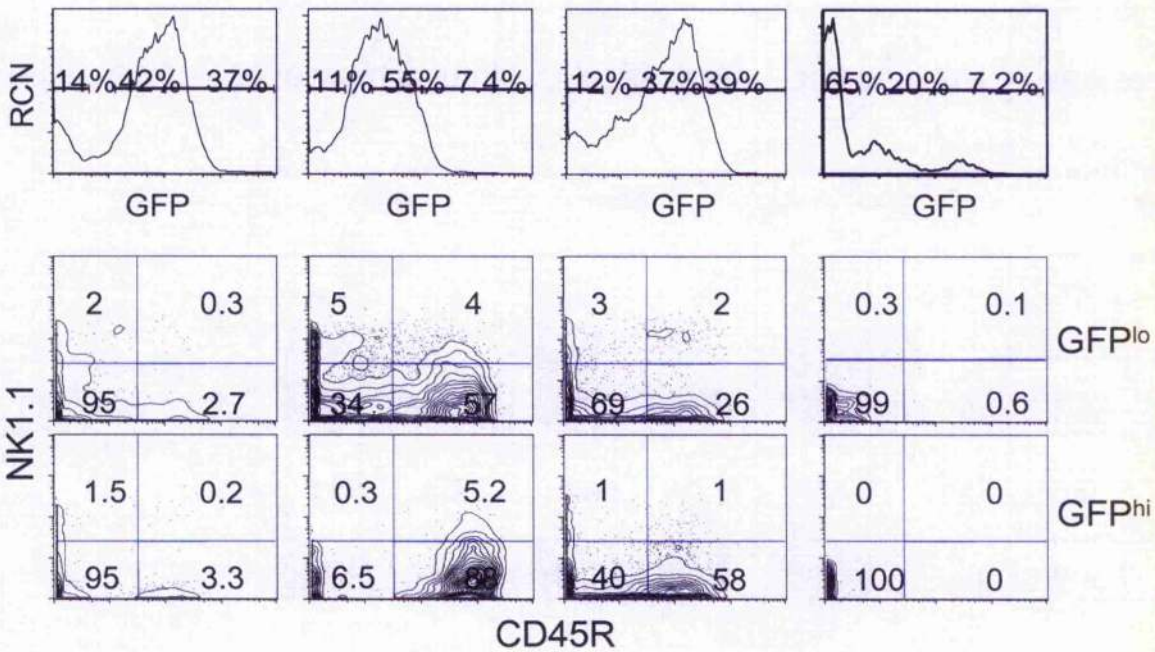

RCN
14% 42% 37%
11% 55% 7.4%
12% 37% 39%
65% 20% 7.2%
GFP
NK1.1
2 0.3 95 2.7
5 4 34 57
3 2 69 26
0.3 0.1 99 0.6
GFP^{lo}
1.5 0.2 95 3.3
0.3 5.2 6.5 88
1 1 40 58
0 0 100 0
GFP^{hi}
CD45R

Figure 3.10 **Retrovirally infected RAG$^{-/-}$ FL-derived HPCs commit and differentiate when cultured on OP9-DL1 cells for 4 days.** FL cells were removed from timed pregnant RAG$^{-/-}$ mice (day 14 of gestation) and retrovirally infected with (MIEV) or Ras^{V12}, Ras^{V12}S35 and Ras^{V12}C40, for 24 hr. After 24 hr the FL cells were incubated on OP9-DL1 cells for 4 days. Day 4 flow cytometric analysis for T cell markers CD117 and CD25 are shown. This data is representative of three separate experiments.

Day 4

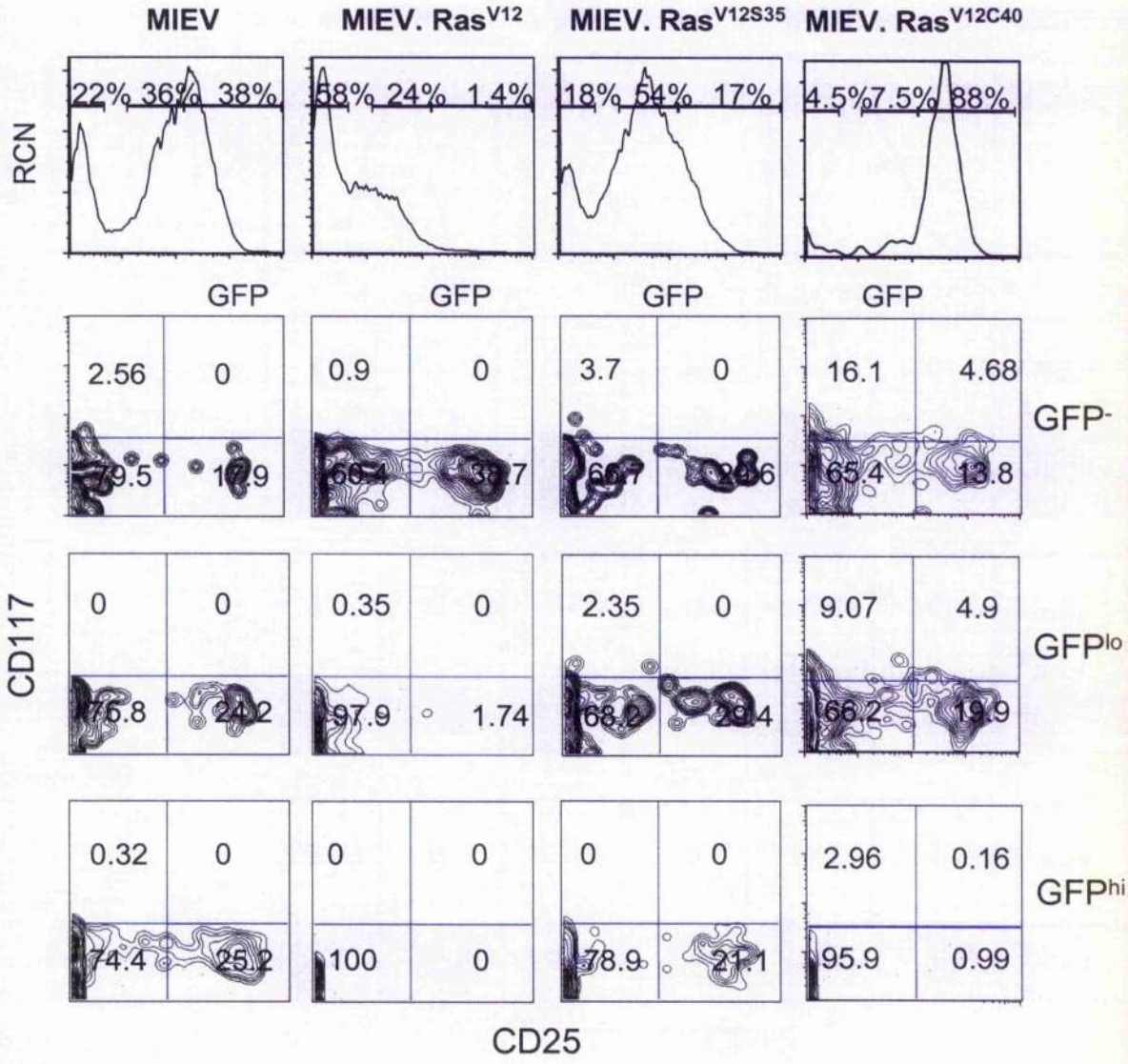

MIEV
MIEV. Ras^{V12}
MIEV. Ras^{V12S35}
MIEV. Ras^{V12C40}
RCN
22% 36% 38%
58% 24% 1.4%
18% 54% 17%
4.5% 7.5% 88%
GFP
GFP
GFP
GFP
2.56
0
79.5
17.9
0.9
0
60.4
37.7
3.7
0
66.7
29.6
16.1
4.68
65.4
13.8
GFP-
CD117
0
0
75.8
24.2
0.35
0
97.9
1.74
2.35
0
68.2
29.4
9.07
4.9
66.2
19.9
GFP^{lo}
0.32
0
74.4
25.2
0
0
100
0
0
0
78.9
21.1
2.96
0.16
95.9
0.99
GFP^{hi}
CD25

Figure 3.11 **Retrovirally infected RAG$^{-/-}$ FL-derived HPCs commit and differentiate when cultured on OP9-DL1 cells for 8 days.** FL cells were removed from timed pregnant RAG$^{-/-}$ mice (day 14 of gestation) and retrovirally infected with MIEV or Ras^{V12}, Ras^{V12}S35 and Ras^{V12}C40, for 24 hr. After 24 hr the FL cells were incubated on OP9-DL1 cells for 8 days. Day 8 flow cytometric analysis for T cell markers CD117 and CD25 are shown. This data is representative of three separate experiments.

Day 8

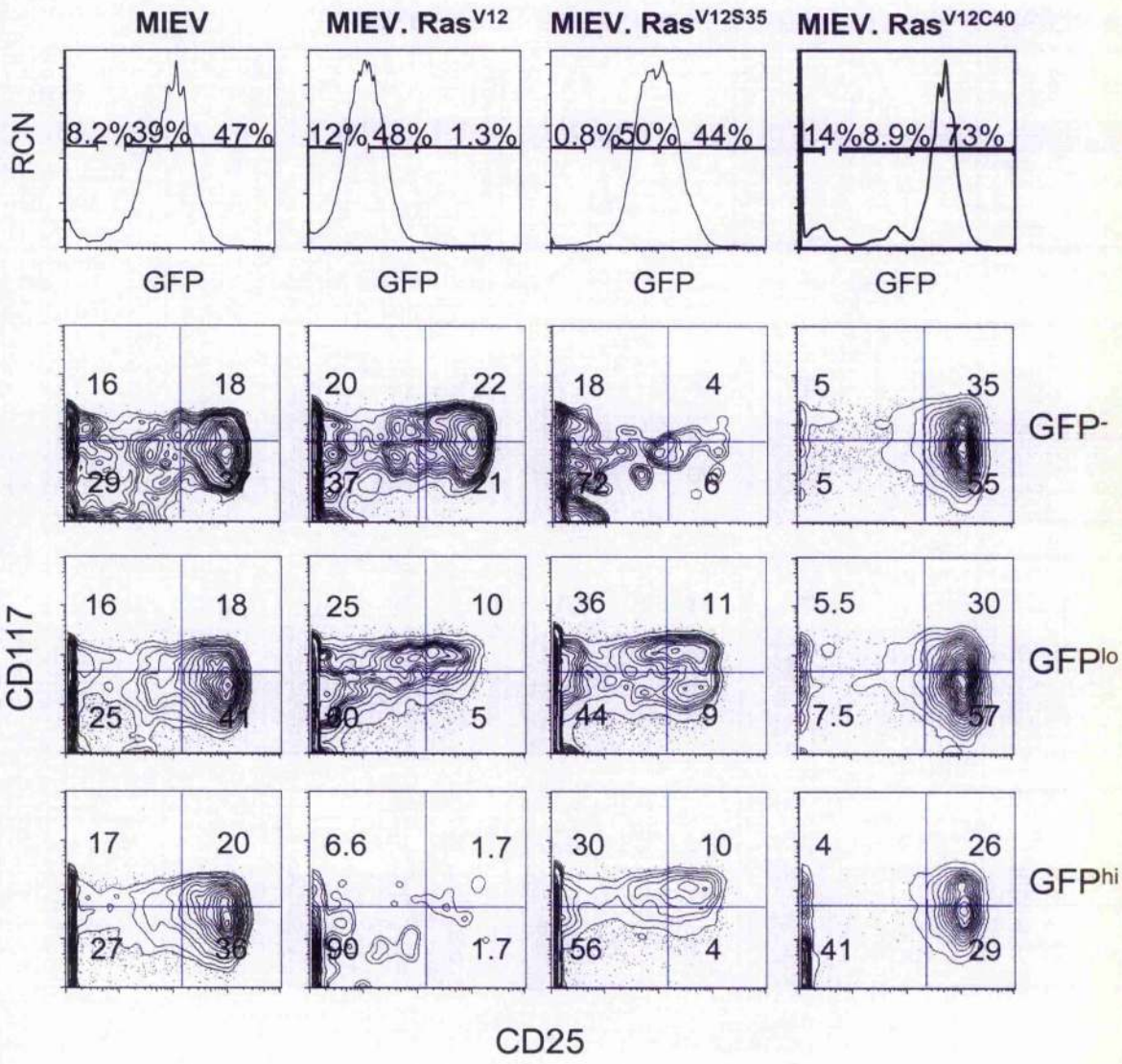
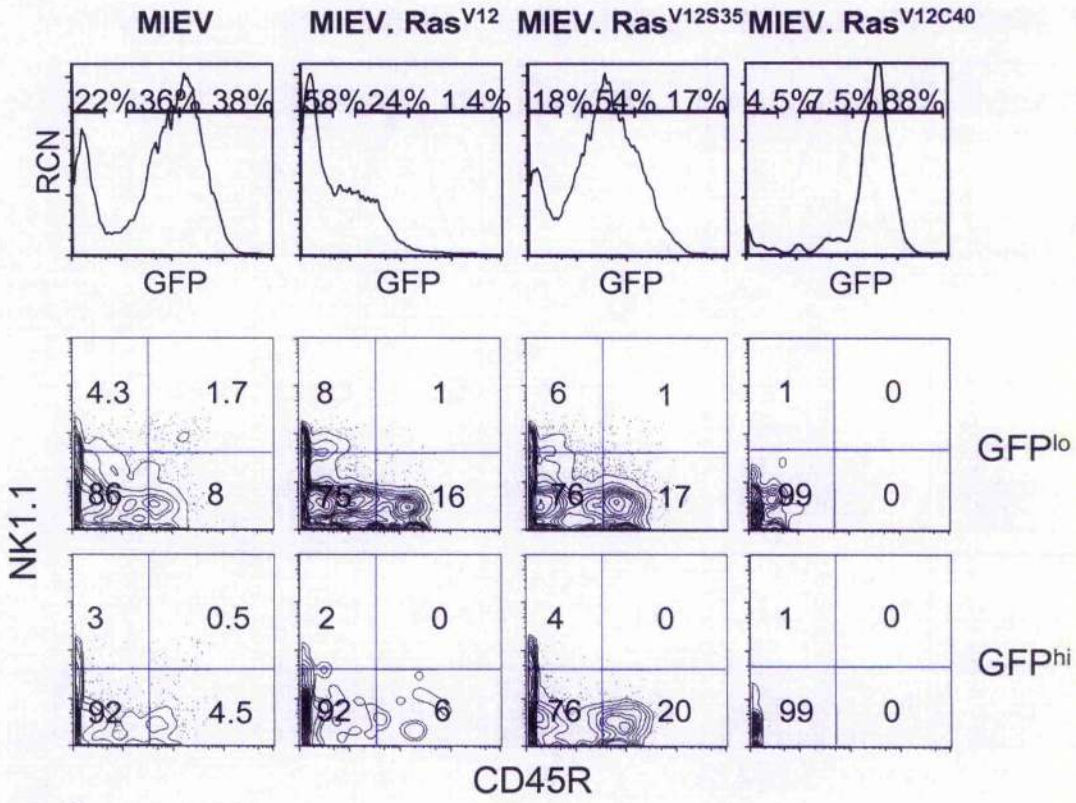

MIEV
MIEV. Ras^{V12}
MIEV. Ras^{V12S35}
MIEV. Ras^{V12C40}
RCN
8.2% 39% 47%
12% 48% 1.3%
0.8% 50% 44%
14% 8.9% 73%
GFP
GFP
GFP
GFP
16 18 29 3
20 22 37 21
18 4 72 6
5 35 5 55
GFP-
CD117
16 18 25 41
25 10 60 5
36 11 44 9
5.5 30 7.5 57
GFP^{lo}
17 20 27 36
6.6 1.7 90 1.7
30 10 56 4
4 26 41 29
GFP^{hi}
CD25

Figure 3.12 **Retrovirally infected RAG$^{-/-}$ FL-derived HPCs commit and differentiate when cultured on OP9-DL1 cells.** FL cells were removed from timed pregnant RAG$^{-/-}$ mice (day 14 of gestation) and retrovirally infected with MIEV or Ras^{V12}, Ras^{V12}S35 and Ras^{V12}C40, for 24 hr. After 24 hr the FL cells were incubated on OP9-DL1 cells for 4 - 8 days. Flow cytometric analysis for cell markers NK1.1 and CD45R are shown. This data is representative of three separate experiments.

Day 4


MIEV
MIEV. Ras^{V12}
MIEV. Ras^{V12S35}
MIEV. Ras^{V12C40}
RCN
22% 36% 38%
58% 24% 1.4%
18% 54% 17%
4.5% 7.5% 88%
GFP
NK1.1
4.3 1.7 86 8
8 1 75 16
6 1 76 17
1 0 99 0
GFP^{lo}
3 0.5 92 4.5
2 0 92 6
4 0 76 20
1 0 99 0
GFP^{hi}
CD45R

Day 8

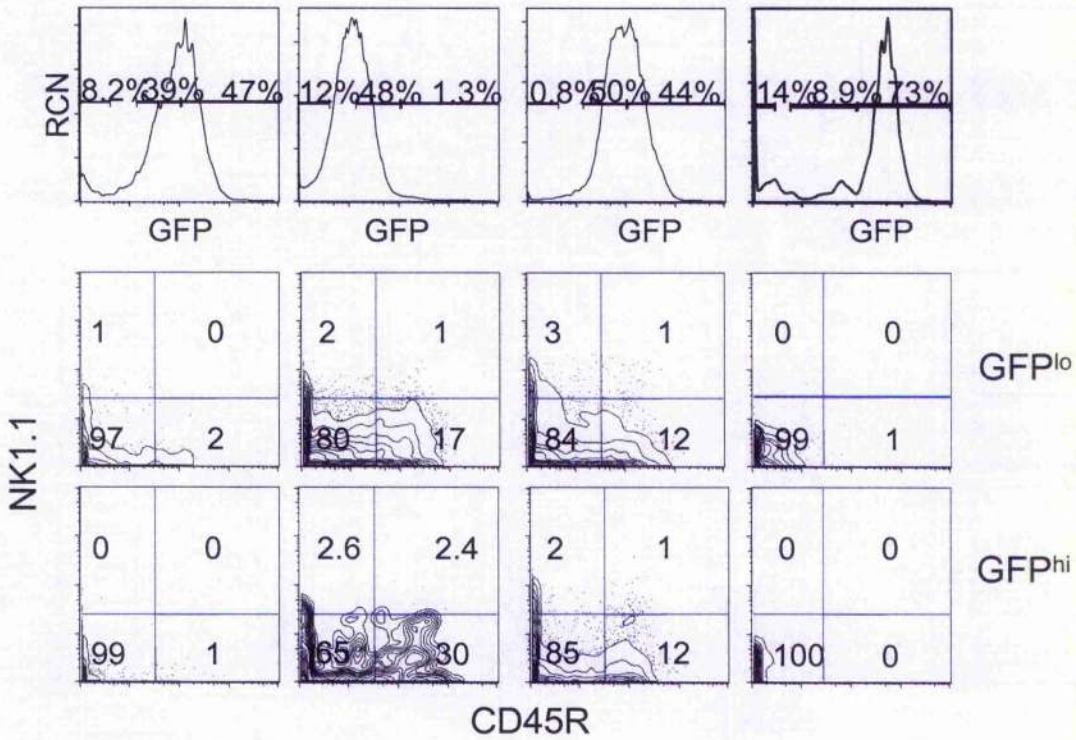

RCN
8.2% 39% 47%
12% 48% 1.3%
0.8% 50% 44%
14% 8.9% 73%
GFP
NK1.1
1 0 97 2
2 1 80 17
3 1 84 12
0 0 99 1
GFP^{lo}
0 0 99 1
2.6 2.4 65 30
2 1 85 12
0 0 100 0
GFP^{hi}
CD45R

Figure 3.13 **Activation of Ras/ERK-MAPK signalling in HPCs results in the reconstitution of thymuses with B cells *in vivo*.** FL cells were removed from timed pregnant wild type mice at day14 of gestation and retrovirally infected with MIEV or Ras^{V12}, $Ras^{V12}S35$ or $Ras^{V12}C40$, for 24 hr. 1 x 10^6 cells/100μl of PBS per condition were IP injected into $RAG^{-/-}$ neonatal mice. Dissecting the mice at week 8, and obtaining flow cytometric analysis on the cell populations present determined organ reconstitution. This data is representative of two separate experiments.

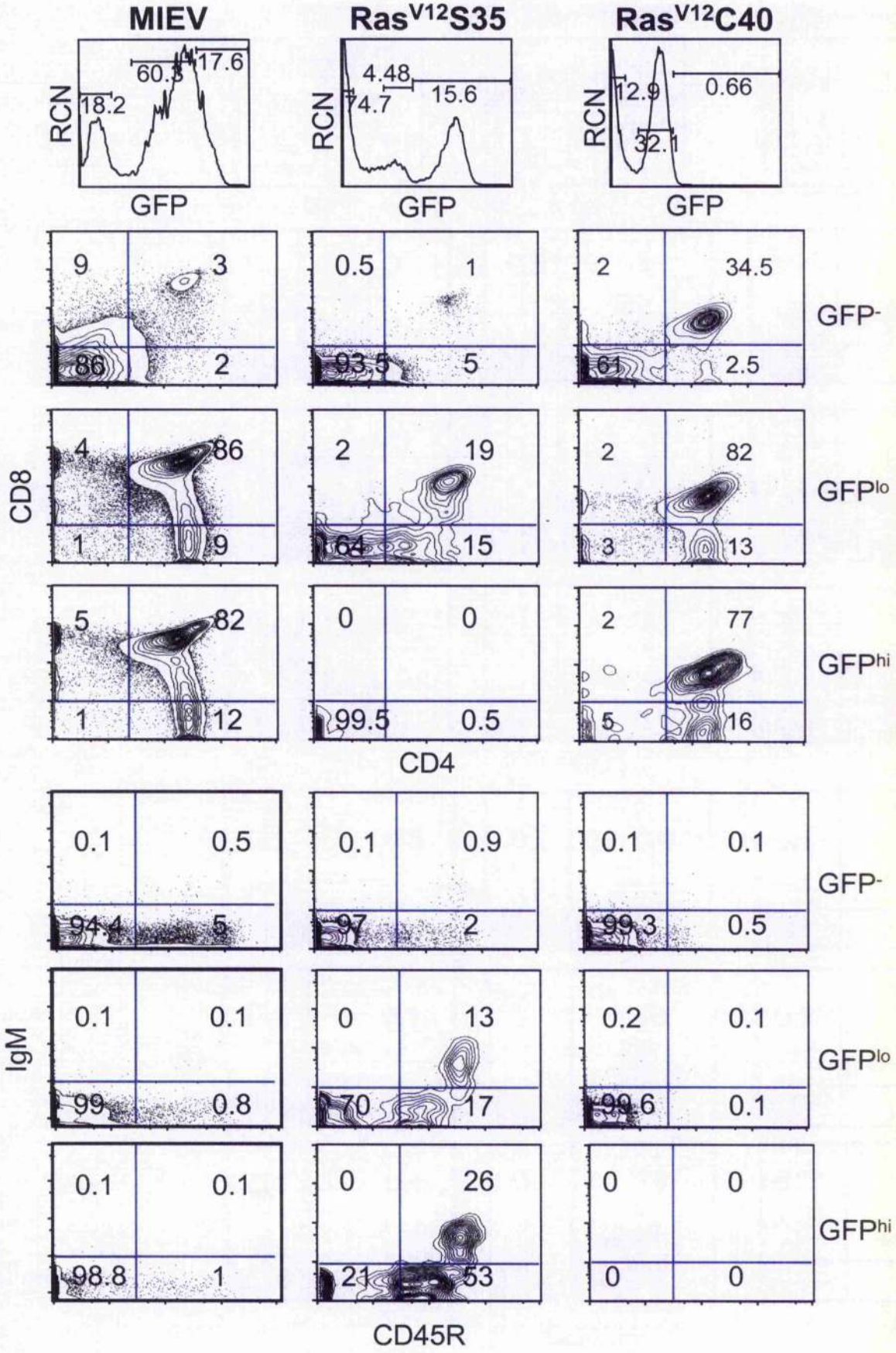
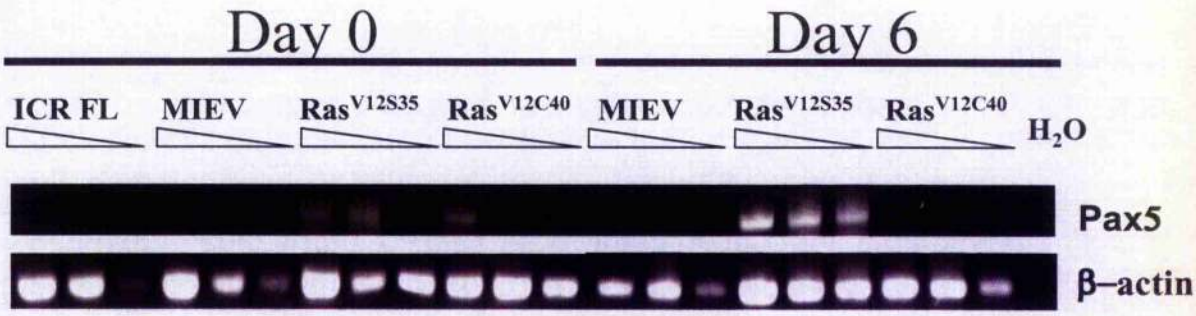

MIEV
Ras^{V12}S35
Ras^{V12}C40
RCN
GFP
18.2
60.3
17.6
4.48
74.7
15.6
12.9
0.66
32.1
CD8
CD4
IgM
CD45R
GFP-
GFP^{lo}
GFP^{hi}
9
3
86
2
0.5
1
93.5
5
2
34.5
61
2.5
4
86
1
9
2
19
64
15
2
82
3
13
5
82
1
12
0
0
99.5
0.5
2
77
5
16
0.1
0.5
94.4
5
0.1
0.9
97
2
0.1
0.1
99.3
0.5
0.1
0.1
99
0.8
0
13
70
17
0.2
0.1
99.6
0.1
0.1
0.1
98.8
1
0
26
21
53
0
0
0
0

Figure 3.14 **Activation of the Ras/ERK-MAPK signalling cascade promotes the up-regulation of Pax5 expression.** RNA extracts were prepared from MIEV-, $Ras^{V12}S35$- and $Ras^{V12}C40$- retrovirally-infected HPCs that were co-cultured with OP9-DL cells for 6 days. Semi-quantitative PCR was performed using the same serially diluted cDNA batches shown for β-actin. Pax5 expression levels present in each culture is shown. This data is representative of one experiment.


Day 0
Day 6
ICR FL
MIEV
Ras^{V12S35}
Ras^{V12C40}
MIEV
Ras^{V12S35}
Ras^{V12C40}
H_2O
Pax5
β–actin

Chapter 4 – General Discussion

The thymus plays a major role in the immune system as it is crucially required for T cell differentiation and repertoire selection (124). These processes are mediated by the thymic stroma, with its complex cellular composition (20). However, the unique functions of the thymus reside mainly in the thymic epithelium, the major sub-compartment of the stroma. Each of the two regions of the stroma, the cortex and medulla, contains several structurally and phenotypically distinct types of thymic epithelial cells (TECs), it is widely thought that these TEC subsets generate discrete intrathymic environments each specialised for mediating a particular aspect of thymocytes development (18, 19, 125-127). The first demonstration of the thymuses crucial role in establishing the development of a normal immune system was provided in 1961, when it was shown the mice thymectomized immediately after birth had poorly developed lymphoid tissues, impaired immune responses and increased susceptibility to infections (128). Although many molecular factors have been identified, such as extracellular matrix components and chemokine receptors and their ligands, the exact mechanisms involved in thymocytes differentiation has yet to be completely characterised (129, 130). As a result of these environmental factors the thymus has a unique ability to support the development of T cells from incoming HPCs derived from either FL or BM.

Some answers have been provided by experiments that demonstrate that although T and B cells might share a HPC, their developmental processes are different and take place in distinct locations (131). Recent studies have shown an important role for Notch signalling at various stages of development (132, 133). In particular, several lines of evidence have indicated a crucial role for Notch signalling in the T versus B cell fate (12, 117, 134, 135).

4.1 Constitutive $Ras^{V12}S35$ signalling interrupts T cell commitment

As a thymic microenvironment is required for T cell commitment and development, we utilised the *in vitro* system most commonly used to study this, the FTOC. In an attempt to elucidate the role of Ras signalling in T lymphopoeisis we utilised the constitutively active Ras^{V12} mutant, along with the MIEV.CMV control plasmid. As Ras proteins are critical components of signalling pathways that link the activation of the cell surface receptors with transcriptional events leading to the control of proliferation, differentiation and apoptosis, we had hypothesised that their may be a drive towards positive selection during T cell development (136, 137). Surprisingly, the results showed a bias towards B cell commitment in the Ras^{V12} culture over that of T cell commitment. As the Ras^{V12} mutant is constitutively signalling through Ras mediated pathways we believe that this may be inhibiting the environmental stimuli within the FTOC and promoting commitment to B cell lineage.

In an attempt to investigate this emergence of B cell commitment in FTOC when the Ras^{V12} pathway is constitutively activated, we utilised the constitutively active mutants $Ras^{V12}S35$ and $RasV^{12}C40$. Interestingly, the results showed that in the HOS-FTOCs reconstituted with Ras^{V12} and $Rasv^{12}S35$ infected HPCs, there was a bias towards B cell commitment (at days 8 and 12) at the expense of T cell commitment. However, in the $Ras^{V12}C40$ reconstituted HOS-FTOC we saw a bias to T cell commitment and minimal B cell commitment. Therefore, we hypothesis that the emergence of B cells in the presence of $Ras^{V12}S35$ and not $Ras^{V12}C40$ may indicate that there is an inhibition of T cell lineage commitment and that it is occurring through the constitutively active ERK-MAPK pathway.

The ability to differentiate T cells on a simple stromal cell monolayer has helped to answer fundamental questions regarding molecular interactions required for the generation of T cells, and also served to create a new system for the study of T cell development. The OP9-DL1 cells are easily propagated and manipulated in culture, by comparison to previously used FTOCs. Therefore we utilised this system to continue our investigation into the role of Ras during lymphocyte

development. FL-derived HPCs cultured on OP9-DL1 cells undergo T cell lineage commitment due to the presence of the Notch ligand delta-like-1 (9, 13, 117). Upon the introduction of FL-derived HPCs retrovirally infected with the Ras^{V12} mutants into co-culture with OP9-DL1 cells, we observed similar data to our FTOC results. These results may imply that constitutive Ras activation of the ERK-MAPK pathway is inhibiting T cell commitment, in particular Notch. Research carried out by Berset *et al* and Sundaram *et al* suggested that Ras and Notch may be working antagonistically in C.elegans, corroborates our results (138, 139). We do not believe that the emergence of B cells within our system is due to an outgrowth of committed pre-B cells, as previous experiments carried out by Schmitt *et al* have shown that HPCs cultured on OP9-DL1 cells resulted in T cell commitment, but failed to give rise to B cells (13, 119). The emergence of B cell commitment in the HOS-FTOC system, which allows us to retrovirally infect HPCs which have never left the thymic environment implies it's not an pre-B cell outgrowth effect. Also, during the preparation of the FL cells we carry out a CD24 kill which will eliminate most pre-B cells present in the sample. However, only cell sorting of our HPCs will allow us to ensure that there are no pre-B cells present in our HPC cell populations upon introduction onto OP9-DL1 cells.

Of particular significance were the results from our *in vivo* adoptive transfer of HPCs, retrovirally infected with the Ras^{V12} mutants, into $RAG^{-/-}$ mice. This system also produced an increase in B cell commitment, at the expense of T cell commitment, when constitutively activated through the ERK-MAPK signalling pathway. These results are particularly important due to being carried out within the *in vivo* thymic microenvironment, in the presence of structural and functional stimuli required for normal thymocyte development. Through the course of this research we have demonstrated that in a variety of assay models we observe this similar trend.

4.2 Notch vs. Pax5

From our data we hypothesise that the generation of B lymphocytes in a T cell environment may be due to constitutive Ras-MAPK induced expression of Pax5 inhibiting Notch1. It has been demonstrated by Souabni *et al* that pan-haemopoietic Pax5 expression strongly promotes B cell development at the expense of T lymphopoiesis (16). They have found that Pax5 interferes with T lineage commitment and early thymocyte development by repressing the transcription of the T cell specification gene Notch1. The analysis of RNA extracts and protein expression of cells arising from our system have shown that Pax5 expression is indeed upregulated in the $Ras^{V12}S35$ retrovirally transfected HPCs grown in co-culture with OP9-DL1 cells.

It has been reported by Ordentlich *et al* (140) that the basic helix-loop-helix (bHLH) transcription factor E47 is a downstream target of Notch signalling, and that activation of Notch1 inhibits its transcriptional activity. E47 is one of the alternative splicing products of the E2A gene. E2A, HEB and E2-2 genes are considered important for B and T cell development, lack of the E2A gene alone is sufficient to block early B cell development and the removal of both E2A and HEB are sufficient for T cell development arrest (141). Since E2A proteins are crucial for the early stages of both B and T cell development, it is thought the modulation of E2A function could be an effective means of controlling lymphocyte differentiation. Le Nie *et al* (141) have reported that the activation of the Notch signalling pathway induces the degradation of E2A proteins in a MAPK-dependent manner. They concluded that high levels of p42/p44 MAPK activities were found in the B cell cultures they analysed, therefore E2A proteins became readily degraded upon signalling through Notch pathways. In contrast the MAPK activities were very low in the T cell cultures they tested, consequently they believe MAPK activity is a limiting factor for E2A degradation in T cells in the presence of Notch.

In our system we propose that Pax5 may be inhibiting Notch1, therefore Notch1 inhibition may be causing a reduction in E2A degradation in the B cell committing

population. Lei Nie *et al* have also hypothesised that B and T cells may have different modes of regulation for MAPKs. This introduces an interesting scenario for the emergence of B cell populations in our constitutively activated Ras/ERK/MAPK OP9-DL1 cultures. The proposed differences in the modes of regulation of MAPKs by B and T cells may be controlling E2A degradation differently in the two cell populations, and therefore be producing favourable conditions for B cell commitment.

The results obtained from the pcDNA.Ras^{V12}C40 electroporation assay, imply that signalling through the PI3K pathway may have an inhibitory effect on ERK activation in SL-12.β12 cells. This may relate to the observation of minimal B cell commitment in the Ras^{V12}C40 retrovirally infected HPCs on OP9-DL1 cells, in FTOC and *in vivo*. We suggest that in these systems a decrease in ERK-MAPK activity may be one of the limiting factors for B cell commitment in this environment. This agrees with Lei Nie *et al* who propose that T cells commit in an environment containing Notch1 and perhaps low levels of MAPKs (141).

4.3 Conclusion and further work

Through this research we have shown that upon constitutive activation of the ERK-MAPK pathway by Ras there is B cell commitment in a T cell microenvironment, implying that exaggerated Ras/ERK/MAPK activity in HPCs results in the production of signalling molecules, such as Pax5, which appear to inhibit T cell lineage commitment and promote B cell lineage commitment. Therefore, future work would include:

- Cell sorting: to sort for distinct subsets of cells i.e. $Scal^{+}$, $CD117^{+}Lin^{-}$, not cells committed to B cell development (pre-B cells).
- Cell sorting: to enable protein and transcriptional determination of GFP^{+} cell populations, thus cells which are expressing our gene of interest.
- Utilising RT-PCR techniques to assess the expression of developmentally regulated genes over time, such as Ets proteins, PU.1, Notch1, Hes1 and the E2A family member E47 in GFP^{+} cultures.
- Utilising western blot techniques for the identification of signalling molecules involved in PI3K, ERK-MAPK and Notch1 signalling.
- Retrovirally introducing the dominant negative Ras mutant Ha-Ras^{N17}, and analysing by flow cytometry for B and T cell surface markers, to determine effect of constitutively knocking down Ras.

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