UNIVERSITY OF CALIFORNIA, SAN DIEGO

Calcium Signaling in Early T Cell Development

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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2012

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LIST OF ABBREVIATIONS

ALL	acute lymphoblastic leukemia/lymphoma
A.U.	arbitrary units
BA	basophil
BCR	B cell receptor
BM	bone marrow
bHLH	basic helix-loop-helix
bp	base pair (nucleotide)
BrdU	bromodeoxyuridine
Ca^{2+}	calcium ion
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CRAC	calcium-activated calcium release channels
Cre	Cre recombination enzyme
Ct	cross threshold
CTL	control
DAG	diacylglycerol
DFNKO	double floxed and single null knockout mice
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive

E47	Tcfe2a (E2A) splice isoform, Tcf3
ED	embryonic day
EDTA	ethylenediaminetetraacetic acid
EO	eosinophil
ER	endoplasmic reticulum
ES	embryonic stem
ETP	early T lineage progenitor
fl	flox, conditional allele
FL	fetal liver
Flpe	enhanced flipase recombination enzyme
Flt3L	Flt3 ligand
FRT	FLP recombination target site
FSC	forward light scatter
GMP	granulocyte-macrophage progenitor
Gy	gray (unit of radioactivity)
Hb	hemoglobin
HCT	hematocrit
HMG	high-mobility group
HSC	hematopoietic stem cell
IC	intracellular (i.c.)
Id	inhibitor of differentiation/DNA binding
Ig	immunoglobulin
IgH	immunoglobulin heavy chain

Igκ	kappa chain of IgL
Igλ	lambda chain of IgL
IgL	immunoglobulin light chain
IgM	immunoglobulin μ heavy chain
IL-7	interleukin-7
i.p.	intraperitoneal
IP_3	inositol triphosphate
IP_3R	inositol triphosphate receptor (protein)
IP_3R-TKO	inositol triphosphate receptor triple knockout mice
ISP	immature single positive
Itpr1	inositol triphosphate receptor type 1 isoform
Itpr2	inositol triphosphate receptor type 2 isoform
Itrp3	inositol triphosphate receptor type 3 isoform
KO	knockout
LEF1	lymphocyte enhancer-binding factor 1
Lin	mature hematopoietic lineage markers
LMPP	lymphoid-primed multipotent progenitor
LoxP	locus of X over P1
LSK	lineage-negative, $Sca1^+$, c-Kit^+
LY	lymphocytes
MAPK	mitogen-activated protein kinase
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration

MCV	mean cell volume
MEP	megakaryocyte-erythrocyte progenitor
MHC	major histocompatibility complex
MPV	mean platelet volume
MO	monocytes
MPP	multiopotent progenitor
mRNA	messenger ribonucleic acid
Neo	neomycin
NE	neutrophils
NK	natural killer cell
NuRD	nucleosome remodeling and histone deacetylase complex
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
PBS	phosphate-buffered saline solution
PCR	polymerase chain reaction
PRC	polycomb repressive complex
PLT	platelets
Pre-BCR	pre-B cell receptor complex
Pre-TCR	pre-T cell receptor complex
qRTPCR	quantitative reverse transcription polymerase chain reaction
Rag	recombination activating gene
RBC	red blood cell
RDW	red cell distribution width

RT	room temperature
SCF	stem cell factor
SCID	severe combined immunodeficiency
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SOCE	store-operated calcium entry
SP	single positive
T-ALL	T cell acute lymphoblastic leukemia
T-LBL	T cell acute lymphoblastic lymphoma
TCR	T cell receptor
TCRα	T cell receptor alpha chain
TCRβ	T cell receptor beta chain
TCRδ	T cell receptor delta chain
TCRγ	T cell receptor gamma chain
TCF1	T cell factor 1 (also called Tcf7)
TG	thapsigargin
TKO	triple knockout
TSP	thymic seeding progenitor
VDJ	variable, diversity, joining gene segment
WBC	white blood cell
WT	wild type
YFP	yellow fluorescent protein

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ABSTRACT OF THE DISSERTATION

Calcium Signaling in Early T Cell Development

by

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Doctor of Philosophy

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Professor Sylvia Evans, Chair

Calcium ions (Ca^{2+}) function as a universal second messenger in all eukaryotic cells, including cells of the immune system. Ca^{2+} signals are crucial for peripheral T lymphocyte activation and effector functions, and in the developing thymus influence thymocyte selection and motility, yet the role of Ca^{2+} signaling in early T lymphocyte development is not well understood. Here, we show that the Ca^{2+} ion channels, inositol triphosphate receptors (IP_3Rs), are required for the differentiation and survival of T lymphocyte precursors in the thymus.

Specifically, signaling via IP_3Rs function to repress *Sox13*, an antagonist of the developmentally important transcription factor Tcf-1. Most importantly, in the absence of IP_3R-Ca^{2+} signaling, the repression of key Notch transcriptional targets, including *Hes1*, fails to occur in post β-selection thymocytes, and IP_3R-deficient mice develop an aggressive T cell malignancy resembling human T cell acute lymphoblastic leukemia (T-ALL). Our data suggest a model where pre-T cell receptor derived Ca^{2+} signals normally act to reinforce Tcf-1 expression and prevent malignant transformation of developing thymocytes. We also uncover previously unknown functions for IP_3R in the development of the red blood cell lineage, implicating IP_3R at the terminal stages of erythrocyte maturation. By establishing the requirement for IP_3R-Ca^{2+} signaling in B cell development at the pre-BCR checkpoint, we have identified a common theme for Ca^{2+} signaling in the regulation of early developmental checkpoints in lymphocytes.

I Introduction

I.1 Overview of lymphocyte development

T cell development is a highly regulated process and is vital to the development of an adaptive immune response. Murine T cell development originates from multipotent hematopoietic stem cells (HSC) in fetal liver and adult bone marrow[1]. As HSCs begin to differentiate, they lose their capacity for self-renewal and migrate from the bone marrow to the thymus. Specification and commitment to the T cell lineage occurs through a series of discrete intermediate stages, each with increasingly restricted alternative lineage potentials. Upon T lineage commitment in the thymus, T cell progenitors mature into a broad repertoire of antigen-specific T cells. This process requires developing thymocytes to produce a functional T cell receptor (TCR) at the cell surface. Expression of a mature TCR requires the sequential and stage-specific gene rearrangement of the TCRβ and α loci, a selection process that ensures the survival of only self-tolerant T cells. This thesis addresses how intrinsic signals within committed $\alpha\beta$ T lineage precursors promote the differentiation and survival of developing T cells.

I.2 T lymphopoiesis

I.2.1 Early T lineage development

T lineage specification and commitment is characterized by the progressive loss of alternative lineage potential and priming of T cell fate. Multipotent HSCs, the source of all blood cell lineages, differentiate in the bone

marrow and lose their capacity for self-renewal. The earliest lymphoid-biased progenitor, the lymphoid-primed multipotent progenitor (LMPP), represents the first step in lymphoid lineage specification[2]. The lymphoid lineage consists of B, T, and natural killer (NK) cells. The LMPP retains the potential for lymphoid, granulocyte, and macrophage differentiation but has lost megakaryocyte and erythroid potential. The LMPP gives rise to two distinct lymphoid precursors: the common lymphoid progenitor (CLP) and the thymic seeding progenitor (TSP)[3]. The CLP is the physiologic progenitor of B cells and retains significant T cell potential but unlike the LMPP does not give rise to the majority of T lineage progenitors present in the bone marrow[4-6]. TSPs migrate from the bone marrow through the circulation to seed the thymus, where upon entering the thymus TSPs encounter microenvironment signals that trigger the loss of alternative lineage potential and promote the differentiation and commitment to the T cell lineage.

In the adult thymus, the earliest thymocyte precursors lack expression of CD4 and CD8 and are termed double negative (DN) cells[7]. Cell surface expression of lineage markers is low or absent on DN precursors (Lineage$^-$). DN cells represent approximately 5% of total thymocytes in the adult mouse, and are subdivided into four developmental stages based on the differential expression of CD44, CD117 (c-Kit), and CD25[8,9]. The most immature thymocyte precursors are found within the DN1 subset (CD25$^-$CD44$^+$) (Figure I.1). This population can be further subdivided into DN1 cells with high expression of CD117, known as early T lineage progenitors (ETPs). ETPs display the ability to proliferate

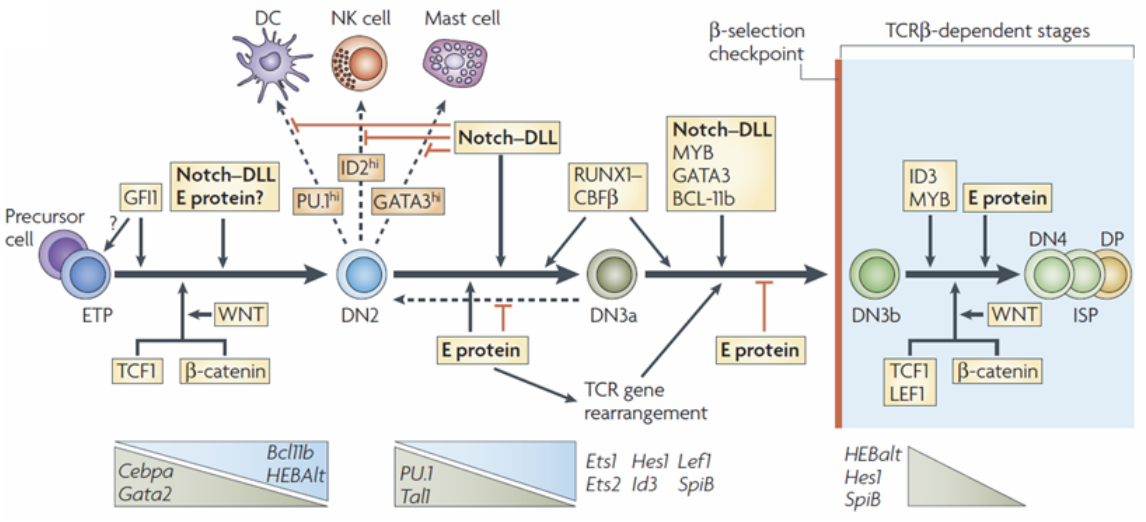


Figure I.1 Genetic regulatory factors in early T cell development.
Summary of the recursive roles of major regulatory factors during the transition from the ETP stage through the β-selection checkpoint. The developmental alternatives that are still available at the DN2 stage, and the potential reversal of the DN2 to DN3 stage transition, are indicated by broken arrows. The β-selection checkpoint is shown at the boundary between T-cell receptor (TCR)-independent developmental stages on the left and the later TCR-dependent developmental stages in the shaded zone on the right. The later developmental changes from DN4 to ISP (immature single positive) to double positive (DP) stages are not shown as discrete transitions. The major increases or decreases in the expression of other transcription factor genes are indicated in the bottom part of the figure. BMP, bone morphogenetic protein; BCL-11b, B-cell lymphoma 11b; ETS, E26-transformation specific; GFI1, growth factor independent 1 transcription repressor; HEBalt, HeLa E-box binding factor, alternative form; Hes1, hairy, enhancer of split 1; ID, inhibitor of DNA binding; Lef1, lymphoid-enhancer-binding factor 1; LRF, leukaemia/lymphoma related factor; MINT, MSX2-interacting protein; SHH, sonic hedgehog; TCF1, T-cell factor 1. Reproduced from Rothenberg, et al., **Nature Reviews Immunology.,** 2008 (8)[10].

extensively and generate all thymocyte populations of the αβ and γδ T cell lineages[11]. ETPs also remain multi-potent, capable of generating other thymus-derived lineages such as natural killer (NK) cells and dendritic cells (DCs), but possess minimal myeloid or B cell potential. Expression of CD25 marks the progression to the DN2 stage ($CD44^+CD25^+CD117^+$). The DN2 subset is T lineage specified giving rise primarily to T cells when transplanted into irradiated recipients or assayed *in vitro*. DN2 cells are highly proliferative, and lack B cell potential but retain limited NK cell and DC potential[12]. The DN2 population can also give rise to αβ and γδ T cells, but the precise stage at which αβ and γδ lineages diverge has not been resolved.

I.2.2 Developmental checkpoints

At the DN3 stage ($CD44^-CD25^+$), T lineage commitment is achieved in parallel with the loss of CD44 and CD117 expression. Extensive rearrangement of the γ, δ, and β T cell receptor (TCR) loci is first detected in DN2 cells, and continues predominantly in noncycling DN3 thymocytes. DN3 cells that successfully rearrange a functional TCRβ chain, which associates with both the CD3 signaling complex and the invariant pre-TCRα (pTα) chain, form the pre-T cell receptor (pre-TCR) complex. DN3 cells that receive a pre-TCR signal are rescued from apoptosis, undergo significant proliferation (six to eight cycles), cease TCRβ locus recombination (allelic exclusion), and differentiate beyond the DN3 stage. This process, known as the β-selection checkpoint, represents a developmental checkpoint to ensure that only cells that have productively

rearranged their TCRβ locus are permitted to differentiate whereas those that fail to express a functional TCRβ chain undergo apoptosis. DN3 cells that express the pre-TCR downregulate surface expression of CD25 and become DN4 thymocytes ($CD44^-CD25^-$). DN4 cells progress through a brief intermediate stage characterized by the expression of CD8, known as the immature single positive (ISP) stage, that will then give rise to immature αβ lineage cells that express surface TCRβ and both the CD4 and CD8 co-receptors, and are termed double positive (DP) cells. DP cells comprise the majority of T lineage cells in the adult mouse thymus (~80%). Cells at this stage initiate rearrangement of the TCRα locus followed by a TCR-dependent selection process. Although the vast majority of thymocytes are DP, only ~5% will survive and mature into $CD4^+$ or $CD8^+$ single positive (SP) thymocytes. DP thymocytes that express a non-productive TCRα chain "die by neglect" as they fail to receive a survival signal from the TCR[13]. Only DP cells with a TCR that interacts with sufficiently low-affinity to major histocompatibility complex (MHC)-self peptide complex present on thymic stromal cells will develop further (positive selection). DP thymocytes with a TCR recognizing MHC-self peptide with high affinity are eliminated through apoptosis (negative selection).

I.3 Transcriptional regulation of early T cell development

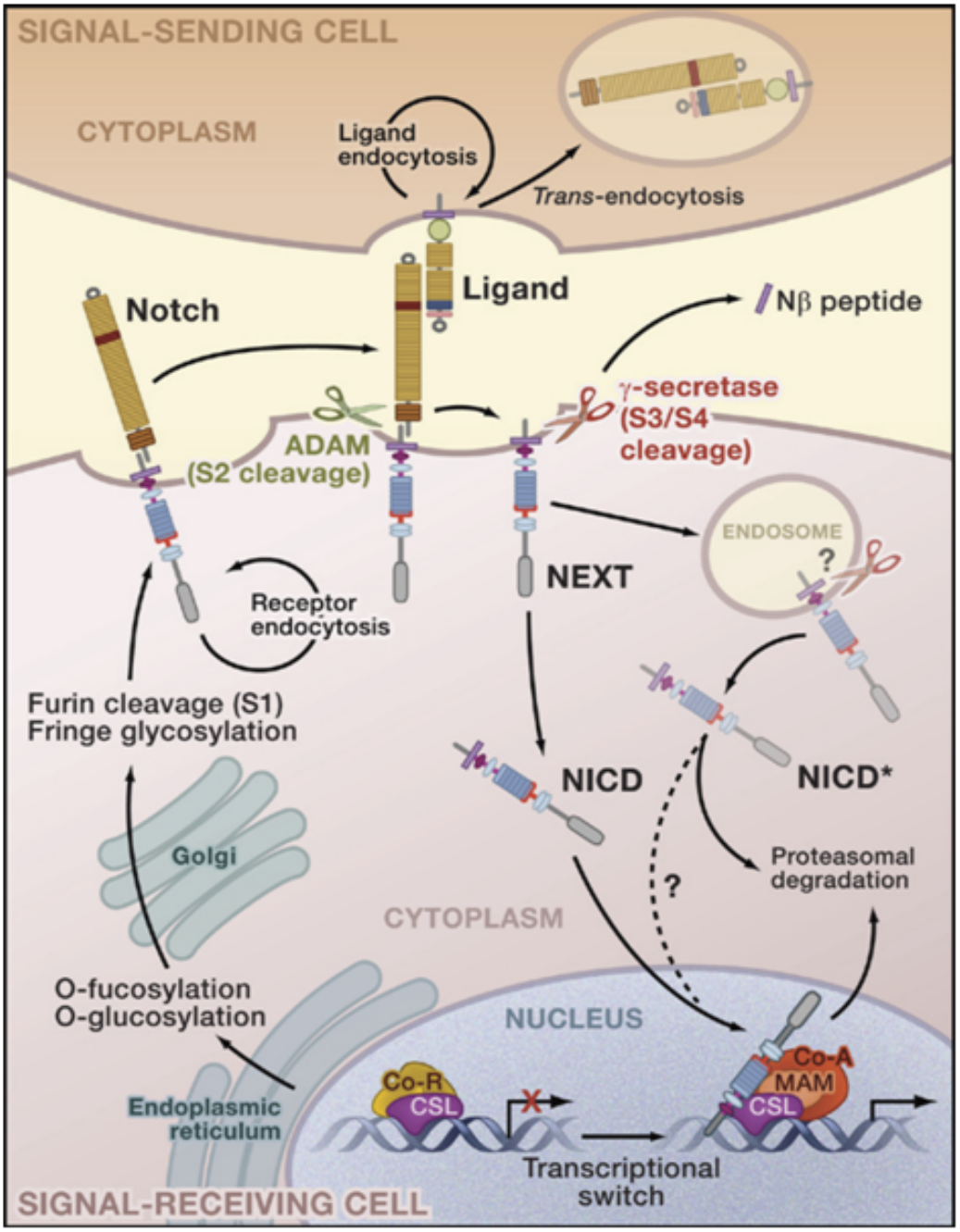
Transcription factors are critical regulators of specification and commitment throughout T cell development. Genetic studies have revealed both a diverse group of core transcription factors that have sustained roles throughout

T cell development and stage-specific factors implicated in lineage plasticity and cell fate decisions[10]. Core-group transcription factors, such as Notch1, Tcf-1 (Tcf7), E2A (Tcfe2a), and Ikaros have different effects throughout T cell development as the function of transcription factors depends on dosage, alternative splicing, and combinatorial interactions with other factors. PU.1 (Sfpi1), an ETS family transcription factor, represents one of the best-studied stage-specific T cell transcription factors. PU.1 function is critical for the generation of early B and T lymphocyte progenitors, and is also a regulator of myeloid differentiation[14,15]. Whereas PU.1 is essential for early thymocyte precursors, high levels of PU.1 can also divert early T cell progenitors to the myeloid lineage indicating PU.1 function must be tightly controlled in developing thymocytes. Indeed, as thymocyte progress to the late DN2 stage, PU.1 acts as a T lineage antagonist and must be silenced[16]. PU.1 belongs to a group of inherited factors expressed prior to thymic entry, including Scl (Tal1), Id2, Gata2, and C/EBPα that are thought to sustain lineage plasticity in early T cell progenitors, and are down-regulated to undetectable levels prior to β- or $\gamma\delta$-selection.

I.3.1 Notch signaling and T cell development

The evolutionarily conserved Notch pathway is essential for cell-fate decisions throughout mammalian development. Notch receptor-ligand interactions serve to communicate signals between neighboring cells through a highly conserved signaling mechanism[17] (Figure I.2). Four mammalian Notch receptors have been identified, Notch1-4; these can interact with five Notch

Figure I.2 The core Notch signaling pathway.
The translated Notch receptor protein is glycosylated, and the mature receptor is produced after proteolytic cleavage by PC5/furin at site 1 (S1). It is then targeted to the cell surface as a heterodimer that is held together by noncovalent interactions. The Notch receptor is activated by binding to a lig7and presented by a neighboring cell. Endocytosis and membrane trafficking regulate ligand and receptor availability at the cell surface. Ligand endocytosis is also thought to generate mechanical force to promote a conformational change in the bound Notch receptor. This conformational change exposes site 2 (S2) in Notch for cleavage by ADAM metalloproteases. Juxtamembrane Notch cleavage at site 2 generates the membrane-anchored Notch extracellular truncation (NEXT) fragment, a substrate for the γ-secretase complex. γ-Secretase then cleaves the Notch transmembrane domain in NEXT progressively from site 3 (S3) to site 4 (S4) to release the Notch intracellular domain (NICD/Notch-IC). γ-Secretase cleavage can occur at the cell surface or in endosomal compartments, but cleavage at the membrane favors the production of a more stable form of NICD. NICD then enters the nucleus where it associates with the DNA-binding protein CSL (RBPjκ). In the absence of NICD, CSL may associate with ubiquitous corepressor (Co-R) proteins and histone deacetylases (HDACs) to repress transcription of some target genes. Upon NICD binding, allosteric changes may occur in CSL that facilitate displacement of transcriptional repressors. The transcriptional coactivator Mastermind (MAM) then recognizes the NICD/CSL interface, and this protein complex recruits additional coactivators (Co-A) to activate transcription. Reproduced from Kopan, et. al, **Cell.,** 2009 (137)[18]


SIGNAL-SENDING CELL
CYTOPLASM
Ligand endocytosis
Trans-endocytosis
Notch
Ligand
Nβ peptide
γ-secretase (S3/S4 cleavage)
ADAM (S2 cleavage)
ENDOSOME
?
NEXT
Receptor endocytosis
Furin cleavage (S1)
Fringe glycosylation
NICD
NICD*
Golgi
Proteasomal degradation
?
CYTOPLASM
O-fucosylation
O-glucosylation
NUCLEUS
Co-R
CSL
Co-A
MAM
CSL
Endoplasmic reticulum
Transcriptional switch
SIGNAL-RECEIVING CELL

ligands [Delta-like (Dll) 1, Dll3, Dll4, Jagged1, Jagged2]. The specificity of receptor-ligand binding is modulated by Fringe family glycosyltransferases such that Notch activation is restriced to Delta ligands[19]. Notch signaling is initiated by ligand-induced proteolysis of Notch receptor by ADAM proteases, removing the extracellular domain (Figure I.2). Presenilin-dependent γ-secretase cleaves Notch within the transmembrane region, releasing the intracellular domain of the Notch receptor (Notch-IC). Cleavage by γ-secretase, the second clevage event upon Notch receptor-ligand binding, appears to depend upon ubiquitination of truncated Notch and its trafficking to endocytic vesicles. Notch-IC is then free to translocate to the nucleus and interact with the transcription factor CSL [CBF-1; recombining binding protein Jκ (RBPJ) in mice], coordinating the CSL complex to switch from a repressor to activator of gene expression through displacement of a co-repressor complex and recruitment of co-activators such as Mastermind-like (MamL) family members to activate transcription[17].

While seemingly simple, the outcomes of Notch signaling pathway activation on cell fate are complex and context dependent. A limited number of Notch target genes have been identified, such as basic helix-loop-helix (bHLH) transcriptional repressors Hairy-enhancer of split (Hes) 1, Hes5, Herp, Deltex, and Nrarp[20,21,22,23]. Tissue-specific Notch target genes such as *Ptcra* and *Cd25* have been identified, and current evidence suggests that specificity in target gene expression occurs via transcriptional co-regulation [24-26]. This feature also extends to universal Notch target genes such as *Hes1*.

Notch signaling is the best-studied T cell lineage positive regulator. Within the thymus, Notch signals are necessary for T lineage specification. Deletion of Notch1, the transcription factor RBPJ, or the Notch ligand Dll4 results in a complete block in T cell development and the differentiation of ectopic B cells in the thymus[27-29]. In contrast, forced expression of the intracellular domain of Notch1 in hematopoietic progenitors leads to the development of immature T cells in the bone marrow and a loss of B cell development, and these mice rapidly succumb to T cell lymphomas[30]. Similarly, ectopic expression of the Notch ligands Dll1 or Dll4 on stromal cell lines support T lineage development while inhibiting B cell generation of cultured hematopoietic progenitors[31]. Interestingly, while multiple Notch receptors and ligands appear to be expressed in the thymus, not all receptor-ligand pairs are physiologically relevant to T lineage development. Genetic inactivation of Dll1, Notch2, or Notch3 has no impact on T cell development, and while Notch2 can support the maturation of T cells up to the DN3 stage from Notch1-deficient hematopoietic progenitors in response to DL1 *in vitro*, unlike Notch1, Notch2 interacts weakly with the most abundant intra-thymic Notch ligand Dll4[32-35]. Thus, we can conclude from reciprocal loss and gain of function studies that Notch1-Dll4 signaling is necessary and sufficient for T lineage specification and commitment, and acts to limit the development of alternative lineages in the thymus.

The expression of Notch receptors throughout thymocyte maturation and in both T cell lineages suggested a role for Notch activity at later stages of development. Initial studies of Notch1 function using chimeric mice repopulated

with Notch1$^{+/+}$ and Notch1$^{+/-}$ bone marrow cells showed Notch1$^{+/-}$ precursor cells contributed less to the αβ lineage than those of Notch1$^{+/+}$, suggesting Notch1 influences the αβ versus γδ lineage decision[36]. Additional reports further suggested a role for Notch1 in CD4 versus CD8 lineage choice and positive selection at the DP to SP transition[37-39]. Interestingly, inactivation of Notch1 in late DN3 thymocytes had no effect on T cell development[40]. As a result, no clear consensus appeared on Notch1 function at these stages. Alternatively, Notch1 may possess non-redundant functions prior to the DN3 stage and expression of the pre-TCR complex, thus loss of Notch1 in late DN3 thymocytes would not disrupt development. In support of this view, conditional inactivation of Notch1 or RBPJ at the DN2 stage with LckCre severely disrupted αβ but not γδ T lineage development[41]. The development of αβ T cells was arrested at DN3, the β-selection checkpoint, and displayed impaired VDJ rearrangement of the TCRβ locus. The presence of normal DJ rearrangements in Notch1-deficient thymocytes strongly suggested that Notch1 plays a key role in controlling rearrangement of the TCRβ locus. Interestingly, of the thymocytes that progressed to the DN4 stage, a significant population lacked intracellular TCRβ expression and thus the capacity to receive pre-TCR signals. Despite the observation that Notch1$^{-/-}$ BM precursors adopt a B cell fate in the thymus, the abnormal DN4 population was not γδ or B lineage cells. These abnormal DN4 cells were presumed to have bypassed the β-selection checkpoint, which normally acts to eliminate cells that lack a functional pre-TCR. Therefore, one major function of Notch1 at the DN3 stage is to eliminate cells with a defective

pre-TCR. Of note, the bypass of the β-selection checkpoint observed in Notch1-deficient thymocytes is also observed in the absence of the transcription factors E47 or Ikaros[42-44].

The deletion of Notch1 in DN3 thymocytes resulted in no effects on αβ versus γδ lineage commitment nor were changes in the CD4/CD8 lineages observed. All mammalian Notch members use RBPJ as a transcriptional mediator, and the potential for redundancy and stage-specific requirements for individual Notch subtype exists. However, conditional deletion of RBPJ at DN2 by LckCre produced the same T lineage developmental phenotypes as observed in Notch1 conditional knockout mice, indicating that RBPJ is not dissociated from Notch1 in T cells. Additionally, ablation of RBPJ at the DN4 stage by CD4Cre does not disrupt the development of $CD4^+$ and $CD8^+$ SP T cell lineages, clearly demonstrating that RBPJ-mediated Notch signaling is dispensable for CD4/CD8 lineage commitment and maturation.

I.3.2 Notch signaling at the β-selection checkpoint

Notch and pre-TCR signals are critical for the continued development of αβ T cells beyond the β-selection checkpoint. Pre-TCR signals and Notch appear to be required concurrently for the functional outcomes of β-selection, including proliferation, differentiation, and rescue from apoptosis[45]. Using an *in vitro* stromal cell culture system that supports T cell differentiation, $Rag2^{-/-}$ DN3 cells which lack rearranged TCRβ and thus a functional pre-TCR were assessed for the ability to differentiate to the DP stage in the presence or absence of the Notch ligand Dll1 after retroviral transduction with TCRβ. After 6 days,

transduced Rag2$^{-/-}$ DN3 cells cultured in the presence of Notch ligand displayed expansion and differentiation to the DP stage. In contrast, transduced Rag2$^{-/-}$ DN3 cells cultured in the absence of Notch ligand failed to differentiate, and in fact cellularity substantially declined. As expected, Rag2$^{-/-}$ DN3 cells transduced with control vector failed to differentiate regardless of the availability of Notch ligand, and survival was severely impaired in the absence of Notch ligand. Thus, both Notch and pre-TCR signals cooperate to ensure the outcome of β-selection.

The mechanisms underlying the requirement for Notch signals at the β-selection checkpoint are mediated through the phosphatidylinositol-3-OH kinase (PI3K) and the kinase Akt/PKB. Active Notch signaling promoted the survival of Rag2$^{-/-}$ DN3 cells independently of pre-TCR mediated survival signals. This effect was mediated by the promotion of glucose uptake and metabolism, and required activation of the PI3K-Akt pathway. Importantly, Rag2$^{-/-}$ DN3 cells expressing both active Akt and a rearranged TCRβ differentiated to the DP stage in the absence of Notch signals, overcoming the requirement for Notch at β-selection. Expression of Akt alone allowed progression to the DP stage only in the presence of Notch ligand. Interestingly, total cell number in short-term cultures of Akt/TCRβ transduced Rag2$^{-/-}$ DN3 cells was similar to that of cells transduced with Akt alone. However, longer-term culture of Akt/TCRβ transduced cells produced a 20 fold increase in DP thymocyte numbers, suggesting that Notch-dependent proliferation signals induced by the pre-TCR are not mediated by Akt. In agreement with this view, Akt1$^{-/-}$Akt2$^{-/-}$ thymocytes have only a mild defect in proliferation upon pre-TCR signaling, yet development is arrested at the DN3

stage and survival is profoundly impaired[46]. Of note, similar to E47 and Ikaros deficient thymocytes, active Akt expression in Rag2$^{-/-}$ thymocytes allows for the development of DP thymocytes that have bypassed the β-selection checkpoint[47].

Activation of the PI3K/Akt pathway is required for the survival, proliferation, and differentiation of αβ T cells beyond the β-selection checkpoint[48]. As Notch signals were shown to affect the activation of the PI3K/Akt pathway, the mechanism responsible for this interaction between Notch and PI3K were examined. Notch1 induction of Hes1 was shown to be necessary to repress Pten, the PI3K-Akt pathway inhibitor, facilitating differentiation induced by pre-TCR signals. Expression of a dominant-negative form of Hes1 or shRNA knockdown in DN3a cells, the *in vivo* equivalent of Rag2$^{-/-}$ DN3 cells, impaired the expansion and development of thymocytes progenitors to the DP cells in the presence of Notch ligand. Similar results were observed in Rag2$^{-/-}$ DN3 cells transduced with TCRβ. Furthermore, the deletion of Pten in DN3 cells allowed T cell differentiation to proceed through the β-selection checkpoint in the absence of Notch signals, although when cultured Pten-deficient DN3 cells fail to expand. Thus, loss of Pten overcame the requirement for Notch signaling in the survival and differentiation of T cell progenitors at β-selection. However, combined ectopic expression of c-Myc and Pten deletion restores the proliferative capacity of DN3 cells through the β-selection checkpoint in the absence of Notch signals. The transcription factor c-Myc is a direct downstream target of Notch signaling, and is thought to mediate proliferation but not developmental progression of T cell progenitors[49-52]. The finding that overexpression of the Notch target c-Myc

overcomes the requirement for Notch signaling to promote the proliferation of thymocytes at β-selection is consistent with the view of c-Myc as a mediator of proliferation.

I.3.3 Transcriptional targets of Notch1: Hes1, Gata3, and Tcf-1

Although Notch signaling is essential to initiate T lineage specification, it is not sufficient to activate T cell lineage genes directly. When cultured for several days on stromal cells expressing the Notch ligand Dll1, thymocytes progenitors at various stages can be reproducibly generated from early hematopoietic progenitors[31,53]. The immediate impact of Notch signaling involves the induction of general Notch targets such as Hes1 and Nrarp, yet expression of key T cell transcription factors Gata3 or Tcf-1 is present in only a subset of these cells. Fewer of these proceed to a DN2 stage, turning on definitive T lineage genes such as CD25, Thy1, and Bcl11b[54]. As only a small subset of precursors activate these T cell genes in response to Notch signaling, it is clear that additional factors are required to enable T cell specification.

The role of Notch1 in T lineage specification is well-established, yet until recently little was known regarding the essential downstream molecular targets required for Notch signaling to initiate T cell development. The basic helix-loop-helix transcription factor Hes1 is one of the best-studied direct Notch transcriptional target genes. Active Notch signaling, including downstream transcriptional targets such as Hes1, can be detected in ETPs making Hes1 a likely candidate for transmitting Notch functions[55]. $Hes1^{-/-}$ embryos develop only a rudimentary thymic structure and die during embryogenesis[56]. When transplanted

into Rag1$^{-/-}$ mice Hes1-deficient fetal liver (FL) hematopoietic progenitor cells fail to undergo T cell differentiation. Only transplantation of high numbers of Hes1$^{-/-}$ FL cells allowed progression to the DN3 stage, although TCR rearrangement was not detected. Thus, Hes1 appears to be required for normal T cell development. However, unlike Notch1 and Dll4 mutant mice, the accumulation of thymic B or myeloid cells was not observed in Hes1$^{-/-}$.

As overexpression of Hes1 in thymocytes enhances proliferation, these findings were thought to suggest that Hes1-deficiency has a greater effect on proliferation and survival than on T lineage commitment[57]. This view was challenged by a conditional gene targeting approach to ablate Hes1 under physiological conditions[58]. Conditional inactivation of Hes1 using the interferon-inducible MxCre in adult bone marrow severely impaired T cell development. In agreement with previous studies, severe thymic hypoplasia was observed in Hes1 mutant mice in steady-state conditions, and immature B or myeloid cells did not accumulate in the thymus. However, neither impaired survival nor proliferation was found to be the cause of thymic hypoplasia in Hes1 mutant mice. Despite the profound reduction in thymic cellularity, T cell maturation in Hes1 mutants was relatively normal. This observation was confirmed using LckCre and Cd4Cre, which inactivate target genes at the DN2 and DN4 stages respectively, to conditionally ablate Hes1. Loss of Hes1 at later stages of T cell development did not reveal any phenotypic changes in T cell maturation or number. Thus, once Hes1-deficient progenitors are committed to the T cell lineage, Hes1 function is dispensable for the further maturation. However, in a

competitive transplant setting a more severe block at the DN1 stage was observed, and immature B cells accumulated in the thymus. Therefore, small numbers of Hes1-deficient progenitors are able to seed the thymus and receive a sufficient Notch1 signal to allow commitment to the T cell lineage and repress the development of the B cell lineage. However, this genetic program is suboptimal, and the majority does not receive a sufficient Notch1-induced genetic program to induce T lineage commitment and develop by default into B cells. This hypothesis was confirmed by exposing Hes1-deficient HSCs to the suboptimal Notch ligand Dll1 resulting in B lineage development, while WT cells retained T lineage potential. Overall, Hes1 appears to be essential for T cell lineage development downstream of Notch1, but is dispensable for Notch1-dependent thymocyte maturation after initial T lineage specification. Similar to Notch1, overexpression of Hes1 in mouse bone marrow inhibits B cell development. Yet ectopic T cell development outside of the thymus was not detected, suggesting that additional factors apart from Hes1 are required to activate the T cell developmental programme[59].

The transcription factor Gata3 is one of the earliest T cell specific genes induced by stimulation of hematopoietic precursor cells with Notch ligand[53], and is highly expressed in ETPs[54]. $Gata3^{-/-}$ mice die early *in utero*, precluding the assessment of T cell development. Therefore, $Rag2^{-/-}$ ES cell blastocyst complementation was utilized to study Gata3 function[60]. $Gata3^{-/-}$ ES cells failed to contribute measurably to the thymocyte populations of $Gata3^{-/-}Rag2^{-/-}$ chimaeric animals, demonstrating an essential role for Gata3 at the earliest stages of T cell

development. Furthermore, in Gata3-deficient fetal liver hematopoietic progenitor cells, the impairment of T lymphopoiesis cannot be overcome with Notch1 alone, requiring both Gata3 and Notch1 for T cell induction[61]. Notch signaling alone, however, appears sufficient to block B cell development in the absence of Gata3. Therefore, Notch signaling is necessary for Gata3 to function in T lineage specification but is not sufficient without Gata3. The role of Gata3 at later stages of thymocytes development was addressed by conditional deletion of Gata3[62]. Ablation of Gata3 with LckCre disrupted T cell development at the DN3 stage. Gata3-deficient DN3 cells expressed reduced intracellular TCRβ, and the few cells that progressed beyond this stage were prone to apoptosis. Expression of a rearranged TCR transgene failed to rescue the DN3 arrest, suggesting Gata3 is required for adequate TCRβ expression and is critical for mediating developmental progression beyond the β-selection checkpoint and events downstream of the pre-TCR. Similarly, ablation of Gata3 with CD4Cre revealed an absolute requirement for Gata3 in the development of $CD4^+$ SP thymocytes. Interestingly, Gata3 does not necessarily confer T lineage identity, as high levels of Gata3 can efficiently drive T lineage precursors to non-T cell lineages[63]. Overexpression of Gata3 in the presence of Notch signaling is antagonistic to survival at the ETP and DN2 stages, and higher levels induced re-specification to the mast cell lineage. Upon removal of Notch signaling, Gata3 overexpression promoted thymocytes progenitor survival and proliferation, suggesting that one function of Notch as thymocytes transition from the DN1 to DN2 stage is to restrain access to the mast cell lineage by limiting the effects of Gata3. This

antagonistic role for Notch was independent of Hes1, despite the ability of Gata3 to up-regulate Hes1 and the positive effect of Hes1 on proliferation.

Despite the understanding that Hes1 and Gata3 are required for T lineage specification, both require additional signals downstream of Notch1 to impose T lineage identity. The high mobility group (HMG) transcription factor Tcf-1 mediates the nuclear response to canonical Wnt signaling, leading to transcriptional activation when bound by β-catenin in response to the binding of secreted Wnt proteins to Frizzled receptors or transcriptional repression when bound by Groucho/Transducin-like enhancer (TLE) proteins[64]. Tcf-1 is up-regulated in ETPs, and is highly expressed when HSCs are exposed to Notch signals[53,65]. When expressed in BM hematopoietic progenitors, Tcf-1 can induce the development of T lineage cells in the absence of Notch signals. To date, Tcf-1 is the only factor outside of Notch1 that is sufficient to impose T cell fate. In support of this view, Tcf-1$^{-/-}$ HSCs cultured on stromal cells expressing Notch ligand fail to generate T lineage cells, however myeloid and B lineage development was inhibited. Tcf-1$^{-/-}$ BM when transplanted in competition with wild-type cells into irradiated recipients are defective in generating ETPs, and later T cell stages are absent. Thymic development in Tcf1$^{-/-}$ mice is also severely disrupted, with greatly reduced thymic cellularity starting at the ETP stage, yet T cell development is not abolished. Hence Tcf-1 is essential for promoting T cell fate but is not involved in Notch1-mediated inhibition of alternative cell fates.

Through combined microarray-based analysis of gene expression and chromatin immunoprecipitation experiments, Tcf-1 was found to directly regulate the expression of numerous T-lineage genes including Bcl11b, Gata3, and Tcf-1 itself. Bcl11b is critical for maintaining T lineage commitment, and loss of Bcl11b diverts committed T cells to the natural killer lineage or results in developmental arrest[66,67]. Interestingly, these functions of Tcf-1 were found to be independent of canonical Wnt signaling, as deletion of β-catenin or expression of a small molecular inhibitor of β- and γ-catenin had no effect on the generation of T lineage cells from bone marrow HSCs. Further demonstrating the instructive role of Tcf-1 in T lineage development, expression of Tcf-1 in HSCs lacking Notch1 allowed the intra-thymic generation of DN3 cells. Therefore, Tcf-1 is also capable of driving early T cell development in the absence of Notch signals. Tcf-1 was also demonstrated to be a direct transcriptional target of Notch1, and Tcf1 expression was found to be dependent upon Notch signaling. Yet separating the function of Tcf-1 from Notch1, forced expression of Tcf-1 in BM hematopoietic progenitors did not induce T cell leukemia unlike overexpression of Notch1-IC, suggesting that the key targets of Notch1 that control proliferation and cellular transformation are not induced by Tcf-1. Overall, these data suggest a model where Notch signals induce Tcf-1, which then imprints T cell fate by up-regulating essential T lineage genes such as Gata3 and Bcl11b. In parallel, the Notch target gene Hes1 promotes T lineage specification and directly inhibits alternative cell fates.

1.3.4 Wnt signaling in the thymus: Essential role of Tcf-1

Wnt proteins are a family of secreted morphogens crucial for various developmental processes including cell-fate specification, proliferation, and control of asymmetric cell division. In the hematopoietic lineages, Wnt proteins function as growth factors and influence cell fate decisions. There are at least three different Wnt pathways: canonical Wnt pathway involving β-catenin and the T-cell factor (Tcf)/lymphocyte-enhancer-binding factor (Lef) family of transcription factors, the planar cell polarity pathway, and the Wnt-Ca^{2+} pathway.

The canonical Wnt pathway is the best characterized pathway in cells of the immune system and appears to be the major pathway for Wnt signaling in developing T cells and will be briefly reviewed here (Figure I.3). In the absence of Wnt ligand bound to its receptor complex, cytoplasmic β-catenin is continuously phosphorylated by casein kinase 1 (Ck1) and glycogen synthase kinase 3β (Gsk3β), leading to binding of the destruction complex and targeted degradation by the proteosome. The destruction complex is composed of the tumor suppressor adenomatous polyposis coli (Apc) and axis inhibition protein 1 (Axin1), along with several scaffolding proteins, Ck1, and Gsk3β. In the nucleus, Tcf/Lef transcription factors are bound by co-repressors such as Groucho/Tle proteins to silence Wnt target genes. Upon binding of Wnt to the Frizzled receptor and the co-receptor low density lipoprotein receptor-related protein (Lrp) at the cell surface, Dishevelled (Dvl) binds to the Frizzed/Lrp complex and promotes the phosphorylation of Lrp by Ck1 and Gsk3β, allowing Axin1 to bind Lrp, pulling away Axin1 from the destruction complex and freeing β-catenin from

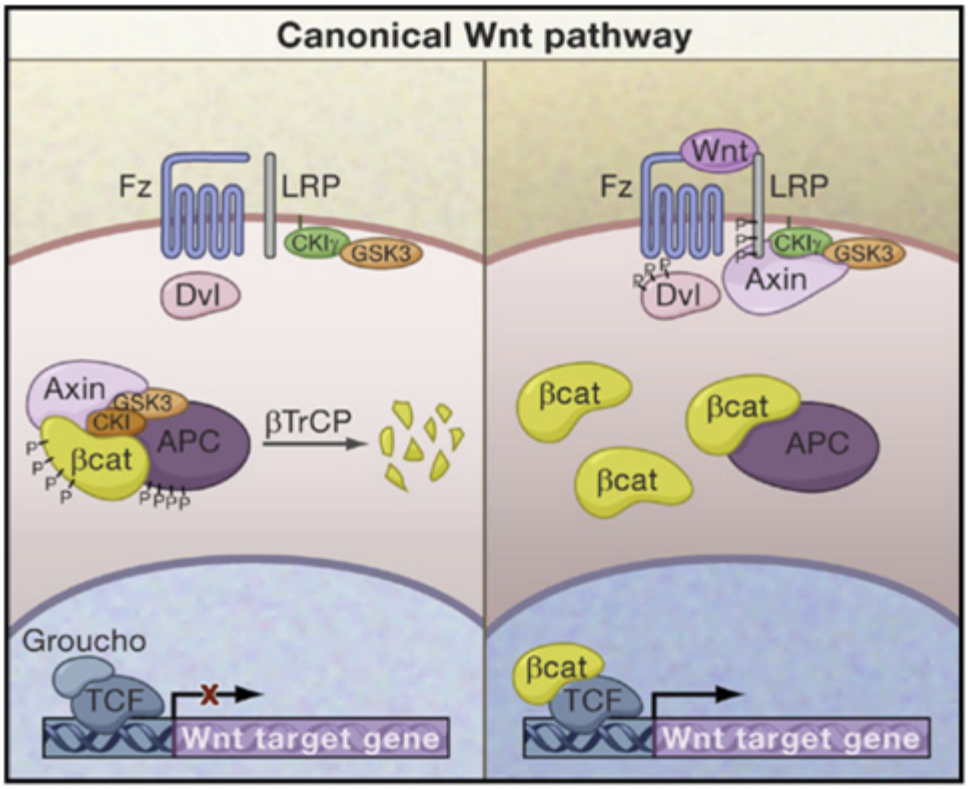


Figure I.3 Canonical Wnt signaling.
(Left panel) When Wnt receptor complexes are not bound by ligand, the serine/threonine kinases, CK1 and GSK3α/β, phosphorylate β-catenin. Phosphorylated β-catenin is recognized by β-TrCP, a component of a dedicated E3 ubiquitin ligase complex. Following ubiquitination, β-catenin is targeted for rapid destruction by the proteasome. In the nucleus, the binding of Groucho to TCF (T cell factor) inhibits the transcription of Wnt target genes. (Right panel) Once bound by Wnt, the Frizzled(Fz)/LRP coreceptor complex activates the canonical signaling pathway. Fz interacts with Dsh, a cytoplasmic protein that functions upstream of β-catenin and the kinase GSK3β. Wnt signaling controls phosphorylation of Dishevelled (Dsh). Wnts are thought to induce the phosphorylation of LRP by GSK3β and casein kinase I-γ (CK1γ), thus regulating the docking of Axin. The recruitment of Axin away from the destruction complex leads to the stabilization of β-catenin. In the nucleus, β-catenin displaces Groucho from Tcf/Lef to promote the transcription of Wnt target genes. Reproduced from Clevers, et. al, **Cell.,** 2006 (127)[68].

phosphorylation and degradation. β-catenin will then translocate to the nucleus to form active β-catenin-Tcf transcription factor complexes activating Wnt target gene expression.

Wnt signaling is important at multiple stages of thymocytes development. Using soluable Frizzled receptors to block active Wnt signaling in fetal thymic organ cultures, thymocyte differentiation was negatively impacted[69]. This was attributed to reduced proliferation, as thymi from $Wnt1^{-/-}$ or $Wnt4^{-/-}$ had low cellularity, and $Wnt1^{-/-}Wnt4^{-/-}$ thymic cellularity was reduced even further[70]. Similarly, mice deficient for the essential downstream transcription factor of canonical Wnt signaling Tcf-1 displayed low thymic cellularity suggesting an essential role for Wnt/Tcf signaling in thymocyte development[71]. Although this interpretation was refined to exclude at least the initial stages of T cell development that are independent of β-catenin[65], deletion of β-catenin impairs the DN3-to-DN4 transition, and DN4 thymocyte proliferation was negatively affected. Similarly, young $Tcf\text{-}1^{-/-}$ mice display fewer cycling DN4 and ISP thymocytes and an incomplete block at the DN3 and immature single positive (ISP) stage, suggesting an essential role for Wnt/Tcf-1 signaling in β-selection and the DN to DP transition[72,73]. However, the impairment of T cell development at these stages in $Tcf\text{-}1^{-/-}$ mice is far more severe than in the absence of β-catenin. Consistent with an essential role for Tcf-1 in β-selection, a significantly increased fraction of $Tcf\text{-}1^{-/-}$ intracellular $TCR\beta^{+}$ DN3 and DN4 cells were apoptotic, and underwent rapid cell death in response to growth factor withdrawal, suggesting that in the absence of Tcf-1 cycling induced by the pre-

TCR is impaired and survival of post β-selection thymocytes is not ensured[74]. Interestingly, induction of pre-TCR signaling in $Rag1^{-/-}$ DN3 thymocytes rapidly induced Tcf-1 mRNA and protein levels. β-catenin appeared to not be affected by pre-TCR signaling, as no change in the abundance of total or active non-phosphorylated β-catenin was observed in response to pre-TCR signals demonstrating that stabilization of β-catenin is not dependent on pre-TCR signals. Thus, induction of Tcf-1 is a critical pre-TCR signaling event, linking pre-TCR with Wnt signals.

In contrast to Tcf-1, $Lef1^{-/-}$ mice have normal T cell development[75]. While T cell development is also normal in mice hypomorphic for Tcf-1, $Lef1^{-/-}$ mice that are hypomorphic for Tcf-1 show a complete block in T cell development at the ISP stage, demonstrating a redundant function for Lef1 and Tcf-1 at the DN to DP transition. A more profound block at the DN stage was observed in the absence of both Lef1 and Tcf-1[76]. In these mice, thymocyte development failed to progress beyond the β-selection checkpoint, and no mature T cells were observed in the periphery. Lef1/Tcf-1 double-deficiency did not further exacerbate the developmental block at the DN1 and DN3 stage as compared to loss of Tcf-1 alone, nor was the rearrangement and expression of intracellular TCRβ or DN3 cell number altered. Yet in contrast to $Tcf\text{-}1^{-/-}$ mice, no increase in intracellular $TCR\beta^{+}$ DN4 cells was observed in Lef1/Tcf-1 double-deficient thymi, suggesting that pre-TCR signaling events are impaired in the absence of Lef1 and Tcf-1. The expression of Tcf-1 and Lef1 are consistent with these data, as Lef1 is rapidly increased at the DN3 stage and both Tcf-1 and Lef1 expression

are maintained at high levels from the DN3 stage onwards. Therefore, Tcf-1 and Lef1 are critical for β-selection and maturation beyond the DN4 stage.

Recent work has also implicated TCF-1 in the development of human T-ALL. Deletions in TCF-1 were observed in a subset of pediatric T-ALLs that resemble ETPs (ETP-ALL), and this subtype was associated with diminished expression of TCF-1[76,77]. Tcf-1$^{-/-}$ mice succumb to precursor T cell lymphoblastic lymphomas that resemble human T-ALL consistent with a role for Tcf-1 as a classical tumor suppressor. The development of T cell lymphomas in Tcf-1$^{-/-}$ mice was dependent upon high Lef1 expression, as Lef1 deletion significantly delayed or prevented malignant transformation. Tcf-1 was demonstrated to directly repress Lef1 in post β-selection thymocytes, and this repression was lost upon loss of Tcf-1[76,77]. Increased Id2 expression was also observed in Tcf-1$^{-/-}$, and repression of Id2 depends on Tcf-1. Whether Id2 is a direct target of Tcf-1 awaits further validation. In contrast to Lef1, loss of Id2 only delayed the onset of lymphoma in Tcf-1$^{-/-}$ mice.

It is interesting to note that T cell development is highly sensitive to Wnt signaling levels. Stabilization of β-catenin leads to pre-TCR independent thymocyte differentiation and survival past the β-selection checkpoint, although thymocyte proliferation is greatly reduced[78]. Deletion of Apc, a negative regulator of Wnt signaling, in early thymocytes using LckCre leads to reduced thymic cellularity and impaired thymocyte development[79]. Loss of Apc promoted extensive proliferation of DN3 cells, and suppressed Notch-dependent transcription and VDJ recombination. Strikingly, Apc-deficient thymocytes that

progressed to DN4 had reduced cycling and lacked expression of intracellular TCRβ suggesting loss of APC promoted thymocyte development in the absence of pre-TCR signaling. Using a similar approach to modulate Wnt signaling through the use of different mutations of Apc, T cell development was shown to have stage-specific sensitivities for Wnt signaling[80]. While intermediate levels of Wnt signaling promoted the DN3-to-DN4 transition and a dramatic expansion of intracellular $TCR\beta^+$ DN4 thymocytes, slightly milder Wnt signaling had no effect. Higher levels of Wnt completely blocked this transition and reduced DN4 cell numbers while allowing intracellular $TCR\beta^-$ DN4 thymocytes to develop to the DP stage. Similarly, using stromal cell cultures to assess T cell development intermediate but not high levels of Wnt signaling increased the number of DP and SP cells. Thus excess Wnt/β-catenin may compensate for lack of pre-TCR signaling and Tcf-1 induction, allowing the survival of pre-TCR-deficient thymocytes. This highlights the need to control Tcf-1 and β-catenin in cells that lack a pre-TCR, and ensure that only β-selected thymocytes receive the prosurvival function of Wnt signaling.

Wnt signaling has also been implicated in thymocyte development at the DP stage. Tcf-$1^{-/-}$ DP thymocyte survival when cultured is severely diminished, undergoing rapid apoptosis[73]. The proportion of apoptotic DP thymocytes in Tcf-$1^{-/-}$ is also greatly increased. The defect in survival can be rescued by full-length but not a truncated version of Tcf-1 that lacks the β-catenin interaction domain, suggesting that Wnt/Tcf-1 signaling mediated through β-catenin supports DP thymocyte survival. The impaired survival of Tcf-$1^{-/-}$ DP thymocytes correlated

with the low expression of Bcl-x_L, a member of the anti-apoptotic Bcl-2 family of proteins, and could be rescued by expression of a Bcl-2 transgene. Expression of full-length but not the truncated version of Tcf-1 in Tcf-$1^{-/-}$ mice restored Bcl-x_L expression. In agreement with these observations, expression of an activated β-catenin transgene increased expression of Bcl-x_L in DP thymocytes[81]. Thus, Tcf-1 interaction with β-catenin ensures the survival of DP thymocytes by up-regulating Bcl-x_L expression, extending their lifespan to allow TCRα rearrangement. The mechanism regulating expression of Bcl-x_L is not mediated directly through Tcf-1/β-catenin, but through the direct Tcf-1 target Rorγt[82].

Rorγt$^{-/-}$ mice have a developmental block at the ISP stage, with a severe impairment in DP thymocyte survival and lack expression of Bcl-x_L[83]. Expression of Rorγt in Tcf-$1^{-/-}$ mice rescues DP thymocyte survival, whereas ectopic Tcf-1 cannot rescue DP thymocyte survival in Rorγt$^{-/-}$ mice. In agreement with a prominent role for Bcl-x_L in DP thymocyte survival, mice deficient in the transcription factor c-Myb had premature DP thymocyte apoptosis, and this was found to be dependent on reduced Bcl-x_L expression[84]. The regulation of Bcl-x_L by c-Myb appears independent of Tcf-1 and Rorγt, as c-Myb expression is normal in mice deficient for these factors. The E protein family transcription factor HEB is also involved in DP thymocyte survival, as DP thymocytes deficient for HEB undergo rapid apoptosis and have reduced Bcl-x_L expression[85]. In contrast to c-Myb, HEB appears to directly regulate Rorγt expression in DP thymocytes.

I.3.5 Sox13 opposes αβ T lineage development by antagonizing Tcf-1

The HMG-box transcription factor Sox13 was identified in a screen of transcription factors that were differentially expressed between αβ and γδ thymocytes[86]. Sox13 expression is restricted to DN thymocytes, and can be detected at high levels in DN1 and DN2 cells. At the DN3 stage, Sox13 expression is rapidly extinguished, and is barely detectable by the DN4 stage. Sox13 expression is significantly increased in immature DN γδ-T cells following γδ-TCR signaling, but is not observed in other lineages[87]. The expression of Sox13 is not linked to TCR rearrangement or expression, as it is detected in both $Rag^{-/-}$ and $TCR\beta^{-/-}$ thymocytes, indicating that Sox13 expression correlates with T cell sub-lineage and not TCR type. The expression of Sox13 in DN2 cells is in agreement with a potential role in γδ T lineage development, as the developmental potential of DN2 cells is heterogeneous and single-cell analysis of Sox13 expression revealed it to be restricted to only a subset the DN2 population.

The function of Sox13 was assessed by generating a transgenic line that overexpressed Sox13 in thymocyte precursors (*Sox13Tg*). Interestingly, Sox13 expression opposed the development of the αβ T cell lineage. In *Sox13Tg* fetal thymi, DP thymocytes were absent and a complete block in αβ T cell lineage development at the DN stage was observed. In adult *Sox13Tg* mice, αβ T cell lineage development remained impaired, with a dramatic reduction in DP thymocyte numbers. DP thymocytes exhibited significantly increased apoptosis in *Sox13Tg* mice, which could be rescued by expression of a Bcl-2 transgene. In

contrast, the absolute number of γδ T-lineage cells in the thymus remained unchanged. The number of DN thymocytes was also decreased, as well as a substantial reduction in DN thymocyte proliferation. As the majority of proliferating DN thymocytes are αβ-lineage pre-TCR^{+} thymocytes, this suggested that Sox13 inhibits the proliferative burst of αβ T cells at the DN-to-DP transition. Conversely, Sox13$^{-/-}$ mice showed impaired γδ T cell development but no defect in αβ T lineage development.

Examination of gene expression in *Sox13Tg* DP thymocytes compared to wild-type revealed increased expression of a set of genes previously identified as differentially expressed between normal αβ and γδ T cells, including TCRγ, which is normally silenced in DP thymocytes. Significantly, expression of TCRγ is also observed in αβ T cells of Tcf-1$^{-/-}$ mice, and the defects in thymocyte development of *Sox13Tg* strongly resemble those observed in Tcf-1$^{-/-}$ mice. Tcf-1 positively regulates Cd4 gene expression at the DN-to-DP transition[88]. Consistent with an interaction between Tcf-1 and Sox13, both Tcf-1$^{-/-}$ and *Sox13Tg* DP thymocytes express diminished levels of CD4. As Sox genes have been shown to influence Wnt signaling[89,90], the possibility of Sox13 antagonizing Tcf-1 function was examined. Sox13 inhibited reporter gene activity controlled by TCF binding sites in a dose-dependent fashion, and down-regulated expression of the Tcf-1 target gene Ly49. Importantly, Sox13 was able to antagonize expression of both β-catenin dependent and independent Tcf-1 target genes. Sox13 was shown to bind Tcf-1 directly, and required the N-terminal β-catenin interaction domain of Tcf-1. No evidence of Sox13 binding to β-catenin was detected. Thus, Sox13 is a

lineage-specific Tcf-1 antagonist that acts by directly binding to Tcf-1 and modifying its activity. The silencing of Sox13 in αβ T cells may represent an initial step in the αβ T lineage developmental programme, and while the γδ-TCR appears to reinforce Sox13 expression, it is unclear whether or how pre-TCR signaling in DN3 thymocytes acts to silence Sox13 thereby controlling the magnitude of Wnt/Tcf-1 signaling.

I.3.6 E protein regulation of T cell development

E proteins are a family of class I bHLH transcription factors that includes three family members: E2A, HEB, and E2-2. The E2A gene encodes two proteins, E47 and E12, due to alternative splicing. E proteins functions as dimers, binding to consensus E-box sites to regulate transcription[91]. In thymocytes, E2A and HEB are the primary E-proteins expressed and form predominantly E2A homodimers and E2A/HEB heterodimers. A second group of HLH proteins expressed in lymphocytes is known to negatively regulate class I bHLH factors. These class V HLH factors include the inhibitor of differentiation (Id) genes Id1-Id4. Id proteins contain the HLH domain that allows dimerization with E-proteins but lack the basic region required for E-box DNA binding thus preveting E-proteins from binding DNA[92].

E proteins are essential for multiple stages of T cell development. E2A is essential for proper development and generation of LMPPs, the BM precursor of ETPs in the thymus[93]. Expression of high levels of Notch1 in LMPPs and ETPs require E2A proteins, and E2A cooperates with Notch1 to activate Notch target genes such as Hes1 and Ptcra, suggesting E2A proteins function prior to and at

the earliest stages of T lineage specification. $E2A^{-/-}$ mice have an incomplete block in T cell development at the DN1 stage and accelerated positive selection during the DP to SP transition. However a T cell specific knockout of E47 failed to reproduce a defect in positive selection[94-96]. E2A is also essential for the complete arrest of thymocyte development in the absence of pre-TCR signaling[42]. $E2A^{-/-}$ thymocytes are unable to prevent the development and proliferation of $TCR\beta^{-}$ DN thymocytes beyond the β-selection checkpoint in pre-TCR signaling mutants. Importantly, E2A also acts to block cell cycle progression prior to the expression of TCRβ, and $E2A^{-/-}$ mice develop thymic lymphomas at a young age.

The E protein HEB is also critical for T cell development. T cell development in $HEB^{-/-}$ mice displays a profound developmental block at the ISP stage and defective DP thymocyte survival[85,97,98]. The ISP block is thought to be related to the interaction of E2A and HEB with the CD4 enhancer[99]. The importance of E2A/HEB heteodimers was demonstrated with a dominant-negative HEB allele, which inactivates both E2A and HEB function[100]. T cell development was arrested completely at the DN3 stage, and thymocytes were found to be deficient for TCRβ VDJ rearrangement. Similar to $HEB^{-/-}$, introduction of a TCR transgene failed to rescue thymocyte development indicating essential functions for E-proteins independent of pre-TCR signaling. However no defects at earlier stages of T cell development were observed indicating E2A/HEB heterodimers function after T lineage specification. In agreement with these studies, conditional ablation of both E2A and HEB also arrested T cell

development at the DN3 stage, and thymocyte proliferation prior to pre-TCR expression was increased[101]. Interestingly, these thymocytes when cultured acquire a DN2-like phenotype even though TCRβ rearrangement had occurred, uncovering a role for E-proteins in promoting normal developmental progression and preventing developmental regression of DN3 cells to the DN2 state.

E2A proteins also play a dose-dependent role during TCRβ rearrangement by increasing the accessibility of TCR Vβ gene segments to the recombination machinery[102]. Feedback regulation by the pre-TCR, through the induction of Id proteins, was implicated in enforcing allelic exclusion. TCR signaling antagonizing E protein function appears to be a common theme, as down-regulation of E2A is observed at both the pre-TCR checkpoint to allow DN progenitors to expand and differentiate to the DP stage and as thymocytes progress from DP to SP stage[43,103]. Notch1 is highly up-regulated at the DN3 stage. However as thymocytes progress beyond DN3, Notch1 is rapidly down-regulated and in post β-selection thymocytes Notch signaling is no longer required for development or survival[104]. This down-regulation of Notch1 requires pre-TCR signaling, and is mediated by Id3 up-regulation following successful β-selection[105]. The up-regulation of Id3 post β-selection antagonizes the positive regulation of Notch1 transcription by E2A. Accordingly, $Id3^{-/-}$ thymocytes maintain E2A-dependent Notch1 transcription, and enforced Id3 expression abolishes Notch1 expression and Notch1-dependent T cell survival. Thus, pre-TCR signaling inactivates Notch1 transcription by inhibiting E2A function. The importance of controlling Notch and E protein activity in thymocytes is highlighted

by the fact that activating NOTCH1 mutations and gain of function mutations in the E protein antagonist SCL/TAL1 are the most common genetic aberrations in pediatric T-ALL[106,107], and loss of E protein function or gain of Notch1 signaling promote T cell leukemia in mice[94,108-110].

At the DP to SP transition, E proteins and Id3 interact in a similar fashion as at the β-selection checkpoint[111]. Id3 is required for thymocyte positive selection, and negative selection is impaired in Id3$^{-/-}$ mice. In the absence of both E2A and HEB, DP thymocytes progress to the SP stage independent of TCR, bypassing the requirement for TCR-mediated selection[112]. Thus, E proteins maintain the DN3 and DP phenotype by enforcing the β-selection checkpoint and TCR-mediated positive selection.

I.3.7 Ikaros and chromatin remodeling complexes in T cell development

Ikaros (Ikzf1), a zinc-finger transcription factor, is essential for the development of the lymphoid lineage from HSCs[113,114]. Ikaros$^{-/-}$ mice exhibit reduced thymic cellularity, a dramatic expansion of DN4 thymocytes, and loss of CD4 silencer activity in DN thymocytes[115]. T cells are also biased toward the CD4 lineage in Ikaros$^{-/-}$ mice[116]. Interestingly, DN thymocytes can differentiate to the DP stage in the absence of pre-TCR signaling in Ikaros$^{-/-}$ mice[44]. Despite the accelerated maturation of DN3 thymocytes to the DP stage, the requirement for pre-TCR signaling in the expansion of post β-selected thymocytes is maintained. T cell lymphomas are also observed in Ikaros$^{-/-}$ mice.

In thymocytes, Ikaros is associated with Mi2β (Chd4), an essential component of the nucleosome remodeling and histone deacetylase (NuRD) co-

repressor complex[117]. Conditional inactivation of Mi2β leads to an accumulation of DP-like thymocytes that lack expression of CD4. A role for Mi2β in positively regulating CD4 transcription through recruitment of HEB and p300, but not HDAC2, was demonstrated. These findings implicated a new role for the NuRD complex as a positive regulator of transcription in the absence of HDACs. The DN-to-DP transition was also disrupted in Mi2β-deficient thymocytes, as DN thymocyte number was expanded and cells accumulated at the DN4 stage. Proliferation and survival of post β-selection thymocytes appeared normal, and expression of pre-TCR signaling components was unchanged. Through examination of fetal and neonatal thymi, where the development of one rather than several waves of thymocyte precursors can be observed, the DN4 accumulation was shown to be a result of inefficient precursor transition. Interestingly, in the absence of Ikaros, the NuRD complex was found to be distributed to transcriptionally poised genes that are not targets of Ikaros, including genes involved in proliferation and metabolism, and induced their reactivation, providing insight into the function of Ikaros in restraining the activity of the NuRD complex[118].

Ikaros was also found to play a role in repressing the transcriptional response to Notch signaling. After β-selection, Notch signaling is no longer required for T cell development, but aberrant activity of Notch at this stage induces malignant transformation. Thus, Notch signaling must be silenced in cells where Notch activity is not required. Ikaros plays a critical role in this process by transcriptionally repressing both Notch1 and the Notch target gene

Hes1 after β-selection[119,120]. Hes1 is a critical Notch1 target and is required for both the development and maintenance of Notch1-induced T-ALL[58]. The repression of Hes1 by Ikaros is first observed in immature thymocytes at the DN4 stage, where Ikaros competes for binding with RBPJκ at the Hes1 promoter. At this stage, thymocytes lose the ability to transcribe Hes1 upon Notch signaling. The requirement for Ikaros in repressing Hes1 was demonstrated in Ikaros-deficient DN4 and DP thymocytes, which retain the capacity to express Hes1 after Notch stimulation. The importance of silencing Notch transcriptional target genes such as Hes1 in post β-selection thymocytes can be observed in transgenic mice that overexpress Hes1[121]. These mice develop T cell lymphomas with low penetrance. Expression of Hes1 significantly accelerates the development of lymphoma in Id1 transgenic mice, which lack E protein function.

Of note, in Ikaros-deficient thymocytes the Hes1 promoter lacks trimethylated H3K27, a repressive chromatin modification placed by the polycomb repressive complex 2 (PRC2). Conditional deletion of Ezh2, a critical component of PRC2, is associated with a profound reduction in thymocyte number, and a complete block in T cell development at the DN stage[122]. These mice also develop frequent γδ T cell lymphomas. Loss of function mutations and deletions of EZH2 and SUZ12 genes, essential for PRC2 activity, are observed in a significant proportion of adult T-ALL samples[123]. Silencing of EZH2 or SUZ12 in human T-ALL cell lines resulted in transcriptional up-regulation of HES1 and other Notch target genes. Activation of NOTCH1 specifically induced the loss of trimethylated H3K27, and loss of PRC2 was shown to increase tumor growth in a

Drosophila Notch-driven tumor model. Thus PRC2 also appears to be involved in the epigenetic silencing of Notch signaling.

Insight into Ikaros-mediated repression of Notch1 came from studies of Ikaros$^{-/-}$ mice in which Notch1 was conditionally inactivated[119]. These studies revealed the presence of alternative and cryptic Notch1 promoters, and identified Ikaros in the repression of ligand-independent Notch1 proteins expressed from cryptic Notch1 promoters. In normal cells, these promoters are silenced, however in Ikaros$^{-/-}$ mice both alternative and cryptic promoters are in a transcriptionally poised state more accessible to the basal transcriptional machinery, marking a transition to a preleukemic state. A feed-forward cycle would then be initiated through both ligand-dependent and -independent Notch1 promoters allowing progressive accumulation of the potent transcriptional enhancer Notch-IC and transition to a leukemic state. The SIRT1-LSD1 corepressor complex is also involved in repression of Notch transcriptional targets in the absence of Notch ligand, however its role in T cell development or Notch1-mediated oncogenesis has yet to be addressed[124].

I.4 Regulation of Ca^{2+} by antigen receptor signaling

In resting cells, the concentration of free intracellular Ca^{2+} is kept low and constant. Engagement of lymphocyte antigen receptors results in the rapid entry of extracellular Ca^{2+} into the cell. Both resting Ca^{2+} and entry of Ca^{2+} into the cell are regulated by a diverse set of mechanisms, and include ion channels involved in the control of membrane potential, Ca^{2+} ATPase pumps present on the plasma membrane and the endoplasmic reticulum, and Ca^{2+} release-activated Ca^{2+}

channels (CRAC) required for entry of extracellular Ca^{2+} into the cell. Cellular organelles such as the mitochondria also play a role in both buffering and in maintaining Ca^{2+} influx. In the remaining sections, we will address the signaling events connecting the T cell antigen receptor to Ca^{2+} entry and the role of Ca^{2+} in regulating the development and function of T cells.

Engagement of the TCR initiates the recruitment of various adaptor molecules and the activation of several layers of protein kinases, leading to the phosphorylation of phospholipase C-γ (Plcγ) and an increase in intracellular Ca^{2+}[125]. Activation of Plcγ by phosphorylation represents the key step in increasing intracellular Ca^{2+} and entry of Ca^{2+} from outside the cell. Plcγ hydrolyzes phosphatidylinositol-3,4-bisphosphate (PIP_2) to the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 diffuses in the cytoplasm and binds to its receptor, the inositol-1,4,5-triphosphate receptor (IP_3R), localized primarily on the endoplasmic reticulum (ER) membrane. The IP_3R is an intracellular ligand-gated Ca^{2+} release channel, and binding of IP_3 to IP_3R induces the release of ER Ca^{2+} stores into the cytoplasm. Ca^{2+} released from intracellular stores results in only a small transient increase in intracellular Ca^{2+} concentration due to the small size of the ER in lymphocytes. The depletion of Ca^{2+} in the ER triggers the entry of extracellular Ca^{2+} across store-operated CRAC channels at the plasma membrane, permitting a sustained increase in intracellular Ca^{2+}(Figure I.4). Plcγ can also function in the regulation of Ca^{2+} signaling through a novel lipase-independent mechanism[126,127], via interaction with Trpc3, a member of the transient receptor potential family of ion

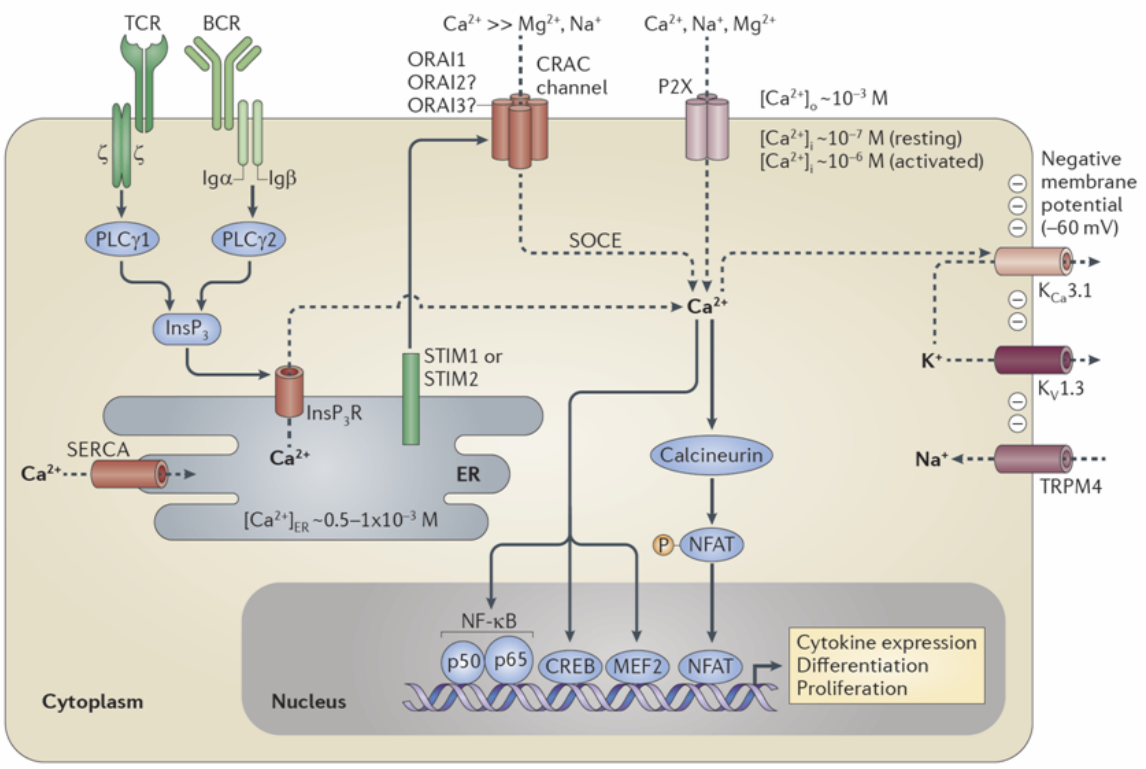


Figure I.4 Ion channels regulating Ca^{2+} signalling in lymphocytes.
Ca^{2+} release-activated Ca^{2+} (CRAC) channels are activated following the engagement of antigen receptors (that is, T cell receptors (TCRs) or B cell receptors (BCRs)). This is mediated through the activation of phospholipase Cγ (PLCγ), the production of inositol-1,4,5-trisphosphate ($InsP_3$) and the release of Ca^{2+} from endoplasmic reticulum (ER) Ca^{2+} stores. The ensuing activation of stromal interaction molecule 1 (STIM1) and STIM2 results in the opening of ORAI1 CRAC channels and store-operated Ca^{2+} entry (SOCE). Sustained Ca^{2+} influx through CRAC channels leads to the activation of Ca^{2+}-dependent enzymes and transcription factors, including calcineurin and nuclear factor of activated T cells (NFAT). P2X receptors, such as P2X4 and P2X7, are non-selective Ca^{2+} channels activated by extracellular ATP. Ca^{2+} influx in lymphocytes depends on the gradient between the extracellular Ca^{2+} concentration (~1 mM) and the intracellular Ca^{2+} concentration (~0.1 μM) and on an electrical gradient established by two K+ channels (namely, $K_V1.3$ and $K_{Ca}3.1$) and the Na^+-permeable channel TRPM4 (transient receptor potential cation channel M4). CREB, cAMP-responsive-element-binding protein; InsP3R, InsP3 receptor; MEF2, myocyte-specific enhancer factor 2; NF-κB, nuclear factor-κB; SERCA, sarcoplasmic/ endoplasmic reticulum Ca^{2+} ATPase. Reproduced from Feske, et. al, **Nature Reviews Immunology.,** 2012 (12)[128].

channels, targeting Trpc3 to the plasma membrane. Additionally, Plcγ can induce Ca^{2+} release into the cell independent of IP_3R through the generation of DAG and activation of transient receptor potential channels (TRPC)[129,130].

Plcγ1 activation in T cells is essential for TCR-mediated Ca^{2+} influx, and links the TCR to IP_3R and SOCE. However, $Plc\gamma1^{-/-}$ embryos die early in gestation, and ES cell complementation assays have identified a requirement for Plcγ1 in hematopoietic stem cell emergence precluding an direct examination of Plcγ1 function in T cell development[131]. The generation of a conditional Plcγ1 knockout mouse has begun to address the limited understanding of Plcγ1 in T cell biology[132]. Ablation of Plcγ1 with CD4Cre, which is active in late DN4 thymocytes after β-selection, impairs thymocyte maturation at the DP stage of development. TCR-mediated Ca^{2+} influx was markedly reduced, as was the activation of Erk and Nfat, a Ca^{2+}-dependent transcription factor. Development of the $CD4^+$ SP T cell lineage was profoundly impaired, and $CD8^+$ SP T cells were reduced albeit to a lesser extent than $CD4^+$ SP T cells. Plcγ1-deficienty in DP thymocytes blocked positive selection, and negative selection was abolished. Thus Plcγ1 activity is essential for T cell development and selection; however the role of Plcγ1 in early stages of development has yet to be directly addressed.

The transmembrane scaffolding protein Lat is essential for recruiting Plcγ1 to the TCR upon engagement of antigen and subsequent Plcγ1 activation. Mice homozygous for a mutation that specifically impairs Plcγ1 activation, LAT^{Y136F}, display a severe block in early T cell development at the DN3 stage and significantly reduced DP thymocyte numbers[133]. As pre-TCR signaling is required

for the DN3-to-DP transition, these data were interpreted to demonstrate a requirement for Plcγ1 in pre-TCR signaling. In these mice, Plcγ1 Ca^{2+} mobilization but not Plcγ1-DAG-mediated Erk activation was found to be specifically impaired. This observation has led to the hypothesis that Plcγ1-mediated Ca^{2+} signaling through the IP_3R is essential for the development of the αβ T cell lineage. This same group examined DP thymocyte selection in LAT^{Y136F} mice, and found similar defects in positive and negative selection as those observed in Plcγ1-deficient DP thymocytes[134], whereby thymocytes that would normally have undergone negative selection exhibited a phenotype resembling positive selection. Together, these data implicate an essential role for Plcγ1-IP_3R-Ca^{2+} signaling in early T cell development.

I.4.1 The inositol triphosphate receptor (IP_3R)

The inositol-1,4,5-triphophate receptors are a family of Ca^{2+} release channels localized predominantly to the endoplasmic reticulum of all cell types. IP_3R functions to release Ca^{2+} into the cytoplasm in response to IP_3 produced by Plc enzymes, generating complex local and global Ca^{2+} signals that regulate diverse cellular processes such as transcription and motility[135]. The IP_3R are passive ion channels, and upon binding of IP_3 will open and allow Ca^{2+} to diffuse down an electrochemical gradient from the ER lumen to the cytoplasm. This gradient is maintained by ER Ca^{2+}-ATPase's that pumps Ca^{2+} into the ER lumen to accumulate to Ca^{2+} to high concentrations. The low concentration of Ca^{2+} in the cytoplasm is maintained by other Ca^{2+} pumps and transporters. Ca^{2+} moves into the cytoplasm by passive diffusion and is buffered by Ca^{2+}-binding proteins,

and the opening of IP_3Rs create localized micro-domains with sharp Ca^{2+} concentration gradients that rapidly dissipate. Thus, the distribution and concentration of IP_3R and Ca^{2+}-binding proteins allow IP_3R-Ca^{2+} signals to have diverse spatial and temporal properties that can be exploited by cells.

The IP_3R receptor was first identified in rat cerebellum extracts[136], and while the IP_3R is expressed at the ER, it can also be observed at the nuclear envelope, plasma membrane, and mitochondria. The IP_3R is encoded by a family of three genes (Itpr1, Itpr2, Itpr3) in mammalian cells, and are highly homologous at critical regions such as the ligand-binding and pore domains. While the IP_3R is ubiquitously expressed, the three channel isoforms have distinct and overlapping expression patterns with most cells expressing more than one type. Further increasing receptor diversity is the significant use of alternative splicing, heteroligomerization among different isoforms when forming IP_3R channels, which exist as both homo- and heterotetramers, and the many unique protein interactions of individual IP_3R isoforms. IP_3R protein levels are regulated by IP_3 as well as Ca^{2+}-dependent post-translational modifications[137].

The functional implications of IP_3R diversity remain largely unexplored. The diversity of IP_3R expression suggests that cells require distinct IP_3R to regulate specific biological functions. For example, cerebellar purkinje neurons express IP_3R1[138], whereas cardiomyocytes express predominantly IP_3R2[139]. $IP_3R1^{-/-}$ mice largely die *in utero*, and those born display severe neurological defects and die by weaning age. In $IP_3R2^{-/-}$, mice are born healthy and viable, and in cardiac atrial myocytes deletion of IP_3R2 abolishes the positive ionotropic

and arrhythmogenic effect of endothelin implicating IP_3R in the initiation and perpetuation of atrial fibrillation. Little is known of IP_3R3 function, however in $IP_3R3^{-/-}$ mice taste perception was reported to be abnormal. IP_3R2 is also expressed in taste buds, but $IP_3R2^{-/-}$ mice have normal taste perception[140].

The association of IP_3R with human disease has been surprisingly rare, considering the numerous consequences of mutations in ryanodine receptors (RyR), the other major family of intracellular Ca^{2+} release channels[141]. Spinocerebellar ataxia 15 is the only human disease caused by inherited mutations of IP_3R, in this instance IP_3R1[142]. The lack of human disease associated with IP_3R suggests that the expression of multiple IP_3Rs in most cell types provides functional redundancy that is essential due to the importance of IP_3R-Ca^{2+} signaling in numerous and diverse cellular processes. Evidence for functional redundancy between the IP_3R channels in specific cells can be seen in $IP_3R2^{-/-}IP_3R3^{-/-}$ mice[143]. In these mice, exocrine secretion in the pancreas is severely impaired, resulting in the inability to digest solid food and early postnatal mortality. This defect was attributed to severely diminished Ca^{2+} signaling in acinar cells of the salivary glands of the pancreas, and required the loss of both IP_3R isoforms, as single knockout mice showed no abnormalities in exocrine pancreas function or impaired survival through early adulthood.

The role of IP_3R in TCR-induced Ca^{2+} signaling is well-established[144]. All three isoforms of IP_3R can be detected in T cells, and expression of IP_3R isoforms appears to be different between T cell subtypes[145-147]. Treatment of T cell lines with chemical inhibitor of IP_3R, or knockdown of IP_3R1, impairs

intracellular Ca^{2+} release and IL-2 production following TCR engagement[148,149]. Yet no defects in T cell development or function are observed in $IP_3R1^{-/-}$ mice[150], suggesting functional redundancy by IP_3R2 and IP_3R3. While genetic evidence has yet to demonstrate a role for IP_3R in T cell development or function, it is clear that Ca^{2+} signaling is vital to these processes[125,128]. Mutations in genes which activate Plcγ-IP_3R, such as Lck and Zap70[151], or are downstream of IP_3R, such as STIM1/ORAI1[152,153], lead to primary immunodeficiency.

Numerous studies have demonstrated an essential function for IP_3R in the initiation of apoptotic signaling in T cells in response to a wide variety of signals[154]. Ca^{2+} appears to regulate both cell survival and cell death through IP_3R, and in T cells Ca^{2+} signaling allows for the discrimination of TCR signals which lead to activation and those that lead to cell death[155,156]. Similar to T cells, expression of all three IP_3R isoforms has been reported in B cells and activation of Plcγ-IP_3R is a major signaling event essential for B cell receptor (BCR) function and Ca^{2+} release[157]. Ca^{2+} signaling, acting through Ca^{2+}-dependent transcription factors such as Nfat and NF-κB, influences B cell fate choices in the periphery in response to antigens and is thought to be critical to early B cell development. As this thesis is primarily concerned with T cell development and function, the role of Ca^{2+} in BCR signaling and development will not be addressed.

I.4.2 Store-operated Ca^{2+} channels

The binding of antigen to the TCR induces the depletion of intracellular Ca^{2+} stores through IP_3R-Ca^{2+} release. This depletion of ER Ca^{2+} leads to the

rapid and sustained influx of Ca^{2+} across the plasma membrane, a process termed store-operated Ca^{2+} entry (SOCE). SOCE is the main mechanism to increase intracellular Ca^{2+} concentrations in lymphocytes. The ion channels responsible for Ca^{2+} entry across the plasma membrane, known as Ca^{2+}-release activated Ca^{2+} channels (CRAC), are activated through binding to the ER Ca^{2+} sensor proteins stromal interaction molecule 1 (Stim1) and Stim2 upon ER Ca^{2+} depletion. The CRAC channel proteins Orai1, Orai2, and Orai3 each form Ca^{2+} channels with similar functional properties, but differ in their expression patterns and pharmacological properties. The importance of Ca^{2+} influx through SOCE is highlighted by the discovery of human patients with severe combined immunodeficiency (SCID) that lack CRAC channel function and SOCE[152]. Mutations in the ORAI1 gene were identified in these patients, and CRAC activity was genetically linked to ORAI1 function[158]. Orai1 is reported to be the predominant CRAC channel isoform in T lymphocytes, and no evidence to date indicates a role for Orai2 or Orai3 in immune cells. Like Orai1, the Stim proteins are essential for SOCE and are expressed in B and T lymphocytes[159,160]. Stim1-deficient T cells have severely impaired SOCE, while Stim2 deficiency had a smaller effect. Interestingly, in T cells lacking Stim1 the development of the αβ T lineage proceeds normally in the thymus, and mice lacking Orai1 also show normal T lineage development despite severely impaired T cell effector functions in the periphery[161,162]. The absence of a T cell development phenotype in Stim1-deficient T cells was not due to compensation by Stim2, as loss of both genes using CD4Cre or VavCre, active in DN4 thymocytes and hematopoietic stem

cells respectively, did not affect thymocyte development[159] (A. Rao, personal communication). Thus, it appears that while SOCE is essential for T cell function in the periphery, this mode of intracellular Ca^{2+} influx is dispensable for thymocyte development. One interesting hypothesis to explain the differential usage of Ca^{2+} signaling modes in developing versus mature T cells is that in the thymus DP thymocytes can encounter antigen gradually over a period of days, and are able to accumulate sufficient small Ca^{2+} signals over this period through repeated encounters with thymic stromal cells. These Ca^{2+} signals would be STIM-independent, presumably IP_3R-dependent, and allow DP thymocytes after accumulating sufficient signal to reach a threshold required for positive selection to differentiation into SP thymocytes. While in the periphery, T cells encounter antigen-presenting cells for hours rather than days and must accumulate a sufficient Ca^{2+} signal to trigger more stable interactions[163]. It is also possible that DP thymocytes use mainly SOCE-independent Ca^{2+} channels during positive selection.

I.4.3 SOCE-independent Ca^{2+} entry in T cells

Aside from SOCE, Ca^{2+} influx pathways through other ion channels have been described in T lymphocytes. Voltage-gated Ca^{2+} channels (Ca_v) are highly Ca^{2+} selective ion channels that mediate Ca^{2+} influx upon depolarization in excitable cells. These channels are composed of a pore-forming α1 subunit, and regulatory subunits $\alpha_2\delta$, β, and γ. Members of the L-type family of Ca_v channels ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$) are expressed in T cells, as well as the regulatory subunits β3 and β4. T cells that lack the β3 or β4 subunits show

impaired Ca^{2+} influx and cytokine production upon TCR engagement[164], and in β3-deficient $CD8^+$ T cells was associated with loss of $Ca_v1.4$ expression[165]. Of note, membrane depolarization does not activate Ca_v channels in T cells, implying the existence of alternative gating mechanisms. It is also unclear in T cells whether Ca_v channels function as Ca^{2+} channels or facilitate Ca^{2+} influx. Considerable debate surrounds L-type Ca_v channels in lymphocytes, from both a mechanistic and functional viewpoint, and further studies are needed to clarify these issues.

The P2x purinoreceptor channels are a family of non-selective ion channels activated by extracellular ATP and allow Na^+ and Ca^{2+} influx. The P2X receptors P2x1, P2x2, P2x6, and P2x7 are expressed in thymocytes, and thymocytes respond to extracellular ATP with increases in intracellular calcium[166]. TCR stimulation induces the release of ATP, which leads to activation of P2x7, which in turn induces Ca^{2+} influx. This autocrine ATP signaling in T cells via P2x7 is thought to amplify weak TCR signals[167]. Knockdown of P2x1, P2x4, and P2x7 decreases Ca^{2+} influx and cytokine production, as well as impaired NFAT activation[168,169]. P2x7 controls the differentiation of T_H17 and regulatory T cells, and thus appears to be pro-inflammatory[170].

The transient receptor potential (TRP) family of ion channels is grouped into seven subfamilies[171]. TRP channels are not ion specific and allow the flow of cations in general. T cells express TRP channels largely belonging to the TRPC and TRPM subfamilies. The function of Trpm2 has been studied extensively in T cells. Trpm2 is a Ca^{2+} permeable channel activated by several intracellular

second messengers, including ADP-ribose (ADPR), nicotinamide adenine dinucleotide (NAD^+), AMP, and cyclic ADPR (cADPR). Trpm2 expression is increased following TCR stimulation, and cADPR is released from the ER following TCR stimulation[172,173]. Trpm7 is a widely expressed non-selective cation channel that is equally permeable to Mg^{2+} and Ca^{2+}. Trpm7 is expressed in T cells, and is the only ion channel identified to date that is required for T lymphocyte development[174]. Trpm7-deficient thymocytes have a block at the DN stage of development, with significantly reduced DP and $CD4^+$ SP thymocyte numbers. Strikingly, in contrast to the requirement for Trpm7 in B cell Mg^{2+} influx and homeostasis[175], Mg^{2+} influx and total cellular Mg^{2+} were normal in Trpm7-deficient thymocytes. Thus, Trpm7 does not appear to be required for Mg^{2+} influx in T cells, and it is unclear how Trpm7 promotes T cell development. As Trpm7 is permeable to Ca^{2+}, it is possible that Trpm7 promotes Ca^{2+} influx in T cells. Several important mechanistic questions remain regarding Trpm7, including how Trpm7 is activated in T cells, and whether Trpm7 activity is linked to TCR signaling or functions in parallel with TCR signaling to promote thymocyte development. Furthermore, Trpm7 possesses kinase activity that is independent of its ion channel activity.

I.5 Regulation of T cell development by Ca^{2+}

The influx of calcium into the cell activates several downstream pathways and leads to a variety of cellular responses. These include the Ca^{2+}-dependent phosphatase calcineurin and its target the transcription factor Nfat, CaMK and its target Creb, Mef2, PKC, NF-κB, and other signaling pathways such as Ras-Mapk

in combination with DAG. Ca^{2+} responses have traditionally been characterized as short-term, rapid response that do not require changes in gene transcription, and long-term modifications that do require new gene transcription such as changes in cell identity. Yet this model is overly simplistic, as short-term increases in Ca^{2+} are able to influence the activity of NF-κB[176]. In the case of CaMK, which regulates the transcriptional activity of Creb and Mef2, a short-term increase in Ca^{2+} can influence CaMK activity for a prolonged period of time due to auto-phosphorylation by CaMK upon activation[177]. This allows CaMK to "remember" a transient increase in Ca^{2+} and function independent of Ca^{2+}-calmodulin and influence transcription even after Ca^{2+} has returned to basal levels.

The calcineurin-Nfat pathway is one of the best characterized Ca^{2+}-dependent signaling pathways. Calcineurin consists of two regulatory subunits, Cnb1 and Cnb2, and three catalytic subunits. Cnb1 is ubiquitously expressed, while Cnb2 is restricted to germline cells. The transcription factor Nfat consists of a family of five transcription factors, Nfat1-5, and are all expressed in immune cells except for Nfat3. Activation of the phosphatase activity of calcineurin by binding to Ca^{2+}-calmodulin leads to de-phosphorylation of Nfat, and rapid translocation to the nucleus. Nfat-driven gene expression is highly dependent upon sustained intracellular Ca^{2+} levels and calcineurin activity, as Nfat is continuously exported from the nucleus due to phosphorylation.

Early studies implicated a role for calcineurin-Nfat in T cell development by chemically inhibiting calcineurin activity[178], which impeded the development of

DP thymocytes to the SP stage. Nfat was also found to translocate to the nucleus upon stimulation of TCR in mature T cells. Examination of pre-TCR signaling revealed a prominent increase in Ca^{2+} mobilization[176]. Nfat and NF-κB transcriptional activity were induced upon pre-TCR signaling, and chemical inhibition of calcineurin in an *ex vivo* model of thymocyte development delayed the appearance of DP thymocytes, suggesting pre-TCR Ca^{2+} signaling through Nfat was important for early thymocyte development. A number of studies examining the individual roles of Nfat in T cell development have revealed essential functions for Nfat at late stages of thymocyte development[125]. At early stages thymocyte development, the role of Nfat remains controversial, one group reported a requirement for Nfat2 at the DN-to-DP transition and another concluded that no changes in thymocyte development were apparent[179,180]. Reasoning that a disruption of calcineurin would reveal a more complete understanding of the roles of Ca^{2+} and Nfat in thymocyte development, a T cell specific deletion of the regulatory subunit of calcineurin via LckCre was generated. Calcineurin b1 deficient thymocyte development is arrested at the DP stage, and is absolutely required for the development of SP T cells. An essential role for calcineurin in positive selection was identified as the cause of the complete block of T cell development at the DP stage. Calcineurin-Nfat appeared to be dispensable for negative selection. A specific defect in Erk activation was also noted, and a minor defect in the progression of DN3 thymocytes to the DN4 stage was observed. This same group went on to describe the mechanism of calcineurin-Nfat action in positive selection[155]. The calcineurin-Nfat pathway

lowers the threshold for Erk activation in pre-selection DP thymocytes. This allows weak TCR signals to induce positive selection. In the absence of calcineurin, the TCR signal intensity needed for positive selection overlaps with that needed for negative selection. To demonstrate this, thymocytes deficient for calcineurin and Bim[181] (which cannot undergo negative selection) were found to undergo positive selection in response to strong negatively selecting TCR signals that would normally induce apoptosis. Conditional inactivation of calcineurin B1 with Mx-1Cre, which is active in HSCs, provided further insight into the role of calcineurin-Nfat prior to the DP stage[182]. In these mice, B cell development is normal and T cell development is blocked at the DP stage as described using LckCre. Yet when placed in a competitive setting with wild-type progenitors, T cell development is blocked at β-selection and B cell development is blocked at the pre-B cell stage, indicative of defects in pre-TCR and pre-BCR signaling. Calcineurin appeared not to be required for γδ T lineage development nor the development of the myeloid lineages. Thus, calcineurin-Nfat signaling has a non-redundant role in the regulation of developmental checkpoints in lymphocytes.

The protein kinase C (PKC) proteins represent a subfamily of the protein kinase A, protein kinase G, and protein kinase C (ACG) serine-theronine kinases[183]. The PKC subfamily includes classical PKC isoforms (α, βI, βII, and γ), which are regulated by Ca^{2+}, DAG, and phospholipids; novel PKC isoforms (δ, ε, η, and θ), which are regulated by DAG and phospholipids; and atypical PCK isoforms which are insensitive to Ca^{2+} and DAG. PKC proteins are the chief targets of DAG in lymphocytes, and PKCβ and PKCθ are essential for NF-κB

activation[184]. In mature T cells, PKCθ is the predominant isoform, and while NF-κB activation is defective in PKCθ$^{-/-}$ mice, T cells develop normally[185]. In contrast to PKCθ, classical Ca^{2+}-dependent PKC isoforms have been implicated in early thymocyte development. Expression of a constitutively active PKCα promotes the differention of DN thymocytes to the DP stage, and bypasses the requirement for TCRβ rearrangement. The PKC target gene Creb was activated upon pre-TCR signaling, and expression of a dominant-negative kinase-dead PKCα impaired progression of DN thymocytes to the DP stage. PKC was also implicated in enforcing allelic exclusion at the TCRβ locus, as constitutively active PKC antagonizes TCRβ VDJ recombination. These data were interpreted as a demonstration that classical PKC isoforms were required for early thymocyte differentiation and expansion. PKCα and PKCβ are the only conventional PKC isoforms expressed in the immune system, yet PKCα$^{-/-}$ and PKCβ$^{-/-}$ mice show no defects in early T cell development[186,187], suggesting functional redundancy between the two isoforms. A role for PKC in pre-TCR mediated thymocyte survival has also been reported[188]. Using transformed thymocyte cell lines of defined developmental stage to dissect events in pre-TCR expressing cells, PKC was found to induce the transcription of the pro-survival Bcl-2 family member Bcl2a1 downstream of the pre-TCR. PKC induction of Bcl2a1 was found to be Plcγ- and Ca^{2+}-dependent, and required the activity of NF-κB. Survival of pre-TCR^{+} thymocyte progenitors upon disruption of Ca^{2+} signaling or chelation of intracellular Ca^{2+} was severely impaired, and this requirement was described at stages prior to β-selection. Thus, PKC also appears to be required for thymocyte

survival downstream of the pre-TCR-mediated Ca^{2+} release. These data were consistent with prior reports linking NF-κB to pre-TCR mediated thymocyte survival[189,190]. Interestingly, Ca^{2+} signaling is also involved in negative selection. The transcriptional induction of Bim, which is required for negative selection[181], in DP thymocytes requires the Ca^{2+}-dependent activation of PKC through the TCR[156].

T cells express multiple Ca^{2+}/calmodulin-dependent protein kinases (CaMK). However the role of CaMK in thymocyte development remains largely unexplored. Of the CaMK family member present in T cells, only CaMKIV appears to be expressed at early stages of thymocyte development and is highest in DP thymocytes. However CaMKIV$^{-/-}$ mice display only mild defects in positive selection[191].

Given the importance of IP_3R to Ca^{2+} signaling in T cell development, and the ability of Ca^{2+} to regulate multiple signaling pathways and transcriptions factors, the biological roles of IP_3R are of considerable interest. Additional functions for Ca^{2+} and individual IP_3R channels are likely to be uncovered using conditional knockout models to dissect early developmental phenotypes.

Chapter 1 Materials and methods

1.1 Mice

The generation of Itpr2^{flox} , Itpr2^{-}, and Tie2Cre mice have been described previously[139,192]. Rag2$^{-/-}$ mice were purchased from Taconic. SCID-Beige mice were purchased from Charles River Laboratories. Rosa26YFP mice were purchased from The Jackson Laboratories. Itpr1$^{fl/fl}$ and Itpr3$^{fl/fl}$ mice were generated as described in Chapter 2.3.1. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Mice were identified by PCR of tail DNA (Itpr1-P1 5'-gagaaaggttaacagcactggatt-3' and Itpr1-P2 5'-actgggcaggcatatatagttagc-3', Itpr2-P1 5'-gctgtgcccaaaatcctagcactg-3' and Itpr2-P2 5'-catgcagaggtcgtgtcagtcatt-3', Itpr3-P1 5'-cctgcctccgtttgttacat-3' and Itpr3-P2 5'-ctgtgcagagtagcggttca-3').

1.2 Generation of *Itpr1*$^{fl/fl}$, *Itpr3*$^{fl/fl}$, *Itpr1*$^{-/-}$, and *Itpr3*$^{-/-}$ mice

The gene targeting strategy used to generate *Itpr1*$^{fl/fl}$, *Itpr3*$^{fl/fl}$, *Itpr1*$^{-/-}$, and *Itpr3*$^{-/-}$ mice is as described in Chapter 2.3.1. Mouse ES cells used were derived from a 129/sv strain. All *Itpr* conditional knockout mice were outcrossed and maintained on an outbred black swiss background.

1.3 Cell preparations, flow cytometry, and cell sorting

Thymocyte suspensions were prepared in PBS, filtered through a 40μm nylon mesh cell strainer (BD Biosciences), and analyzed individually. Intracellular staining was done by 2% paraformaldehyde fixation followed by permeabilizing in

05% saponin. Apoptosis was assessed by Annexin-V staining according to manufacturer's instructions (Annexin-V FITC Kit, BD Pharmingen). Antibodies were purchased from eBioscience or BD Pharmingen. Antibodies were directly conjugated to biotin, fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy7, peridinin-chlorophyll-protein complex (PerCP), PerCP-Cy5.5, allophycoerythrin (APC), or APC-Cy7. The lineage (lin) cocktail included antibodies against B220 (RA3-6B3), CD19 (1D3), Ter119 (Ter-119), CD11b/Mac1 (M1/70), Gr1 (8C5), CD11c (HL3), pan-NK/CD49b (DX5), γδ-TCR (GL-3), CD3 (2C11), and CD4 (GK1.5). Additional antibodies used include antibodies against CD8α (53-6.7), TCRβ (H57), c-Kit (2B8), CD25 (PC61.5), CD27 (LG.7F9), CD44 (IM7). Biotinylated antibodies were stained with a streptavidin-conjugated secondary antibody. Cell sorting was performed on a FACSAria I/II (BD Biosciences). Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences), LSR II (BD Biosciences), and FACSCanto (BD Biosciences). Data were analyzed using FlowJo (TreeStar).

1.4 *In vivo* BrdU labeling

Mice were injected intraperitonealy (i.p.) with 200ul of 10mg/mL bromodeoxyuridine (BrdU) in sterile 1X PBS for a total of 1mg BrdU/mouse. Analysis of thymocytes was done 4 hours post-injection. Thymocytes were stained with antibodies, and BrdU incorporation was assessed by flow cytometry according to manufacturer's instructions (BrdU-APC Kit, BD Pharmingen).

1.5 Western blot analysis

Thymocytes were lysed in SDS loading buffer and fractionated by SDS-PAGE gel electrophoresis, transferred to nitrocellulose membrane, and immunoblotted with antibodies purchased from Cell Signaling Technology, Sigma-Aldrich, or Santa Cruz Biotechnology. Antibodies used for protein analysis include total Notch1, cleaved Notch1 (V1744/NICD1), Notch3, c-Myc, Ikaros, Chd3, Chd4, Rbap46, Mta1, Mbd3, Hdac1, Hdac2, Ezh2, Suz12, Tcf-1, Lef1, Sox13, E47, HEB, Id2, Id3, total Erk1/2, phopho-Erk1/2, total Akt1/2, phosph-Akt1/2, Bim, Bcl2, Mcl-1, Bcl-xL, and Gapdh. HRP-conjugated secondary antibodies were used to detect antibody-protein complexes on nitrocellulose membranes as per manufacturer's instructions (Pierce).

1.6 Quantitative RT-PCR

RNA was purified from sorted cells using the RNeasy MiniKit (Qiagen) and reverse transcribed to cDNA using SuperScript II Kit (Invitrogen). Real-time PCR was performed with Brilliant II SYBR Green PCR Master Mix (Stratagene) or SSoFast EvaGreen Supermix (Bio-Rad) and analyzed on MX-Pro (Stratagene) or Roche LightCycler (Roche). Relative transcript abundance was determined by ΔΔCt or ΔCt method after normalization with 18s. All samples were run in duplicate. Error bars represent S.D. Primers were designed using PrimerBank software[193]. The following primer pairs were used for PCR: Itpr1-for 5'-gggtcctgctccacttgac-3' and Itpr1-rev 5'-ccacatcttggctagtaaccag-3', Itpr2-for 5'-cctctacattggggacatcgt-3' and Itpr2-rev 5'-ggcacaccttgaacaggca-3', Itpr3-for 5'-gggcgcagaacaacgagat-3' and Itpr3-rev 5'-gaagttttgcaggtcacggtt-3', Tcf-1-for 5'-

caaggcagagaaggaggctaag-3' and Tcf-1-rev 3'-ggcagcgctctccttgag-3', Sox13-for 5'-gatgcaccaacgctaaagc-3' and Sox13-rev 5'-ttgcggttgaagtccaggc-3', Hes1-for 5'-tccttggtcctggaatagtgcta-3' and Hes1-rev 5'-actgagcagttgaaggtttattatgtct-3', 18s-for 5'-taaaggaattgacggaaggg-3' and 18s-rev 5'-ctgtcaatcctgtccgtgtc-3'.

1.7 Microarray gene expression studies

RNA was extracted from sorted cells and submitted to UCSD Microarray Core Facility, and the quality of RNA was tested with an Agilent RNA bioanalyzer. All protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. GeneChips used were Affymetrix Mouse Gene1.0ST. Data and statistical analysis were analyzed using Partek Genomics Suite (Partek).

1.8 OP9-DL1 cell culture

OP9-DL1 cells were provided by the Traver laboratory at UCSD. Thymocyte progenitors (DN3) were plated on confluent OP9-DL1 monolayers and grown with IL-7 and Flt3L, and fresh media and cytokines were provided every 3 days for a period of 7 days. Cells were analyzed at day 7 by flow cytometry.

1.9 Transplantation of leukemic T cells

Control (*Itpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$) and transformed T lymphocytes from thymic tumors (*Tie2CreItpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$) were collected and transplanted (2 million cells per mouse) retro-orbitally into young male (4-6 week) sub-lethally irradiated (300 cGy) SCID-Beige mice. Mice were observed over a period of 8 weeks for signs of morbidity, and analyzed post-mortem for the presence of tumors.

1.10 Histology and hematology

Tissues were harvested, fixed in 4% paraformaldehyde, and placed in 20% sucrose solution in PBS overnight. Samples were frozen in OCT freezing medium and stored at -80C until sectioned at 10μm using a microtome. Cryosections were washed with PBS and stained with hematoxylin and eosin following standard protocols. For peripheral blood smears and complete blood counts, blood was collected retro-orbitally in EDTA-containing microtainer tubes (BD Biosciences) and processed according to standard protocols at the UCSD Hematology Core Facility.

1.11 Statistical analysis

Kaplan-Meier analysis was used to compare overall survival among different genotypes. Statistical significance was assessed by the two-tailed Student's *t*-test.

Chapter 2 Generation and characterization of IP_3R triple knockout mice

2.1 Introduction

Calcium ions (Ca^{2+}) function as universal second messengers in all eukaryotic cells, including cells of the immune system. Ca^{2+} signals are crucial for peripheral B and T lymphocyte activation and effector functions, influence thymocyte selection and motility in the developing thymus, and control pro-inflammatory responses in innate immune cells. Yet the role of Ca^{2+} signaling in hematopoietic development is not well understood. The inositol triphosphate receptor (IP_3R) is a Ca^{2+} permeable ion channel encoded by a family of three genes[135] (*Itpr1*, *Itpr2*, and *Itpr3*). While the IP_3R is ubiquitously expressed, the three channel isoforms have distinct and overlapping expression patterns with most cells expressing more than one type. Receptor diversity is significant, as individual IP_3Rs display extensive alternative splicing, and individual IP_3R isoforms have many unique protein interactions. The IP_3R channel exists as homo- and heterotetramers, with allowing different IP_3R isoforms to be present within one IP_3R ion channel. The diversity of IP_3R expression suggests that cells require distinct IP_3R to regulate specific biological functions, yet the functional implications of IP_3R diversity remain largely unexplored. Complicating the study of IP_3Rs, functional redundancy between IP_3R isoforms and postnatal mortality in the absence of single and multiple *Itpr* genes has been observed[138,194].

Given the importance of IP_3R to Ca^{2+} signaling to cells of the immune system, and the ability of Ca^{2+} to regulate multiple signaling pathways and transcriptions factors, the biological roles of IP_3R are of considerable interest. Additional functions for Ca^{2+} and individual IP_3R channels are likely to be uncovered using conditional knockout models to dissect early developmental phenotypes. Therefore, we will examine the requirement for IP_3R signaling in hematopoietic lineages by generating conditional *Itpr* double and triple knockout mice utilizing Tie2Cre, which mediates excision in hematopoietic stem cells throughout ontogeny[192].

2.2 Chapter aims

Through the generation of a hematopoietic-specific conditional knockout of all three Itpr genes, I aimed to characterize the combined role IP_3R in hematopoiesis. Analysis of the role of the IP_3R's in lymphoid and myeloid cell development will be assessed by immunostaining and flow cytometry. Combined, these studies are expected to reveal new functions for these ion channels in hematopoietic cell development.

2.3 Results

2.3.1 Targeting and generation of *Itpr1*$^{fl/fl}$, *Itpr3*$^{fl/fl}$, *Itpr1*$^{-/-}$, and *Itpr3*$^{-/-}$ mice

Conditional alleles for *Itpr1* and *Itpr3* were generated by the Chen laboratory at UCSD. The targeting strategies will be described here. The generation of conditional and global null alleles for *Itpr2* have been described previously[139]. Targeting constructs were generated by flanking exon 5 of *Itpr1* or

exon 3 of *Itpr3* with Cre-recombinase recognition sites (LoxP sites) (Figure 2.1). Deletion of exon 5 of *Itpr1* or exon 3 of *Itpr3* creates both a frameshift mutation and premature stop codon, such that any residual transcript is subjected to nonsense-mediated decay and in the event decay does not occur the residual protein product is truncated and non-functional. The targeting vectors also contained a Neo expression cassette flanked by FLPe recombinase recognition sites (FRT sites) for positive selection and enrichment of successfully targeted ES cells. ES cells were electroporated and selected for neomycin resistance using standard techniques. Genomic DNA (gDNA) isolated from neomycin positive ES cell clones were screened by DNA Southern blot analysis to detect correctly targeted clones. To detect successful targeting of the *Itpr1* targeting vector, the Itpr1 5' probe (Figure 2.1a) was hybridized with Acc65I digested gDNA, detecting either a 10.3kb wild type or 5.3kb targeted allele (Figure 2.1b). To detect successful targeting of *Itpr3* targeting vector, the Itpr3 5' probe (Figure 2.1a) was hybridized with NotI digested gDNA, detecting either a 13.2kb wild type or 5.5kb targeted allele (Figure 2.1d). Successfully targeted heterozygous mouse ES cells were used to generate mice heterozygous for targeted *Itpr1* and *Itpr3* alleles by blastocyst injection. These mice were bred to generate homozygous cohorts and are henceforth denoted as $Itpr1^{fl/fl}$ and $Itpr3^{fl/fl}$. Null alleles for *Itpr1* ($Itpr1^{-}$) and *Itpr3* ($Itpr3^{-}$) were also generated by crossing with mice expressing a ProtamineCre transgene, where Cre expression occurs in the male germ line, allowing for germ line recombination and transmission of the recombined allele[195]. Homozygous $Itpr1^{-/-}$ and $Itpr3^{-/-}$ mice were generated and

viable at birth. Loss of Itpr1 and Itpr3 protein was confirmed by western blot analysis in neonatal brain and placental tissue of *Itpr1*$^{-/-}$ (Figure 2.1c) and *Itpr3*$^{-/-}$ (Figure 2.1e) mice, respectively.

2.3.2 Strategy for the generation of IP_3R-TKO mice carrying the hematopoietic specific Tie2Cre transgene

We examined the requirement for IP_3R-Ca^{2+} signaling in hematopoiesis by generating conditional triple knockout mice (TKO). *Itpr2* conditional and null alleles have been described previously[139]. *Itpr1*$^{fl/fl}$, *Itpr2*$^{fl/fl}$, and *Itpr3*$^{fl/fl}$ mice were crossed to generate triple homozygous floxed mice (*Itpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$). Triple homozygous floxed mice were then crossed with a Tie2Cre transgenic line, and crossed to generate compound heterozygotes (*Tie2CreItpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/+}$)[192]. Subsequent generations were PCR genotyped for floxed alleles and for the presence of Tie2Cre (Figure 2.2a). Conditional triple knockout mice (*Tie2CreItpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$), herein referred to as IP_3R-TKO, were generated through breeding triple homozygous floxed mice with compound heterozygotes. IP_3R-TKO mice were born at expected mendelian ratios and viable at birth.

Expression of Cre in the Tie2Cre line is controlled by a Tie2 transgene, and is active in all hematopoietic stem cells throughout both embryonic and adult life. This strategy should result in the highest possible excision efficiency for all six conditional alleles prior to the earliest stages of lymphocyte development. Tie2Cre activity at the HSC level was demonstrated by crossing with the Rosa26YFP Cre reporter line[196] and analyzing YFP (yellow fluorescent protein) expression in total bone marrow fraction and the LSK (Lineage$^-$, Sca1$^+$, c-Kit$^+$)

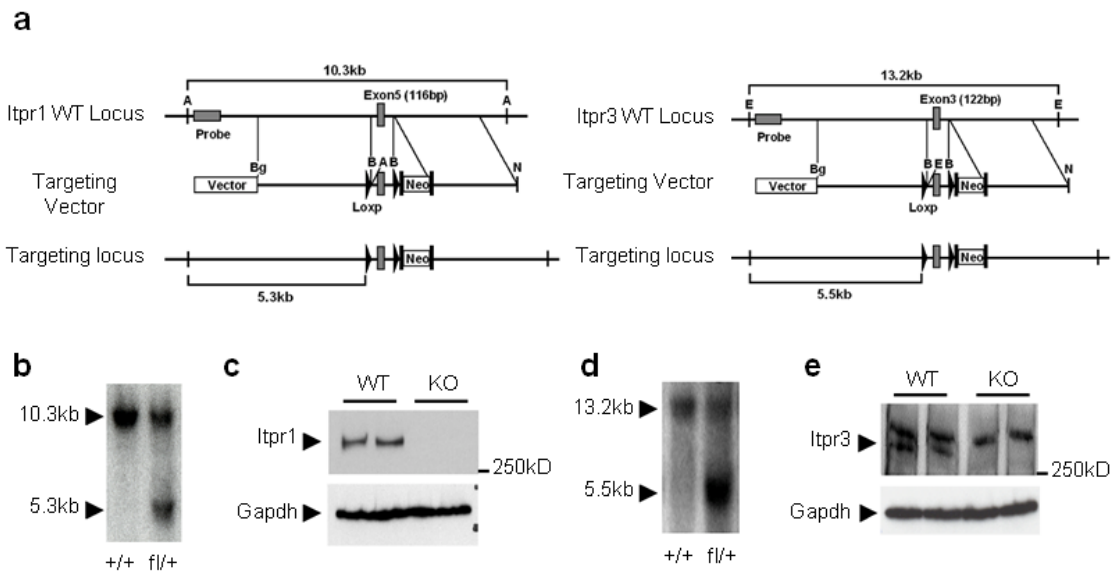


Figure 2.1 Gene targeting strategies to generate *Itpr1 and Itpr3* knockout mice.

(**a**) Targeting strategies for *Itpr1* and *Itpr3* genes. A restriction map of the relevant genomic regions (top), the targeting vectors (middle) and the targeted locus after recombination (bottom) is shown. The targeting construct was generated by flanking exon 5 of *Itpr1* (**a**) and exon 3 of *Itpr3* (**d**) with loxP sites, while frt sites flank the Neo-cassette. A, *Acc65I*; B, *BamHI*; Bg, *BglII*; E, *EcoRV*; N, *NotI*. Neo represents the neomycin resistance gene; while the arrowheads represent LoxP sites and the long boxes represent frt sites. (**b**) Detection of wild type (WT) and targeted alleles for the *Itpr1* gene by DNA Southern blot analysis. DNAs isolated from neo positive electroporated ES cell clones were digested with *Acc65I* and analyzed by DNA blot analysis with the probe as shown in (**a**). The 10.3- and 5.3-kb bands represent the WT and targeted alleles, respectively. (**c**) Detection of Itpr1 by protein analysis. Proteins were prepared from the brains of neonatal WT and Itpr1-knockout (KO) mice, and analyzed with Itpr1 antibodies. (**d**) Detection of WT and targeted alleles for the *Itpr3* gene by DNA Southern blot analysis, DNAs were digested with *NotI*. The 13.2- and 5.5-kb bands represent WT and targeted alleles, respectively. (**e**) Detection of Itpr3 by protein analysis. Proteins were prepared from the placenta of WT and Itpr3-null (KO) mice, and analyzed with Itpr3 antibodies.

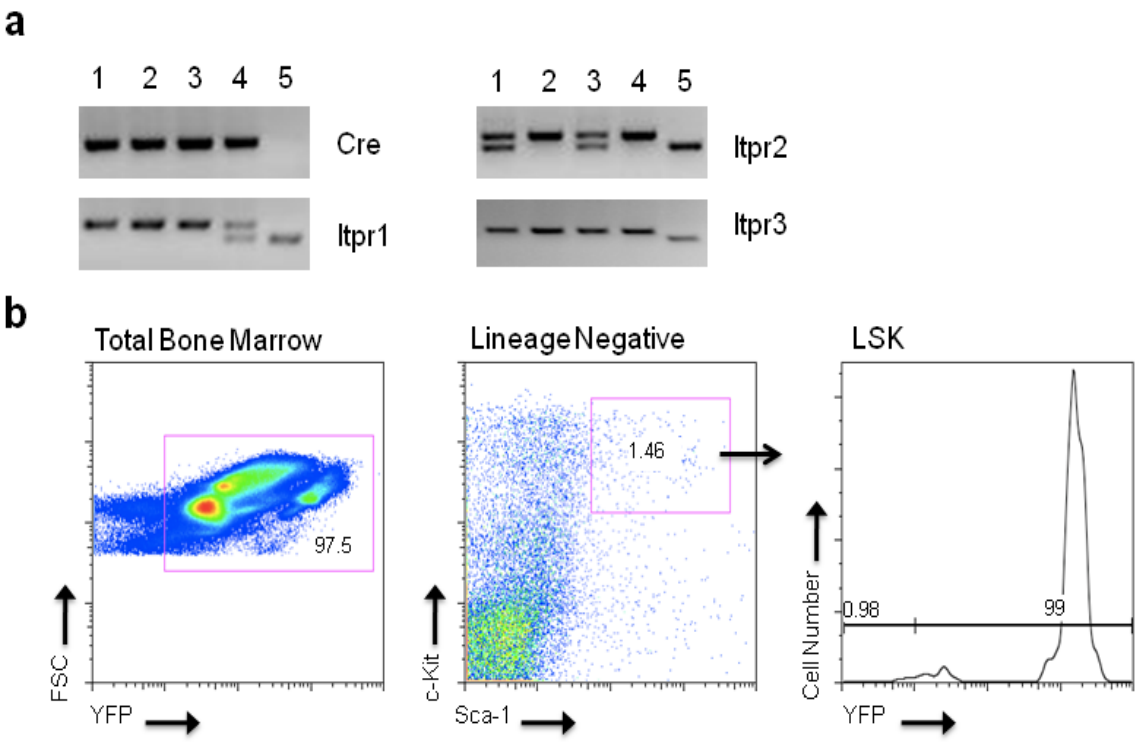


Figure 2.2 Genotyping of IP$_3$R-TKO and Tie2Cre activity in HSCs.
(**a**) Genotyping PCR of IP$_3$R-TKO DNA using primers specific for the Tie2Cre transgene and conditional alleles for *Itpr1*, *Itpr2*, and *Itpr3*. Lanes; (1) *Tie2Cre;Itpr1*$^{fl/fl}$*Itpr2*$^{fl/+}$*Itpr3*$^{fl/fl}$, (2) *Tie2Cre;Itpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$ (IP$_3$R-TKO), (3) *Tie2Cre;Itpr1*$^{fl/fl}$*Itpr2*$^{fl/+}$*Itpr3*$^{fl/fl}$, (4) *Tie2Cre;Itpr1*$^{fl/+}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$, and (5) wild-type. (**b**) Rosa26YFP Cre reporter activity in total bone marrow and LSK fraction of adult Tie2Cre mice. Data are representative of a minimum of three independent experiments.

fraction (Figure 2.2b). The LSK fraction contains all hematopoietic stem cell activity in the mouse. The total YFP^+ population of the bone marrow consistently exceeded 95%. Similar results were observed within the LSK fraction, demonstrating Tie2Cre activity in the hematopoietic stem cell compartment and in virtually all hematopoietic cells of the bone marrow.

Cre recombinase has been reported to have cytotoxic effects on mammalian cell lines and transgenic mouse models[197,198]. The Tie2Cre transgene is expressed at the earliest stages of definitive hematopoiesis. The presence of the Tie2Cre transgene in wild-type (WT) mice did not affect thymic T lymphopoiesis, B lymphopoieis, or myeloid cell development in the bone marrow (Figure 2.3). Similarly, the development of the aforementioned hematopoietic lineages is normal in *Itpr* triple homozygous floxed mice (*Itpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$). From herein targeted mice that carry *Itpr* conditional alleles in the absence of Tie2Cre will be referred to as control.

2.3.3 Incomplete deletion of *Itpr* in IP_3R-TKO thymocytes

Cre expression beginning in the hematopoietic stem cell via the Tie2Cre transgene should result in conditional LoxP recominbation and deletion of all six *Itpr* conditional alleles present in IP_3R-TKO mice. To detect successful Cre recombinase excision of *Itpr* conditional alleles, we assessed T cells of Control and IP_3R-TKO mice for *Itpr* mRNA expression (Figure 2.4a). Quantitative reverse-transcription PCR of sorted DN and ISP thymocyte progenitors revealed a significant reduction (<10% of Control mRNA levels) of *Itpr1*, *Itpr2*, and *Itpr3*

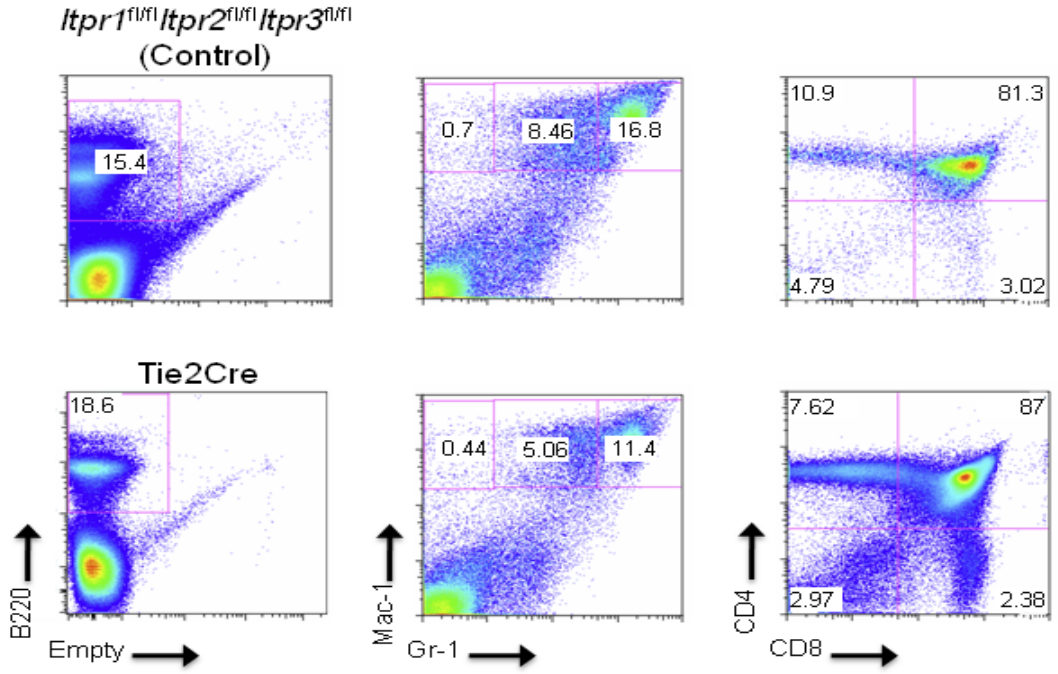


Figure 2.3 Normal hematopoietic development in Tie2Cre and Control mice. Flow cytometry analysis of Control (*Itpr1*$^{fl/fl}$*Itpr2*$^{fl/fl}$*Itpr3*$^{fl/fl}$) and Tie2Cre mice for bone marrow expression of B lymphocyte marker B220 and myeloid markers Mac-1 and Gr-1, and for thymocyte for expression of T lymphocyte markers CD4 and CD8. Data are representative of a minimum of three independent experiments.

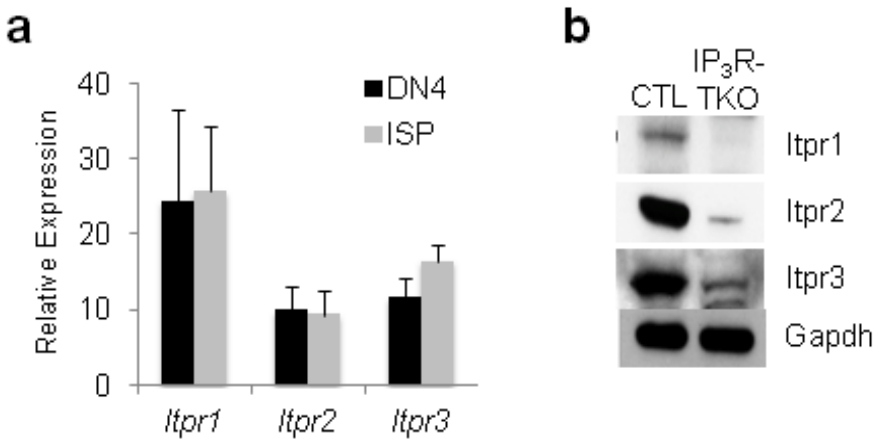


Figure 2.4 Conditional deletion of *Itpr* in mouse thymocytes.
(**a**) Neonatal gene expression of *Itpr* in IP_3R-TKO as measured by qRT-PCR in sorted DN4 and ISP thymocytes normalized to *18s* rRNA expression. Values represented are fold reduction relative to Control (CTL). (**b**) Western blot analysis of Itpr expression from ED 17.5 thymocytes from Control and IP_3R-TKO mice. Data are representative of three independent experiments.

mRNA transcripts. Deletion of IP_3R protein was then evaluated by western blot using whole cell protein extracts of total thymocyte populations at embryonic day 17.5 (Figure 2.4b). IP_3R protein levels were significantly reduced or absent (<15% of Control protein levels), confirming that Tie2Cre results in significant deletion of the targeted proteins in IP_3R-TKO thymocytes. However, residual Itpr2 and Itpr3 protein were detected suggesting deletion of all *Itpr* alleles was incomplete.

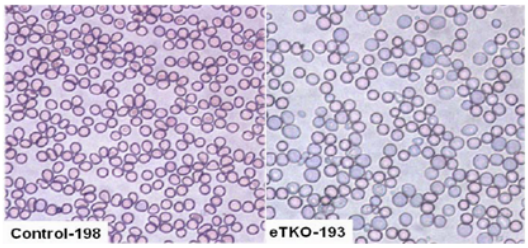
2.3.4 Multiple defects in hematopoietic development of IP_3R-TKO mice

To determine the effect of loss of all IP_3R alleles on hematopoietic lineage development, we analyzed adult (4-8 weeks) peripheral blood by automated complete blood count (Figure 2.5a). Peripheral blood (PB) leukocytes counts revealed moderate leukopenia, with a 20% reduction in white blood cell (WBC) cellularity of IP_3R-TKO mice as compared to Control. Absolute lymphocyte (LY) cellularity was also reduced (>2.2 fold as compared to Control). Neutrophil (NE) cellularity, while moderately increased, was within the normal physiological range. Other myeloid lineages such as basophils (BA), eosinophils (EO), and monocytes (MO) displayed little to no change in cellularity. Interestingly, IP_3R-TKO mice showed several signs of macrocytic anemia including; moderate decreases in red blood cell (RBC) number, hematocrit (HCT), and total hemoglobin (Hb), as well as increased red cell distribution width (RDW) and mean cell volume (MCV). To further characterize the changes in leukocyte and red blood cell distribution, we examined Wright Giemsa stained PB blood smears by light microscopy (Figure 2.5b). Extensive polychromasia was observed in

a

		Control	IP_3R-TKO	
Leukocytes	WBC #	8.12 ± 1.15	6.00 ± 1.12	P<0.01
	NE #	1.84 ± 0.33	2.84 ± 0.18	P<0.01
	LY #	6.06 ± 1.25	2.70 ± 0.92	P<0.01
	MO #	0.20 ± 0.06	0.43 ± 0.15	P<0.01
	EO #	0.02 ± 0.02	0.03 ± 0.01	P>0.05
	BA #	0.00 ± 0.00	0.01 ± 0.01	P>0.05
	NE %	23.3 ± 6.5	48.4 ± 6.8	P<0.01
	LY %	74.1 ± 6.6	44.1 ± 7.1	P<0.01
	MO %	2.43 ± 0.52	7.05 ± 2.08	P<0.01
Erythrocytes	RBC	9.34 ± 1.04	7.10 ± 0.50	P<0.01
	Hb	11.8 ± 1.3	9.90 ± 0.71	P<0.01
	HCT	43.6 ± 3.7	40.3 ± 3.2	P>0.05
	MCV	46.9 ± 2.7	56.8 ± 4.2	P<0.01
	MCH	12.6 ± 1.1	14.0 ± 1.1	P<0.05
	MCHC	27.0 ± 1.7	24.6 ± 1.1	P<0.01
	RDW	20.0 ± 1.7	25.5 ± 1.2	P<0.01
Thrombocytes	PLT	653 ± 110	708 ± 96	P>0.05
	MPV	4.84 ± 0.18	4.60 ± 0.14	P<0.05

b



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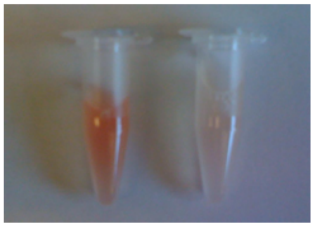


Figure 2.5 Lymphopenia and anemia in adult IP_3R-TKO peripheral blood. (**a**) Complete peripheral blood count of Control and IP_3R-TKO mice. Age of animals at collection was 6-8 weeks. (n>10) Significance determined by student's t-test. (**b**) Peripheral blood smears of Control and IP_3R-TKO. Polychromatic reticulocytes appear as large spherical cells with various shades of blue and pink present in cytoplasm. (**c**) Total bone marrow collected from Control and IP_3R-TKO. Data are representative of two independent experiments. White blood cell (WBC), neutrophils (NE) basophils (BA), eosinophils (EO), monocytes (MO), lymphocytes (LY), red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV). Significant determined by student's t-test.

IP_3R-TKO smears, appearing as a dimorphic population of polychromatic reticulocytes (light blue) that vary in size and lack the cleared central pallor and uniform pink color of mature erythrocytes. In contrast, Control smears displayed mature pink erythrocytes that were uniform in color and size with a cleared central pallor. The appearance of polychromasia in the PB is consistent with a physiological attempt to compensate for anemia. To determine the origin of the anemia, we examined the bone marrow (BM), the primary site of murine erythropoiesis. IP_3R-TKO BM appeared dull pink, a shift in color from bright red marrow observed in Control mice, suggesting a potential defect in erythroid maturation within the BM of IP_3R-TKO mice (Figure 2.5c).

We next sought to determine the origin of the lymphopenia observed in IP_3R-TKO mice. Adult PB leukocytes from Control and IP_3R-TKO mice were analyzed by flow cytometry (Figure 2.6). Strikingly, flow cytometric analysis of adult IP_3R-TKO PB revealed a significant decrease (>5 fold reduction as compared to Control) in the percentage of both $B220^+$ B lymphocytes and $CD3^+$ T lymphocytes. In contrast, the percentage of myeloid lineage cells such as macrophages ($Mac\text{-}1^+Gr\text{-}1^-$) and neutrophils ($Mac\text{-}1^+Gr\text{-}1^+$) were increased in a compensatory fashion (>4 fold increase as compared to Control). Collectively, these data suggest that loss of the IP_3R's inhibits multiple aspects of hematopoietic cell development. Myeloid cell development, however, appears relatively unaffected.

2.3.5 Ineffective erythropoiesis in the bone marrow and extramedullary erythropoiesis in spleen of IP_3R-TKO mice

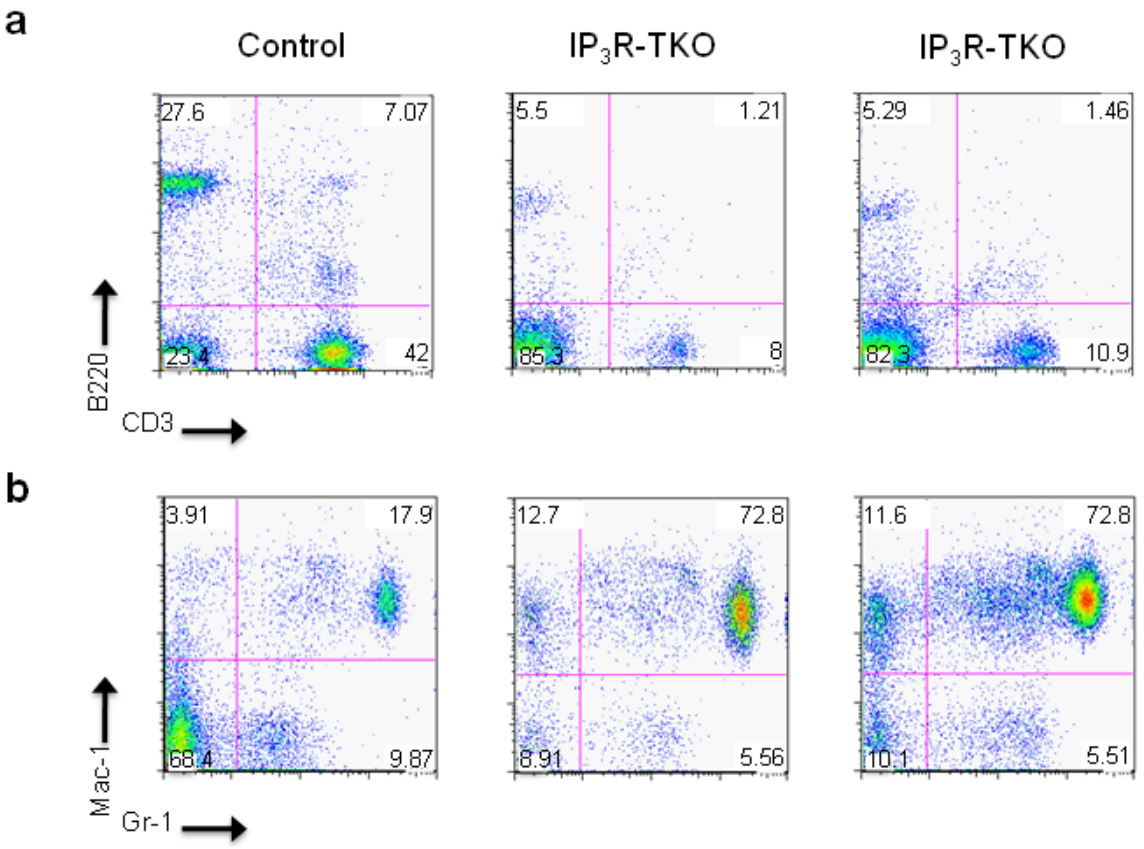


Figure 2.6 Severe reduction of T and B cells in IP_3R-TKO peripheral blood. Peripheral blood leukocytes from Control and IP_3R-TKO mice were analyzed by flow cytometry for expression of B and T lymphocyte markers (a) B220 and CD3, and myeloid markers (b) Mac-1 and Gr-1. Data are representative of three litters analyzed (n>10).

Given the appearance of polychromasia in the PB, anemia, and the change in the appearance of harvested BM, we examined erythroid maturation in the BM of IP_3R-TKO mice. We first noted that the cellularity of adult (6-8 weeks) BM in IP_3R-TKO mice was similar to Control (Figure 2.7a). Phenotypic assessment by flow cytometry revealed an expansion of c-Kit^+ proerythroblasts ($CD71^{high}Ter119^-$/Stage II) in IP_3R-TKO BM (Figure 2.7b and Figure 2.8). Strikingly, despite slightly elevated numbers of early erythroblasts ($CD71^{high}Ter119^+$/Stage III), the percentage of late erythroblasts ($CD71^{low/-}$ $Ter119^+$/Stage IV) was reduced 3 fold as compared to Control. We also examined a more immature and uncommitted population of erythroid progenitor, termed the megakaryocyte-erythrocyte progenitor (MEP). MEPs were also observed to be slightly increased (Figure 2.9a). The differentiation of polychromatic erythroblast to orthochromic erythroblast occurs as cells transition from the $CD71^{high}$ to $CD71^{low}$ fraction of $Ter119^+$ cells[199]. This post-mitotic transition is followed by enucleation, loss of CD71 expression, and terminal maturation. The above data suggest a potential defect in the maturation or survival of late orthochromic erythroblasts upon transition from the early polychromatic erythroblast stage in IP_3R-TKO mice.

The spleens of adult (4-8 weeks) IP_3R-TKO mice were also pale and slightly enlarged as compared to Control (data not shown). Phenotypic analysis by flow cytometry revealed extensive extramedullary erythropoiesis occurring in

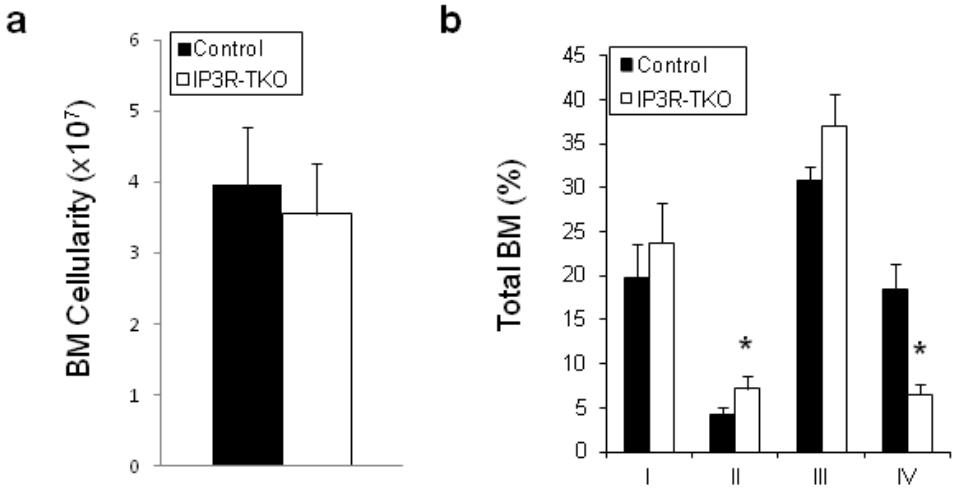


Figure 2.7 Deletion of IP_3R causes ineffective erythropoiesis in the BM.
(**a**) Total BM cellularity of adult 4-8 week IP_3R-TKO and Control mice (n>5). (**b**) Percentages of erythroid lineages in the BM as assessed using surface markers CD71 and Ter119 (n>3). *P < 0.05.

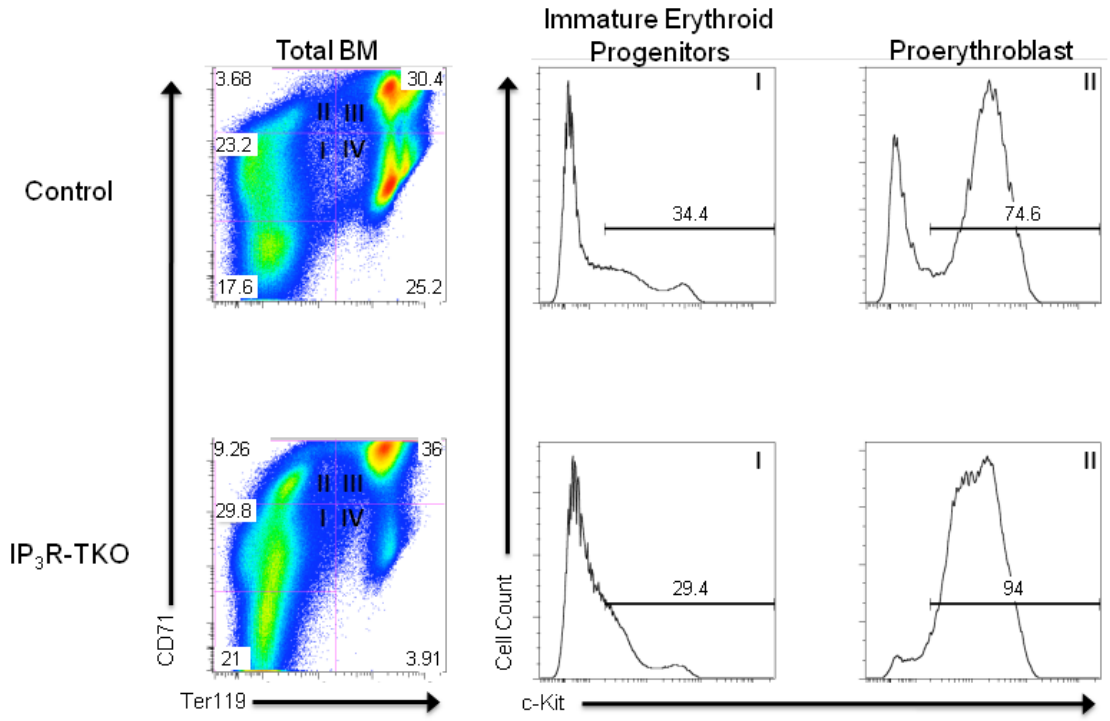


Figure 2.8 Impaired erythroid development in IP_3R-TKO BM.
Total BM from adult 4-8 week IP_3R-TKO and Control mice were analyzed by flow cytometry for erythroid lineage markers CD71 and Ter119, and precursor subsets I (immature erythroid progenitors, $CD71^{+}Ter119^{-}$) and II (proerythroblasts, $CD71^{high}Ter119^{-}$) were gated and analyzed for c-Kit expression. Data are representative of a minimum of three independent experiments.

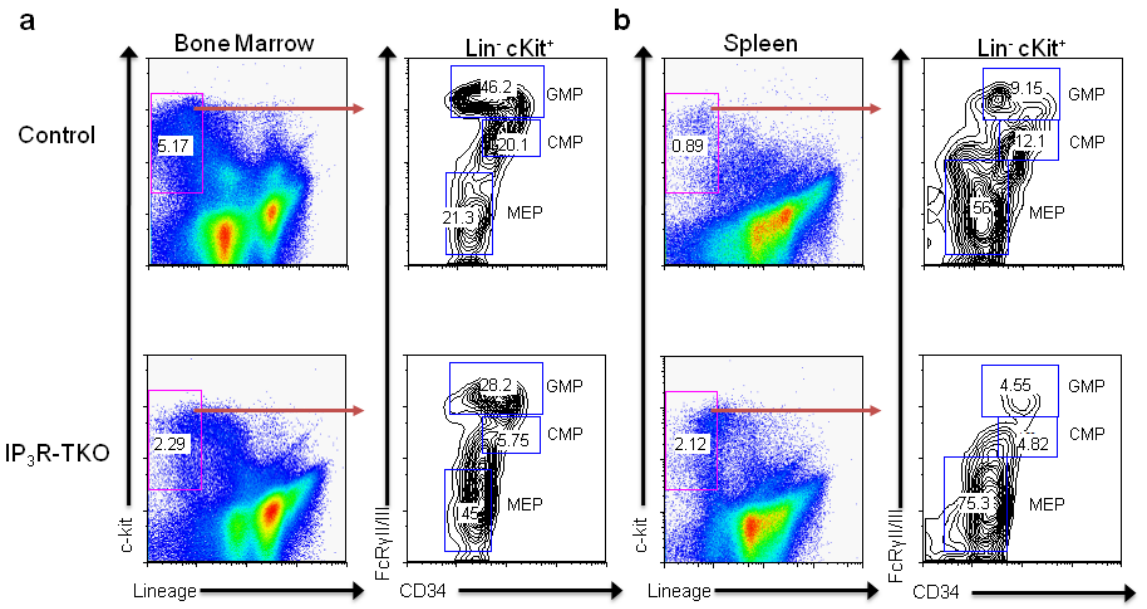


Figure 2.9 Expansion of megakaryocyte-erythrocyte progenitors (MEPs) in IP_3R-TKO BM and spleen.

Cells from (**a**) BM and (**b**) spleen were analyzed by flow cytometry for surface expression of mature lineage markers and c-Kit. The c-Kit+ Lineage- fraction were gated and analyzed for expression of CD34 and FcRγII/III. Megakaryocyte-erythrocyte progenitor (MEP), common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP). Data are representative of three independent experiments. Data are representative of a minimum of three independent experiments.

the spleens of IP_3R-TKO mice, with a greater than 5 fold increase in the early erythroblast population ($CD71^+Ter119^+$/Stage III) (Figure 2.10a,b). Post-mitotic erythroid precursors ($CD71^{low}Ter119^+$/Stage III) were also increased. Interestingly, while not reaching significance the mature erythrocyte population ($CD71^-Ter119^+$/Stage IV) was consistently lower in IP_3R-TKO mice. Consistent with extensive extramedullary erythropoiesis, we also observed an expansion of uncommitted MEPs in IP_3R-TKO spleens (Figure 2.9b). Wright Giemsa staining of single-cell suspensions prepared from spleens revealed a significant expansion of nucleated erythroblasts and a striking reduction in the number of mature erythrocytes in IP_3R-TKO spleens (Figure 2.10c). Collectively, these data suggest that the maturation or survival of late erythroid progenitors and mature erythrocytes in the BM and spleen of IP_3R-TKO mice is impaired, and is consistent with a phenotype of ineffective erythropoiesis upon loss of the IP_3R's. From the above data it is not clear the mechanism of reduced generation or loss of mature erythrocyte. However, we may infer from the reduced weight (Figure 2.11) and bloody stool observed from birth (data not shown) in IP_3R-TKO mice that both diet and gastrointestinal bleeding may contribute or further compound the ineffective erythropoiesis and anemia.

2.3.6 Loss of IP_3R inhibits B lineage development

B cell development is characterized by the differential expression of marker proteins and stepwise gene rearrangement of the immunoglobulin loci[200,201] (Figure 2.12a). Commitment to the B lineage from multipotent

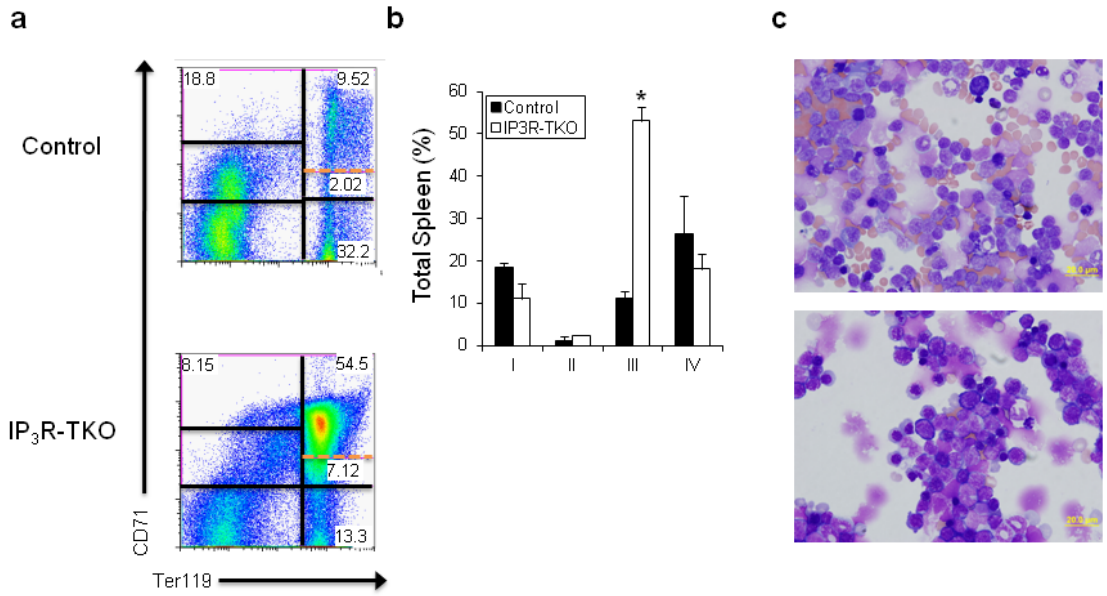


Figure 2.10 Extensive extramedullary erythropoiesis in the spleen of IP_3R-TKO mice.

(**a**) Single cell suspensions prepared from spleens of adult 4-8 week IP_3R-TKO and Control mice were analyzed by flow cytometry for erythroid lineage markers CD71 and Ter119. (**b**) Percentages of erythroid lineages in the spleen as assessed using surface markers CD71 and Ter119 (n>3). *P < 0.05. (**c**) Wright Giemsa staining of single cell suspensions prepared from spleens. Data are representative of a minimum of three independent experiments.

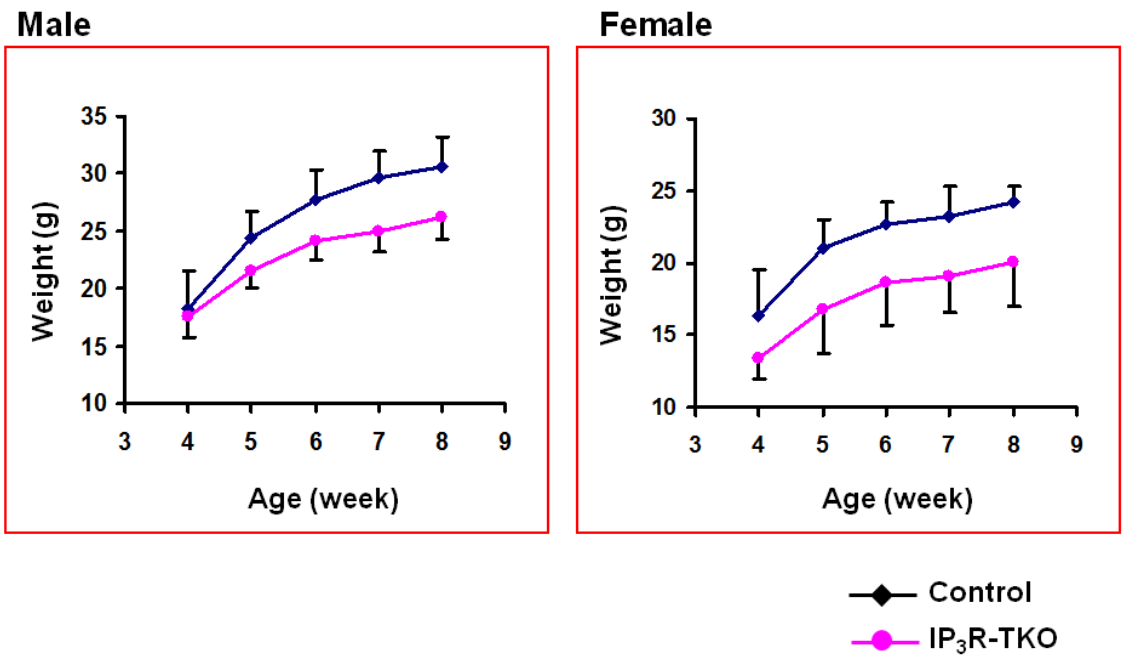


Figure 2.11 Decreased body weights in IP_3R-TKO mice.
Adult littermate Control and IP_3R-TKO mice, male and female, were monitored weekly for total body weight starting at 4 weeks of age. Data are representative of at least two independent litters.

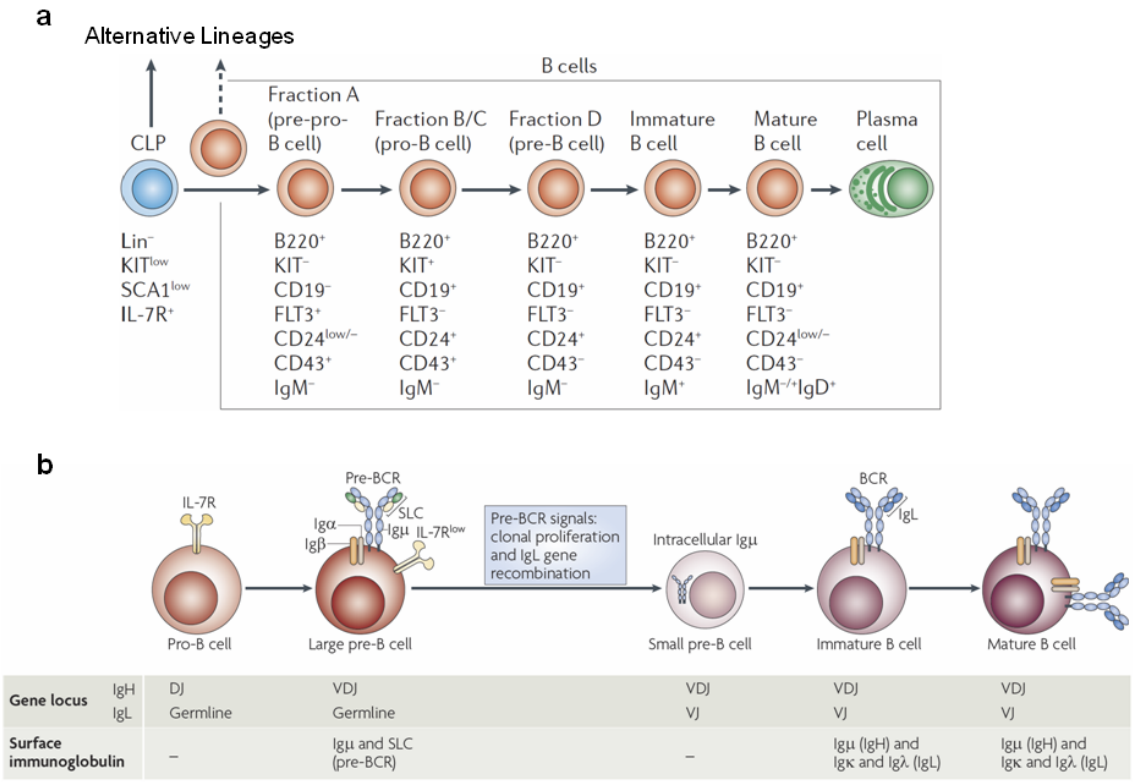


Figure 2.12 Early B cell development.
(**a**) The earliest non-committed B cell progenitor is the common lymphoid progenitor (CLP), which can give rise to B and T cells but not myeloid lineages. B cell precursors that are negative for cell-surface immunoglobulin (Ig) but positive for the B cell lineage marker B220 can be divided into four fractions according to their differential expression of a cell surface markers during development. These fractions are A (pre-pro-B cells), B (pro-B cells), C (pro-B cells) and D (pre-B cells). Immature B cells, which are generated from fraction D cells, exit the bone marrow and reach the spleen, where they mature into peripheral mature B cells and plasma cells. (**b**) Rearrangement of the immunoglobulin heavy chain (IgH) locus is initiated at the CLP or pro-B cell stage and, if successful, gives rise to the Igμ chain that is expressed on the cell surface in the form of the pre-B cell receptor (pre-BCR) at the large pre-B-cell stage. Signalling from the pre-BCR provides rapid feedback about the functionality of the recombined Igμ and induces clonal proliferation, downregulation of pre-BCR components and recombination of immunoglobulin light chain (IgL) genes. In-frame IgL gene rearrangements in small pre-B cells result in the expression of a BCR that is composed of two Igμ chains and two Igκ (the κ-chain of IgL) or Igλ (the λ-chain of IgL) chains on immature B cells. These immature B cells are subjected to selection processes and eventually enter the pool of long-living mature B cells. Reproduced from (**a**) Nagasawa, et. al, **Nature Reviews Immunology.,** 2006 (6)[201] and (**b**) Herzog, et al., **Nature Reviews Immunology.**, 2009 (9)[200].

progenitor cells occurs at the early pro-B cell stage, and is followed at later pro-B cell stages by rearrangement of the immunoglobulin heavy chain (IgH) locus. Productive IgH locus recombination results in the expression of Igμ (IgM), which is expressed by large $CD25^{lo}$ pre-B cells as part of the pre-B cell receptor (pre-BCR) complex (Figure 2.12b). The expression of the pre-BCR serves as an important checkpoint in B cell development. Signals from the pre-BCR are required for proliferation and recombination of the immunoglobulin light chain (IgL) locus in small $CD25^{+}$ pre-B cells, which are essential for the continued differentiation of pre-B cells to the surface IgM^{+} immature B cell stage. Surface IgM^{+} B lymphocytes that have undergone this selection process and express a functional BCR (both IgH and IgL) will migrate through the blood and enter the spleen, where they pass through additional BCR-mediated developmental checkpoints.

Our analysis of PB revealed a significant decrease in circulating $B220^{+}$ B lymphocytes in IP_3R-TKO mice. To identify the stage at which B lymphopoiesis is first disrupted, we analyzed B lymphocyte development in the BM and spleen of IP_3R-TKO mice by flow cytometry (Figure 2.13). B lymphopoiesis was significantly reduced in IP_3R-TKO BM, with a greater than 5 fold reduction in the total population of $B220^{+}$ B lymphocytes. While the ratio of small ($B220^{+}IgM^{-}CD25^{+}$) and large ($B220^{+}IgM^{-}CD25^{+}$) pre-B cells was similar to control, pre-B cells were nearly absent in IP_3R-TKO BM (>10 fold reduction as compared to control), whereas the $B220^{+}IgM^{-}CD25^{-}$ population, which contains pro-B and pre-pro-B cells, was expanded. We confirmed the percentage of pro-B cells ($CD19^{+}$c-

Kit^+) in IP_3R-TKO BM was increased two fold as compared to control BM (Figure 2.14). This apparent increase in pro-B cells may represent a real increase in pro-B cell number or alternatively be the result of a decrease in the number of small pre-B and immature B cells. As total cellularity was not significantly reduced in IP_3R-TKO BM as compared to control, we determined the increase in pro-B cells represents an actual increase in pro-B cell number, and suggests that B cell development was impaired after lineage commitment as pro-B cells transition to the large pre-B cell stage. We also observed a decrease in the percentage of immature B cells ($B220^+IgM^+$) present in IP_3R-TKO BM, and consistent with the loss of circulating B lymphocytes in the PB, mature $B220^{hi}IgM^+$ B lymphocytes in the BM were nearly absent.

Analysis of splenic B lymphocyte development showed a 10 fold reduction in the proportion of total splenic $B220^+$ B lymphocytes in IP_3R-TKO mice (Figure 2.14). We noted that while IP_3R-TKO total splenic B lymphocytes were reduced in number, the relative proportions of transitional T1 (IgM^+IgD^-) and T2-Marginal Zone (IgM^+IgD^+) $B220^+$ B lymphocytes were similar to Control. In contrast, the proportion of follicular mature ($IgM^{lo}IgD^+$) $B220^+$ B lymphocytes was reduced over 3 fold. Thus, B lymphocyte maturation in the spleen is also severely disrupted in the absence of the IP_3R. Collectively, the above data demonstrate that IP_3R-Ca^{2+} is essential for the development of pre-B cells, and is required for late stages of B cell maturation in the spleen.

2.3.7 Loss of peripheral T cells in IP_3R-TKO mice

Our analysis of PB also revealed a profound decrease in circulating $CD3^+$

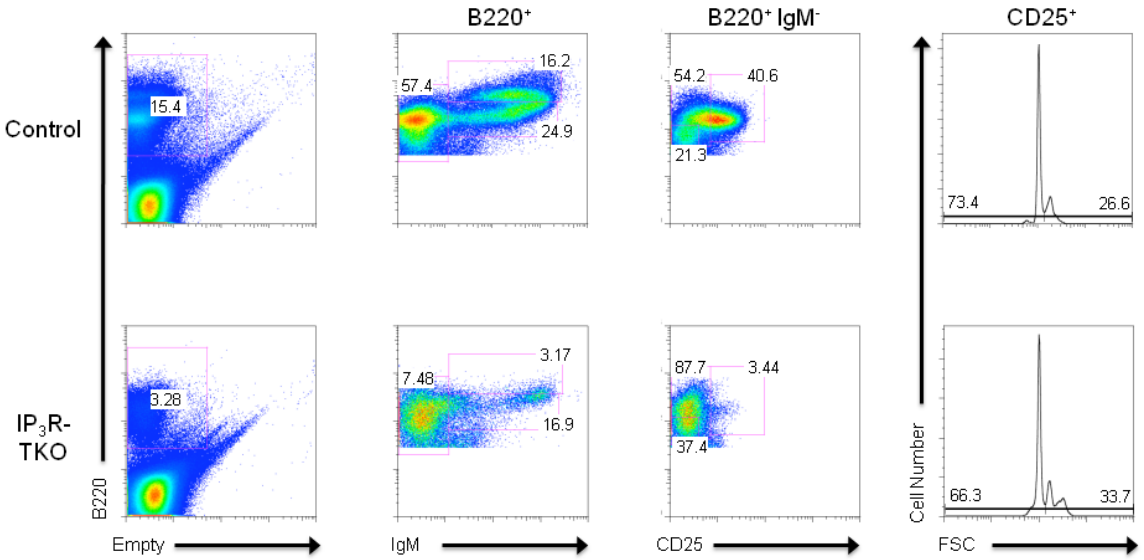


Figure 2.13 Disruption of B cell development in IP_3R-TKO BM. Single cell suspensions prepared from BM of adult 4-8 week IP_3R-TKO and Control mice were analyzed by flow cytometry for the pan B cell lineage marker B220. $B220^+$ cells were gated and analyzed for expression of IgM. $B220^+IgM^-$ cells were further analyzed for expression of CD25, and the size distribution of $B220^+IgM^-CD25^+$ pre-B cells was analyzed based on forward light scatter (FSC). Data are representative of a minimum of five independent experiments.

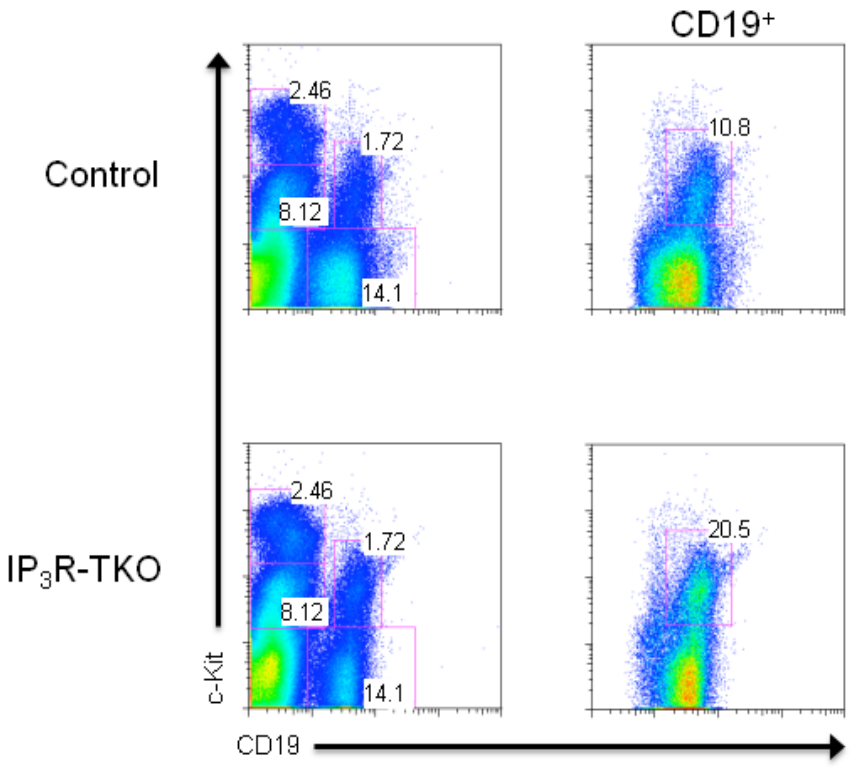


Figure 2.14 Accumulation of pro-B cells in IP_3R-TKO mice.
Single cell suspensions prepared from BM of adult 4-8 week IP_3R-TKO and Control mice were analyzed by flow cytometry for the definitive B cell lineage marker CD19 and c-Kit (left). $CD19^+$ cells were gated (right) and analyzed further to distinguish $CD19^+$c-Kit^+ pro-B cells. Data are representative of three independent experiments.

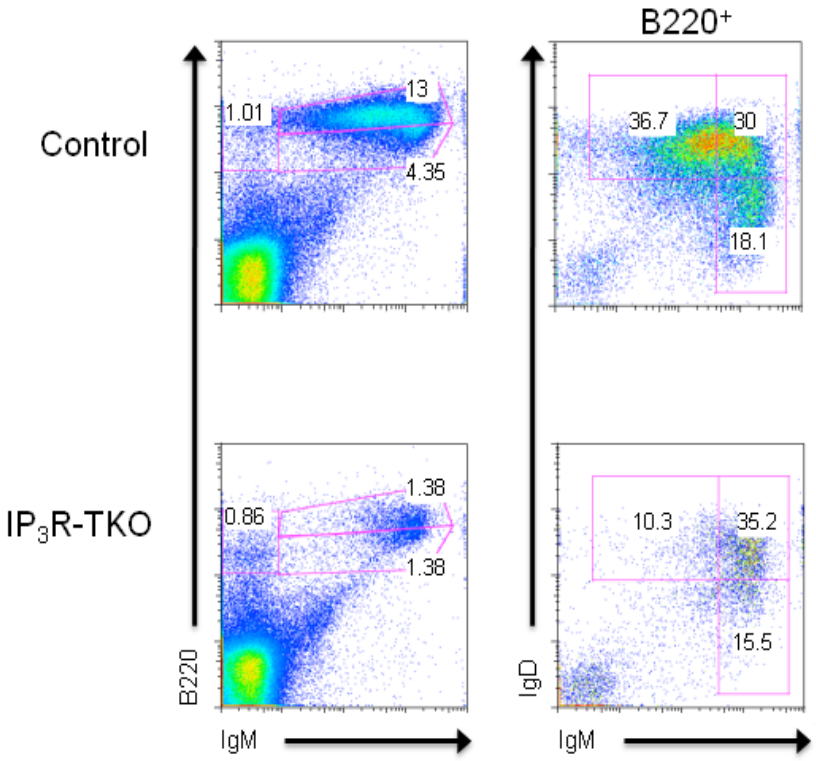


Figure 2.15 Loss of splenic B cells in IP_3R-TKO mice.
Single cell suspensions prepared from spleens of adult 4-8 week IP_3R-TKO and Control mice were analyzed by flow cytometry for the B cell lineage markers B220 and IgM (left). $B220^+$ cells were gated (right) and analyzed further for expression of IgM and IgD. Data are representative of three independent experiments.

T lymphocytes in IP_3R-TKO mice. We next sought to further characterize the peripheral T lymphocyte population in the spleen of IP_3R-TKO mice by flow cytometry (Figure 2.16). The $CD4^+$ SP T cell population was nearly absent in IP_3R-TKO spleens (>9 fold reduction as compared to control). $CD8^+$ SP T cells, while less affected than their $CD4^+$ SP T cell counterparts, were also significantly reduced (>3.9 fold reduction as compared to control). Examination of surface TCRβ revealed an abnormal $CD4^+$ population that contained a significant proportion of $TCR\beta^-$ cells. While the decrease in splenic and peripheral blood T cells could result from decreased proliferation or increased cell death in the periphery, a more likely explanation would be an earlier defect in thymic T cell development (see Chapter 3).

2.3.8 Reduced hematopoietic stem and progenitor cells in IP_3R-TKO neonates

Mammalian hematopoietic development undergoes major transitions as the needs of the developing animal change[202]. In the mid-gestation embryo, the need to expand the progenitor cell pool is paramount, and fetal liver HSCs proliferate extensively. As the embryo develops further its needs change, as do the sites of hematopoietic cell development, requiring the migration of HSCs to a new niche, the bone marrow. A further transition in HSC activity can be observed as neonate's transition to adulthood. We examined hematopoietic cell development in adults and did not observe significant changes in early hematopoietic progenitor cell number (data not shown). However, this does not necessarily preclude a defect at early stages of development, as moderate and

severe defects in hematopoietic progenitor cell homeostasis can be corrected over time. Thus, we examined neonate bone marrow hematopoietic stem and progenitor cell populations in IP_3R-TKO and Control animals. Interestingly, in neonates the LSK population[203], which contains the earliest hematopoietic stem and progenitor cells, was decreased up to 50% (Figure 2.17a). Total BM cellularity was reduced by one-third in IP_3R-TKO mice, consistent with the diminished LSK population (Figure 2.17b). The LSK population is heterogeneous and contains several progenitor cell populations with varying degrees of self-renewal and lineage potential[204,205]. We observed a similar decrease in both the $Flt3^{+}$LMPP and $Flt3^{lo/-}$MPP/HSC populations, consistent with a defect in total HSC number. The LMPP generates both T and B lymphocyte progenitors, and in the case of T cell progenitors the LMPP gives rise to a cell that will seed the thymus[3]. Examination of the most primitive T cell precursor, the early thymic progenitor (ETP), revealed a similar decrease in total cell number which was maintained in the progeny of the ETP, the DN2 thymocytes. Of note, T cell progenitor numbers appear to recover at the stage immediately after DN2 (see chapter 3.3.3), although the requirement for IP_3R at later stages of T cell development precludes a full recovery of thymic cellularity. We also observed a similar decrease in the earliest physiological B cell progenitor, the common lymphoid progenitor, although it did not reach statistical significance (data not shown). Together, these data implicate IP_3R-Ca^{2+} in regulating early hematopoietic cell number, likely at the HSC stage. The proportional decrease in ETPs is consistent with reductions observed in the BM LSK and LMPP

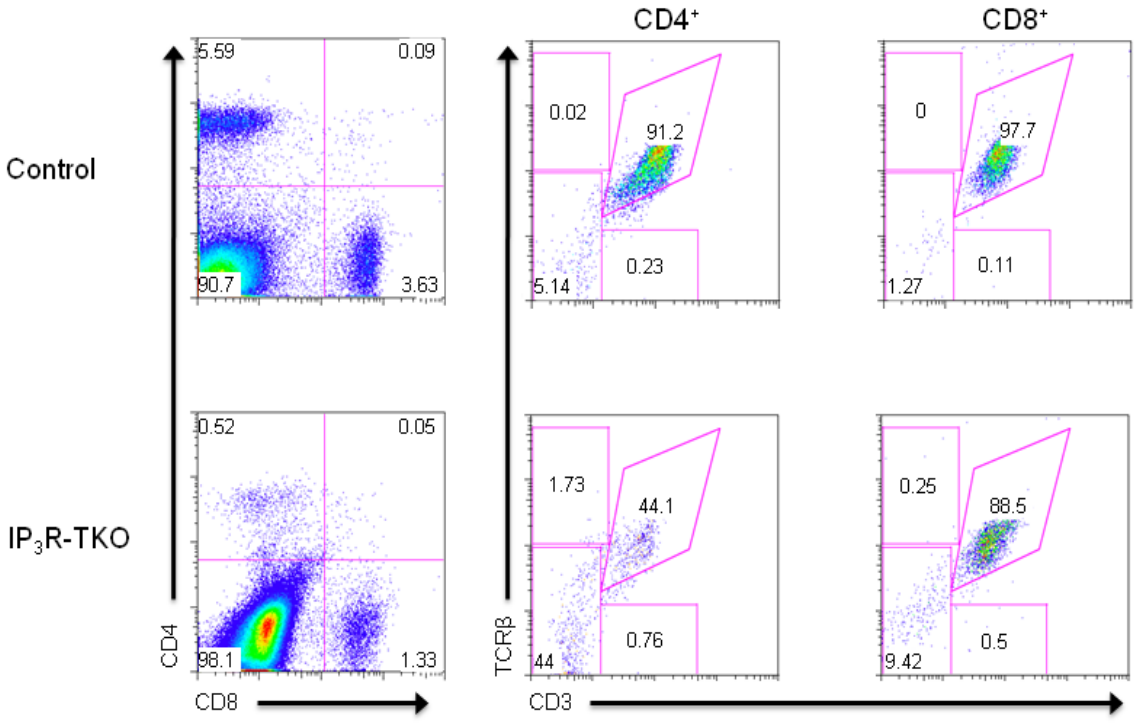


Figure 2.16 Loss of splenic T cells in IP_3R-TKO mice.
Single cell suspensions prepared from spleens of adult 4-8 week IP_3R-TKO and Control mice were analyzed by flow cytometry for the T cell lineage markers CD4 and CD8 (left). $CD4^+$ (middle) and $CD8^+$ (right) T cells were gated and analyzed further for expression of TCRβ and CD3. Data are representative of three independent experiments.

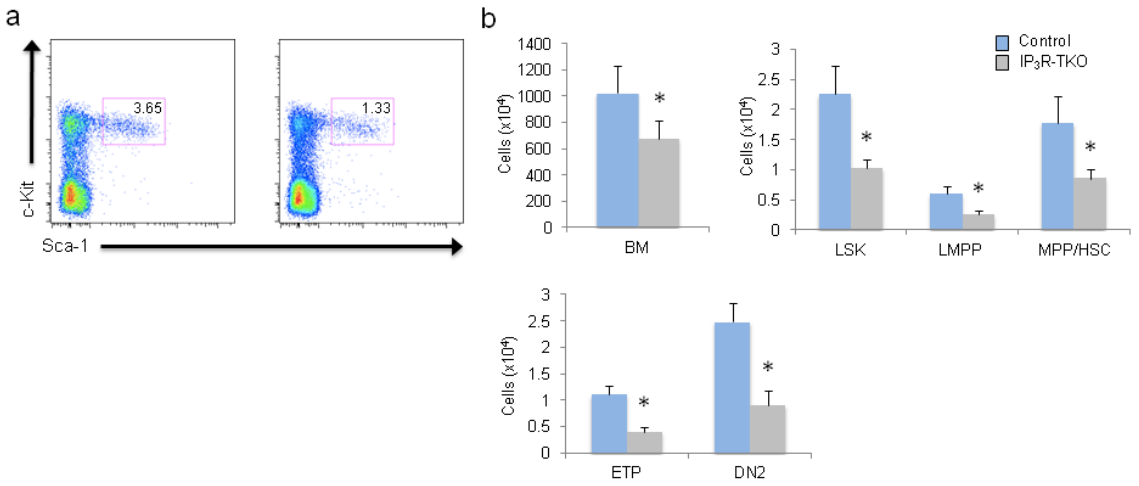


Figure 2.17 Reduced BM LSK population in IP_3R-TKO mice.
(a) Single cell suspensions prepared from BM of neonate (3-7 days) IP_3R-TKO and Control mice were analyzed by flow cytometry. The mature lineage marker negative (Lineage-) fraction was gated and analyzed for expression of c-Kit and Sca-1. (b) Total cellularity of neonate progenitor populations from BM and thymi (mean ± SEM). $Lineage^-c\text{-}Kit^+Sca1^+$ (LSK), lymphoid primed multipotent progenitor (LMPP), multipotent progenitor (MPP), hematopoietic stem cell (HSC), early thymic progenitor (ETP). Total Data are representative of three independent experiments. * $P<0.05$.

populations, and thus likely reflects a defect prior to seeding of the thymus.

2.4 Discussion

We have established a hematopoietic-specific conditional knockout model for all three *Itpr* genes. Loss of IP_3R leads to multiple defects in the development of the hematopoietic lineages. As expected, severe lymphopenia was observed in the PB of IP_3R-TKO mice. B lymphocyte development was significantly impaired in IP_3R-TKO BM. Consistent with IP_3R-Ca^{2+} release being a major signaling event downstream of the pre-BCR in developing B cells[206], we observed a significant block in B cell development at the pro-B to pre-B cell transition, indicating a block at the pre-BCR checkpoint. Plcγ2 is the major isoform of Plcγ found in B cells linking the BCR to IP_3R, although Plcγ1 is also expressed. The B cell developmental phenotype of IP_3R-TKO mice is more severe than in Plcγ2$^{-/-}$ mice, and largely resembles Plcγ1$^{+/-}$Plcγ2$^{-/-}$ compound mutants[207]. In Plcγ1$^{+/-}$Plcγ2$^{-/-}$ compound mutants, early B cell development is disrupted at the pro-B to pre-B cell transition due to defective pre-BCR signaling. Similar to IP_3R-TKO mice, Plcγ1$^{+/-}$Plcγ2$^{-/-}$ splenic B cells are also significantly reduced and development of IgM^{+}IgD^{+} T2-marginal zone and IgM^{-}IgD^{+} follicular mature B cells is diminished. Thus, our IP_3R-TKO model faithfully reproduces the phenotypes observed with diminished Plcγ activity, providing a developmental setting in which were able to validate the loss of function of the IP_3R's. While we did not observe a defect in myeloid cell development, BM hematopoietic progenitor differentiation as assessed by *in vitro* differentiation in methylcellulose revealed changes in colony size and cell migration (data not shown), indicating a

potential role for IP_3R in granulocyte or macrophage migration. The development of conditional IP_3R alleles and the IP_3R-TKO model will be highly informative in future studies aimed at elucidating IP_3R function in the regulation of myeloid cell differentiation and function. A reduction in the hematopoietic stem cell compartment was also observed in neonate IP_3R-TKO mice. Activation of IP_3R through Plcγ1 can occur in response to chemokine signaling and downstream of several G-protein coupled receptors. While an intrinsic role for IP_3R-Ca^{2+} in regulating HSC number is possible, a more likely explanation is a defect in HSC adhesion or migration from the fetal liver. Future studies to address these possibilities are under consideration.

We also observed a disruption in red blood cell development. Little is known of the potential role for IP_3R-Ca^{2+} in erythrocyte maturation or function. While stimulation of the erythropoietin receptor (EpoR) can induce Ca^{2+} mobilization[208], no definitive function of Ca^{2+} has been identified, nor is there evidence that Ca^{2+} is required for EpoR function. The block in erythrocyte development observed in IP_3R-TKO is not consistent with a defect in EpoR signaling, and is more consistent with a defect in the terminal maturation of erythroblasts. Future studies are needed to more closely examine the processes associated with terminal maturation of erythrocytes including determination of erythrocyte lifespan and enucleation. Studies of erythroid enucleation in IP_3R-TKO mice may prove particularly enlightening, as this process requires the removal of organelles through autophagy[209]. IP_3R-Ca^{2+} is a well-established

regulator of autophagy[210], and is known to influence the expression and activity of Bcl-2 family members[154], also known to regulate autophagy[211].

Experiments presented in this chapter identified a specific reduction in both $CD4^+$ SP and $CD8^+$ SP αβ T cells in peripheral blood and secondary lymphoid organs. The disruption of both T cell lineages, and the near abolishment of $CD4^+$ SP T cells, is suggestive of a requirement for IP_3R-Ca^{2+} in thymic T cell development. The following chapter will examine the role of the IP_3R in intra-thymic T cell development.

2.5 Acknowledgements

Chapter 2, in part is currently being prepared for submission for the publication of material. Gomez-Amaro, R.L.; Ouyang, K.; Stachura, D.S.; Traver, D.; Evans, S.; Chen, J. The dissertation author was the primary investigator and co-first author of this material along with Ouyang, K. and Stachura, D.S..

Chapter 3 Dissecting IP_3R-Ca^{2+} function in murine T cell development

3.1 Introduction

Signaling through the pre-TCR is essential for the development, proliferation, and survival of thymocytes through the double-negative (DN) to double-positive (DP) transition[212]. Mobilization of intracellular Ca^{2+} stores, attributed largely to the PLCγ1-dependent generation of inositol triphosphate (IP_3), has long been thought essential for pre-TCR^+ T cell development[176,133]. Consistent with a role for Ca^{2+} in pre-TCR signaling, Ca^{2+}-dependent signaling pathways such as calcineurin-Nfat, PKCα, and NF-κB have been shown to influence T cell development or survival at and subsequent to the β-selection checkpoint[182,188,213,214]. However, these effects are modest, and little is known about the genes and signaling pathways influenced by Ca^{2+} outside of these pathways. Furthermore, the physiological mechanisms that influence or control Ca^{2+} release downstream of the pre-TCR in developing thymocytes is limited[128,159,161,174].

The IP_3R is a Ca^{2+} permeable ion channel encoded by a family of three genes[135] (*Itpr1*, *Itpr2*, and *Itpr3*). Functional redundancy between IP_3Rs and postnatal mortality in the absence of single and multiple *Itpr* genes have been observed[138,194]. IP_3R channels are functionally diverse; different cells and tissues utilize distinct IP_3Rs to regulate specific biological functions[143]. In the thymus, all three IP_3R subtypes are expressed, suggesting potentially redundant functions

between isoforms. Identifying and separating the individual roles of the IP_3R's using conventional genetic approaches has been difficult due to genetic redundancy and early postnatal lethality in the absence of multiple IP_3R's. Thus, our understanding of how IP_3R-Ca^{2+} release and individual IP_3R ion channels influence T cell biology has been largely restricted to *in vitro* studies using cell-permeable but non-specific Ca^{2+} chelators. We therefore examined the requirement for IP_3R signaling in T cell development by generating conditional *Itpr* double and triple knockout mice utilizing Tie2Cre, which mediates excision prior to the double negative (DN) 1 stage of thymocyte development[192].

3.2 Chapter aims

Using a hematopoietic specific conditional knockout of all three Itpr genes, I aimed to characterize the combined and individual roles IP_3R in intra-thymic T cell development. Analysis of the roles of the IP_3R's in numerous aspects of T cell development will be assessed by immunostaining and flow cytometry. Biochemical analysis of conditional knockout mice will also be performed. Combined, these studies are expected to reveal both unique and redundant functions of these ion channels.

3.3 Results

3.3.1 Loss of IP_3R inhibits αβ T lineage development

We observed a significant reduction in αβ T lineage cells in the PB of IP_3R-TKO mice. Analysis of splenic T lymphocyte development revealed near ablation of $CD4^+$ T cells and reduced $CD8^+$ T cells, suggesting T lineage development in the thymus may be severely impaired. To determine the role of

IP_3R in early thymocyte development, we analyzed neonate and adult thymocytes from IP_3R-TKO mice by flow cytometry.

IP_3R-TKO thymi were noticeably smaller, with a consistent reduction in cellularity as compared to those of littermate controls (Figure 3.1a). The distribution of thymocyte subsets was also abnormal. In IP_3R-TKO neonates, a significant expansion was observed in DN and $CD8^+CD3^-TCR\beta^-CD24^+$ immature single positive (ISP) T cells, a transient progenitor cell present between the DN-to-DP transition (Figure 3.1a, b). DP thymocyte numbers were reduced by 50%, and a significant reduction in $CD4^+$ single-positive (SP) thymocytes was observed. Unexpectedly, mature $CD8^+$ SP thymocytes were present in numbers similar to controls. Developing T lymphocytes can be divided into two major subsets based upon which TCR genes have undergone somatic recombination, αβ or γδ. To address whether the increase in DN thymocytes in IP_3R-TKO mice resulted from aberrations in a specific lineage, we analyzed the expression of CD3, CD24, and $\gamma\delta$ TCR in DN thymocytes (Figure 3.2a,b). $\gamma\delta$ T cell number was reduced four-fold in IP_3R-TKO thymi (Figure 3.2c), indicating that the development of both $\gamma\delta$ and $\alpha\beta$ T cell lineage subsets were impaired in IP_3R-TKO mice. Examination of thymocyte development in adult IP_3R-TKO mice revealed a further accumulation of ISP T cells and a compensatory reduction in DP thymocytes (Figure 3.1c).

To confirm that the reduction of IP_3R expression resulted in a functional decrease in IP_3R-mediated Ca^{2+} release, we examined T cell receptor (TCR)-mediated Ca^{2+} influx in thymocytes after crosslinking of the TCR with an anti-CD3

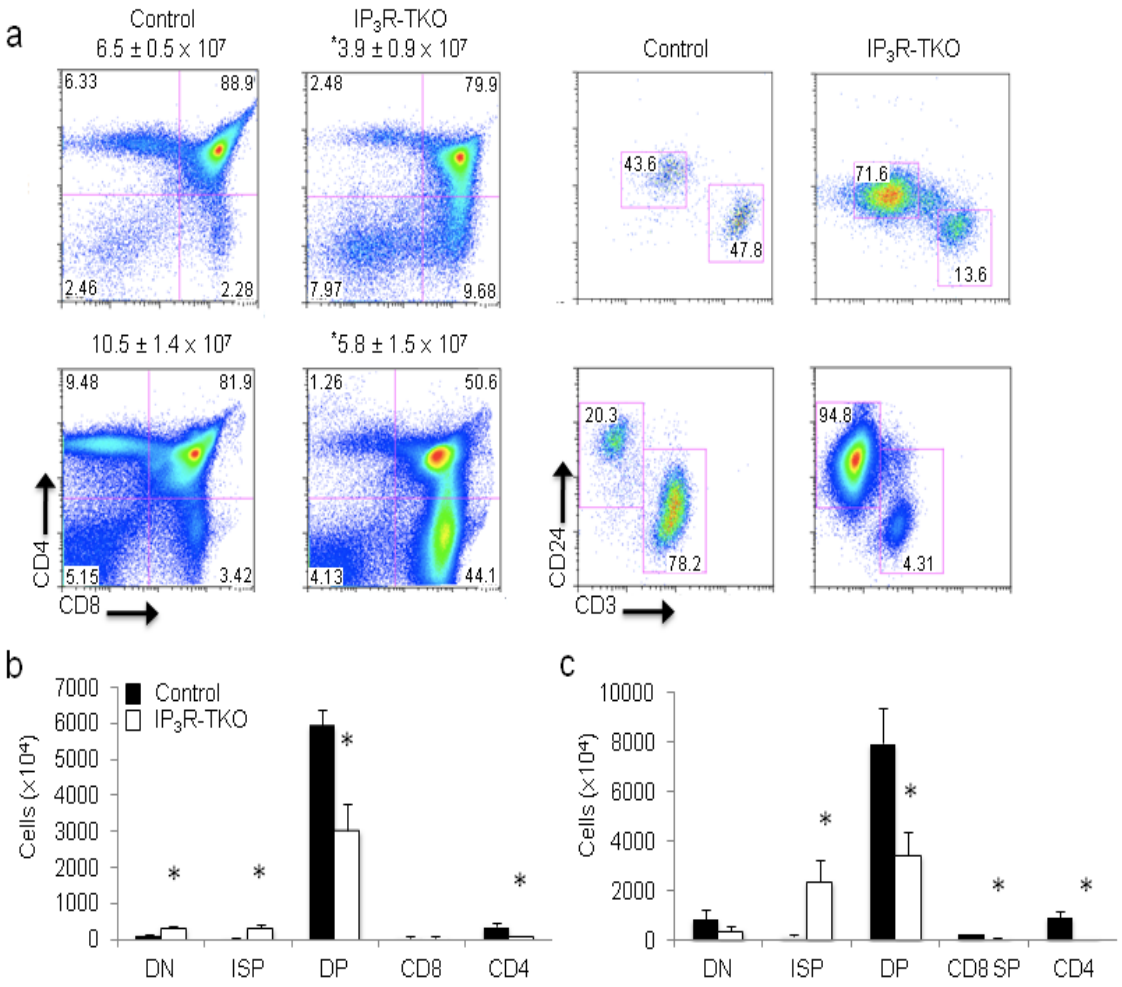


Figure 3.1 IP_3R-Ca^{2+} influx is essential for the DN to DP transition.
(**a**) Expression of surface CD4 and CD8 (left) in neonate (1wk, top) and adult (6wk, bottom) thymi of IP_3R-TKO and control mice. Total thymic cellularity is shown above plots (mean ± SEM). $CD8^+CD4^-$ thymocytes of neonatal and adult mice were subgated (right), and surface expression of CD24 and CD3 was examined. Numbers in plots indicate percentage of cells. (**b**) Absolute cellularity of thymic subsets in neonatal and (**c**) adult thymii (mean ± SEM). Data are representative of a minimum of five (**a**-**c**) independent experiments. *P < 0.05.

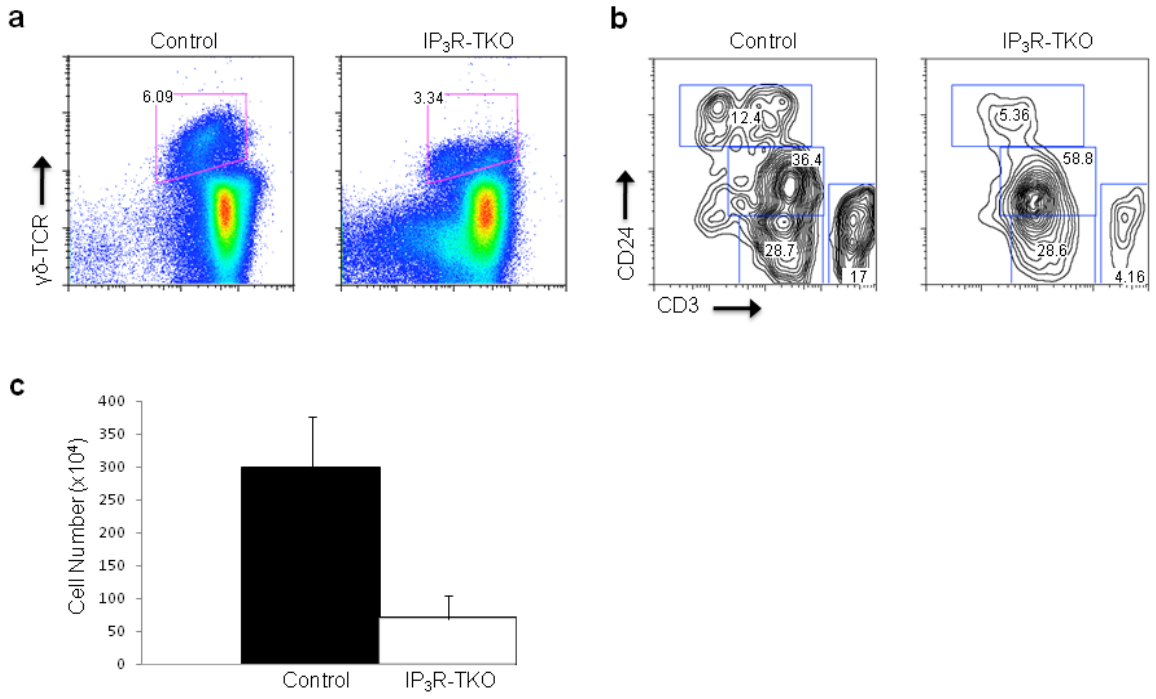


Figure 3.2 γδ T cell development is disrupted in IP_3R-TKO mice.
(**a**) Expression of surface γδ-TCR of control and IP_3R-TKO. (**b**) CD3 in DN thymocytes of control and IP_3R-TKO. (**c**) Total thymic cellularity of γδ-TCR^+ T cells in control and IP_3R-TKO mice. Data are representative of three independent experiments (mean ± SEM).

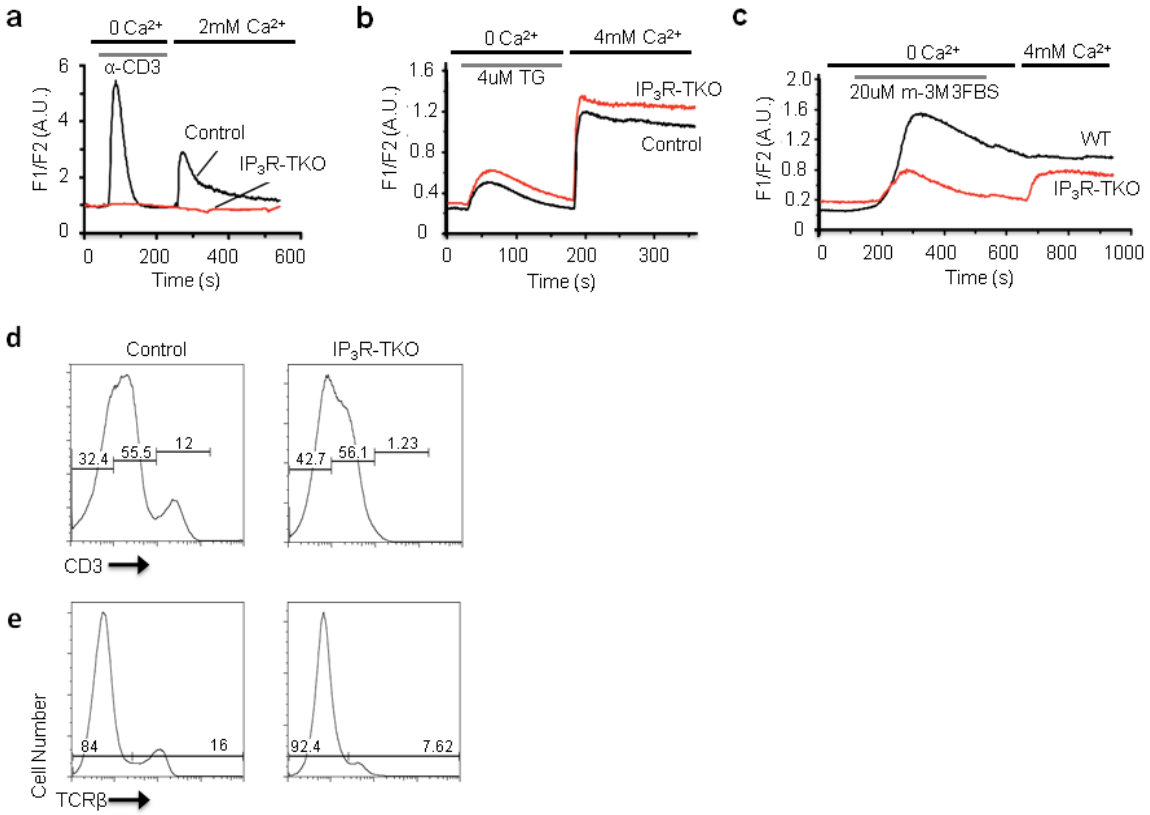


Figure 3.3 TCR-Ca^{2+} influx in IP_3R-TKO thymocytes is significantly reduced. (**a**) Ca^{2+} influx in adult control and IP_3R-TKO thymocytes in response to crosslinking of TCR with anti-CD3 antibody or (**b**) after passive depletion of intracellular Ca^{2+} stores by thapsigargin (TG), followed by reintroduction of extracellular Ca^{2+} or (**c**) in response to treatment with the PLCγ agonist m-3M3FBS, followed by reintroduction of extracellular Ca^{2+}. Expression of surface (**d**) CD3 and (**e**) TCRβ in adult control and IP_3R-TKO thymocytes. Relative Ca^{2+} levels were determined by ratiometric measurement with Fluo-5 (F1) and Fura-red (F2) Ca^{2+} indicator dyes. A.U., arbitrary units. CTL, control. Data are representative of a minimum of three independent experiments.

antibody. IP_3R-TKO thymocytes showed little or no Ca^{2+} influx after TCR crosslinking (Figure 3.3a). Passive depletion of endoplasmic reticulum Ca^{2+} stores with thapsigargin (Figure 3.3b) led to a large and sustained influx of Ca^{2+} similar to control, indicating that the loss of Itpr did not impair the machinery required for store-operated Ca^{2+} entry. Diminished TCR-mediated Ca^{2+} influx in IP_3R-TKO thymocytes might be caused by reduced surface expression of CD3 and TCRβ. Therefore, we examined their respective surface expression in adult thymocytes. TCRβ^{hi} thymocytes were significantly reduced in IP_3R-TKO thymocytes. However, TCRβ^{lo} and TCRβ^{-} populations were present in proportions similar to WT (Figure 3.3c). Similar results were observed for CD3 surface expression (Figure 3.3d). As TCR crosslinking did not induce significant changes in Ca^{2+} influx, we sought to directly activate Ca^{2+} influx by treatment with the PLCγ agonist m-3M3FBS[15]. Treatment of IP_3R-TKO thymocytes with m-3M3FBS produced a small, transient influx of Ca^{2+}, in contrast to the sustained increase in Ca^{2+} observed in WT (Figure 3.3e). Reintroduction of extracellular Ca^{2+} resulted in an increased influx of Ca^{2+}, but did not approach WT thymocyte levels. Collectively, these results indicate that Ca^{2+} influx in response to TCR signaling is significantly impaired in IP_3R-TKO thymocytes.

Despite the extensive reduction of all IP_3R proteins by western blot in IP_3R-TKO thymocytes, deletion was incomplete and raised the possibility of a strong selective pressure for cells to retain one or more intact *Itpr* alleles (Figure 2.4). To address this concern, we reduced the number of conditional alleles by replacing the *Itpr2*^{flox} alleles with *Itpr2*$^{-/-}$. *Itpr1*$^{fl/fl}$*Itpr2*$^{-/-}$*Itpr3*$^{fl/fl}$*Tie2*Cre double floxed

and single null mice (IP_3R-DFNKO) were born healthy and at expected Mendelian ratios. However, no phenotypic differences were observed between IP_3R-TKO and IP_3R-DFNKO (Figure 3.1a and Figure 3.4a). As breeding additional global Itpr null alleles would introduce both a significant delay in our analysis of IP_3R function in T cells and concern for embryonic and postnatal viability, we utilized the IP_3R-TKO line for further investigation.

Taken together, the above data demonstrate a requirement for IP_3R-mediated Ca^{2+} signaling during the development of both the $\alpha\beta$ and $\gamma\delta$ T cell lineages, and highlight the essential role of Ca^{2+} ion channel IP_3R in thymocytes throughout the DN to DP transition.

3.3.2 IP_3R-TKO mice, but not double knockout mice, displayed severe defects in thymocyte development

In the thymus, we observed *Itpr2* and *Itpr3* to be the predominantly expressed isoforms. We therefore examined the requirement for distinct IP_3Rs in T cell development by generating conditional *Itpr* double-knockout mice with Tie2Cre. We observed no changes in T cell development throughout the DN-to-DP transition in *Itpr1*$^{fl/-}$*Itpr3*$^{-/-}$*Tie2*Cre, *Itpr1*$^{fl/fl}$*Itpr2*$^{-/-}$*Tie2*Cre, or *Itpr2*$^{fl/-}$ *Itpr3*$^{fl/fl}$*Tie2*Cre thymii (Figure 3.5a). Similarly, heterozygosity of *Itpr3* in the absence *Itpr1* and *Itpr2* did not affect T cell development up to the DP stage regardless of the presence of *Itpr2* null or floxed alleles (data not shown). However, *Itpr1*$^{fl/fl}$*Itpr2*$^{-/-}$*Tie2*-Cre and *Itpr2*$^{fl/-}$*Itpr3*$^{fl/fl}$*Tie2*-Cre thymii displayed increased proportions of $CD4^+$ SP and $CD8^+$ SP T cells (Figure 3.5b). Together,

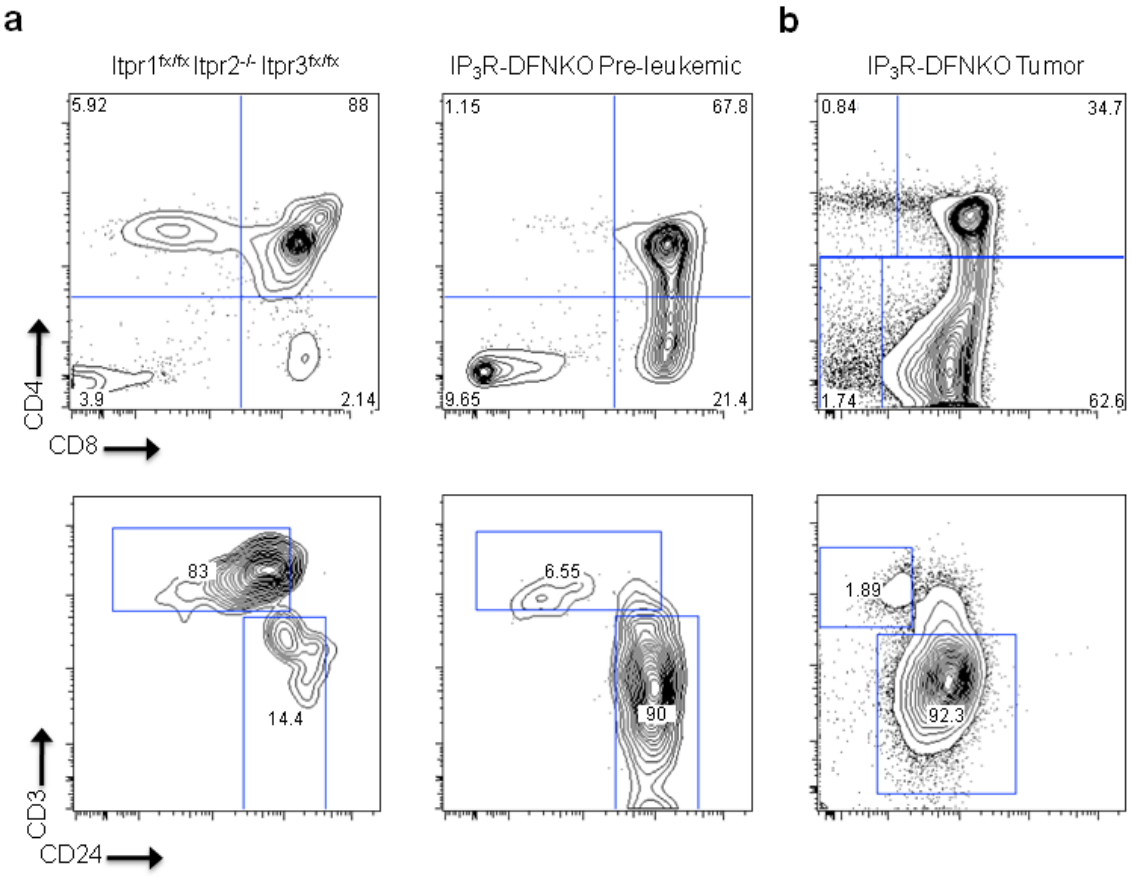


Figure 3.4 Accumulation of ISP thymocytes in pre-leukmic and tumor-burdened IP_3R-DFNKO thymii.
CD4 and CD8 surface expression (top) in thymii of preleukemic (**a**) and tumor-burdened (**b**) IP_3R-DFNKO adult mice. $CD8^+CD4^-$ thymocytes were subgated and surface expression of CD24 and CD3 (bottom) was examined. Numbers in plots indicate percentage of cells. Data are representative of a minimum of ten independent experiments.

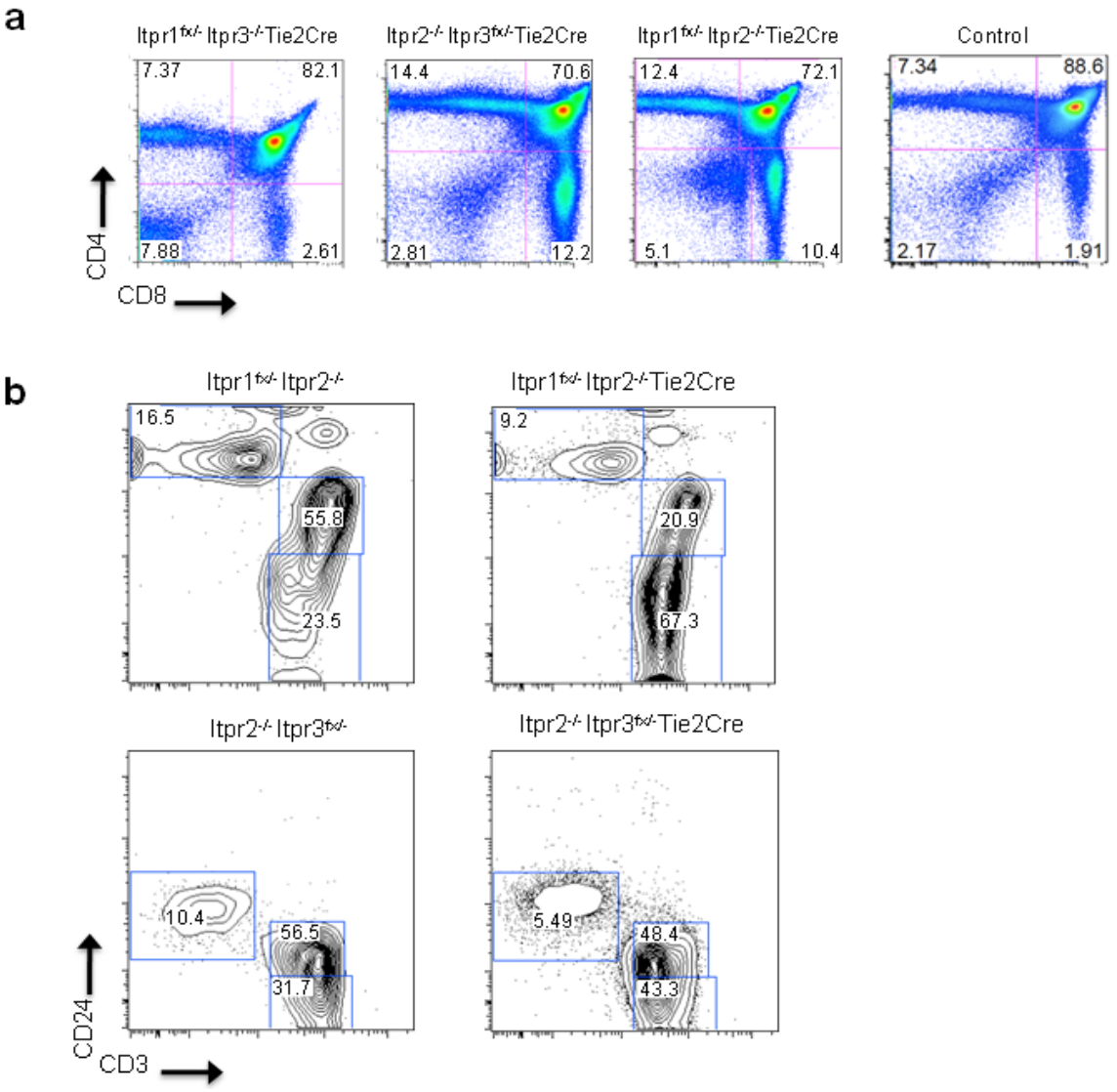


Figure 3.5 Increased proportions of mature CD4$^+$ and CD8$^+$ T cells and normal DN to DP transition in IP$_3$R double knockout mice.
(**a**) Expression of surface CD4 and CD8 in adult thymi of Itpr1$^{fx/-}$ Itpr3$^{-/-}$Tie2Cre, Itpr2$^{-/-}$ Itpr3$^{fx/-}$Tie2Cre, Itpr1$^{fx/-}$ Itpr2$^{-/-}$Tie2Cre, and Control mice. (**b**) CD8$^+$CD4$^-$ thymocytes were subgated, and surface expression of CD24 and CD3 was examined. Numbers in plots indicate percentage of cells. Data are representative of a minimum of three independent experiments.

these results provide genetic evidence that *Itpr* genes are functionally redundant throughout the DN to DP transition of thymocyte development, and reveal a unique and non-redundant role for *Itpr2* after the DP stage.

3.3.3 Loss of IP_3R-Ca^{2+} influx impairs pre-TCR mediated proliferation and survival

To further characterize the developmental defect observed at the DN stage in IP_3R-TKO neonates, we examined the lineage-negative thymocyte distribution between the DN1 to DN4 stages. The successful rearrangement and expression of intracellular TCRβ (i.c.TCRβ) can be detected between DN2 and DN3. This process, also known as the β-selection checkpoint, ensures the formation of a functional pre-TCR and is required for development beyond the DN3 stage[215]. Consistent with a defect in pre-TCR signaling[41], we observed a an accumulation of DN3 thymocytes that were $CD25^{bright}$(Figure 3.6a). However, DN3 cellularity was only slightly elevated when compared to control (Figure 3.6b). Importantly, IP_3R-TKO mice displayed a marked increase in DN4 thymocytes. We next examined the expression of intracellular (ic) TCRβ in thymocytes pre- and post β-selection. Expression of icTCRβ in IP_3R-TKO DN3 and DN4 thymocytes was similar to that of controls (Figure 3.6c). IP_3R-TKO thymocytes express low amounts of Ikaros and E2A, and increased Id2, an E protein antagonist (see chapter 3.3.4). As $Ikaros^{-/-}$ and $E2A^{-/-}$ thymocytes develop independent of pre-TCR signaling thus bypassing β-selection we sought to exclude the possibility that IP_3R-TKO T cells have bypassed the β-selection

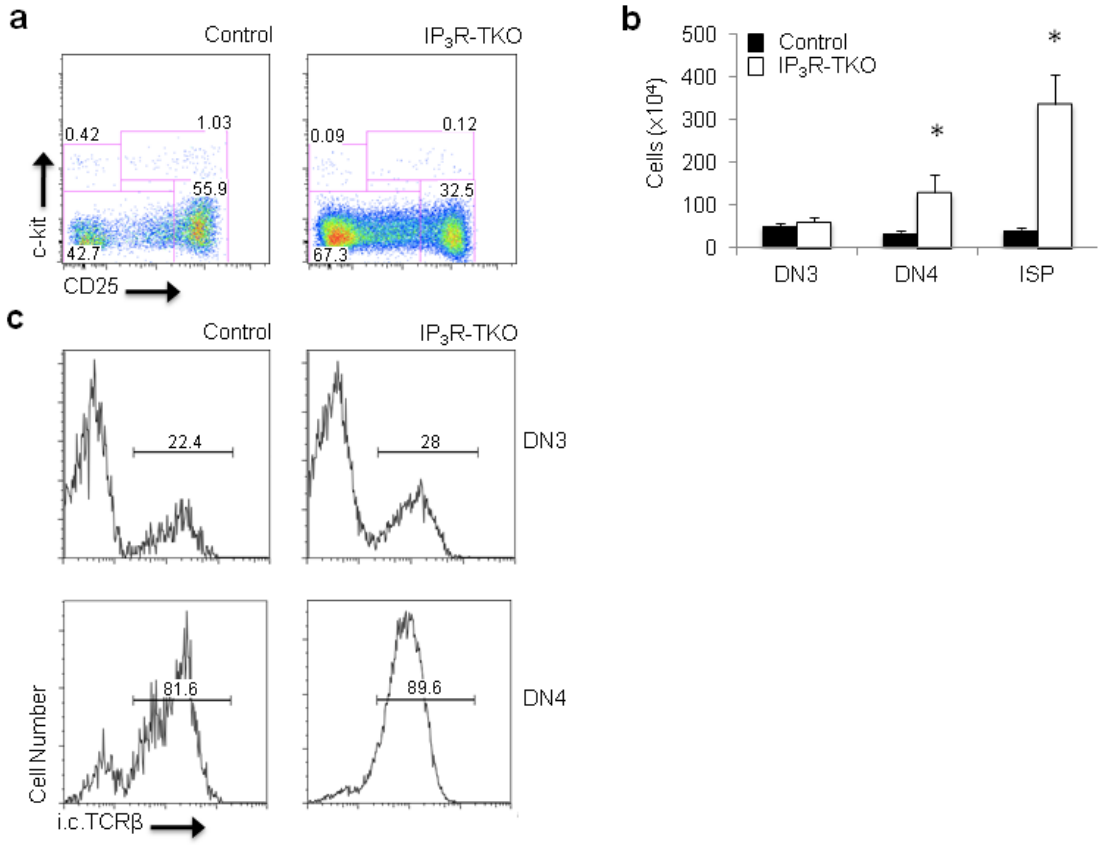


Figure 3.6 Accumulation of DN4 thymocytes in IP_3R-TKO mice.
(**a**) C-Kit and CD25 surface expression in lineage$^-$ thymocytes of control and IP_3R-TKO mice. (**b**) Total cellularity of populations depicted in (**a**) (mean ± SEM)**.** (**c**) Intracellular TCR-β (i.c.TCRβ) chain expression in DN3 and DN4 thymocytes of control and IP_3R-TKO mice. Data are representative of a minimum of three independent experiments. *P< 0.05.

checkpoint. IP_3R-TKO mice were bred into a $Rag2^{-/-}$ genetic background. $Rag2^{-/-}$ thymocytes cannot undergo TCRβ rearrangement and thus do not receive pre-TCR signals, arresting thymocyte development at the β-selection checkpoint[216]. T cell development was completely blocked at the DN3 stage in $Rag2^{-/-}IP_3R$-TKO thymi, indicating that the β-selection checkpoint remained intact in the absence of pre-TCR-mediated Ca^{2+} release (Figure 3.7). These data indicate that IP_3R-Ca^{2+} is not required for TCRβ rearrangement or expression, and is not required to enforce the β-selection checkpoint. Importantly, thymocyte development is specifically impaired at pre-TCR dependent stages of development in IP_3R-TKO mice, demonstrating a requirement for IP_3R-Ca^{2+} in pre-TCR signaling.

We next sought to determine the cause of the significantly decreased thymic cellularity and accumulation of DN4 and ISP thymocytes in the IP_3R-TKO mice. Decreased thymic cellularity can be the result of reduced proliferation or increased cell death. We examined thymocyte proliferation in neonatal IP_3R-TKO and control mice by injecting mice with Bromodeoxyuridine (BrdU) to determine the percentage of cells in S phase of the cell cycle. As thymocytes do not undergo endoreduplication, this method is appropriate for estimating proliferation. IP_3R-TKO thymocyte proliferation was modestly reduced at both DN4 and ISP stages (Figure 3.8a). This inhibition of proliferation was limited to the highly proliferative post β-selection stages of development as proliferation in the normally quiescent DN3 and DP thymocyte populations was similar to control, suggesting IP_3R-TKO thymocytes retain the normal mechanisms that control cell

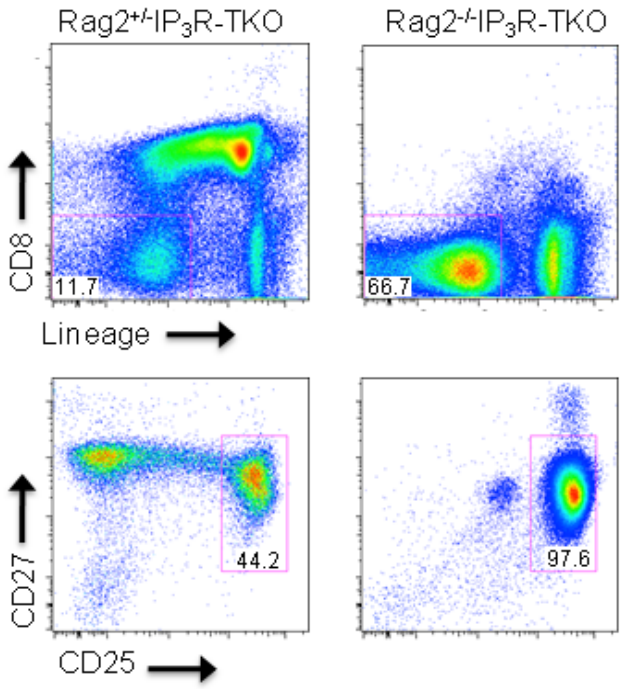


Figure 3.7 IP_3R-Ca^{2+} influx is not required for the enforcement of β-selection.
Thymocytes from IP_3R-TKO $Rag2^{+/-}$ and IP_3R-TKO $Rag2^{-/-}$ were stained for lineage markers (CD3, CD4, Mac1, Gr1, Ter119, B220, γδ-TCR) and surface CD8 expression (top). $CD8^-Lineage^-$ thymocytes were then subgated and examined for surface CD27 and CD25 expression (bottom). Data are representative of three independent experiments.

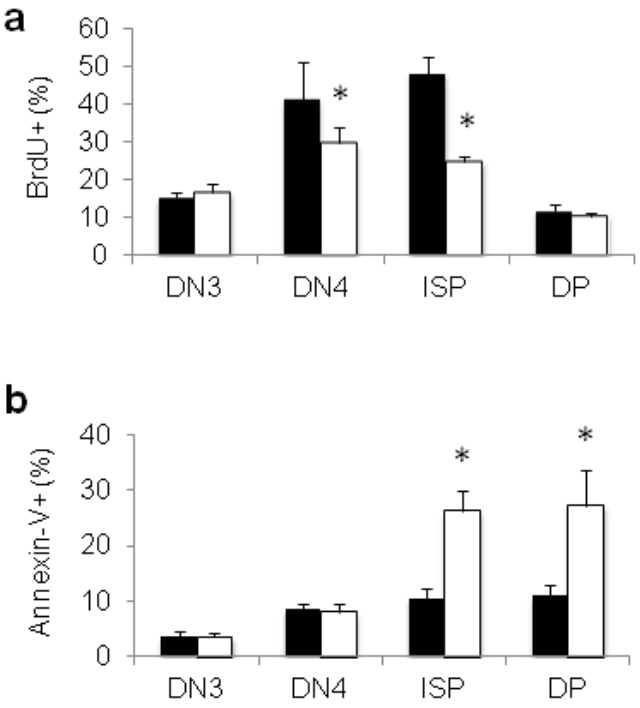


Figure 3.8 IP_3R-Ca^{2+} influx is essential for pre-TCR mediated proliferation and survival.

(**a**) Thymocyte proliferation measured by BrdU-incorporation in Control and IP_3R-TKO mice. (**b**) Apoptosis measured by annexin-V staining. Data are representative of a minimum of three independent experiments. *P < 0.05.

cycle progression and quiescence at the critical TCR selection checkpoints. To determine if loss of IP_3R affects cell survival, we examined surface Annexin-V expression, an early marker of apoptosis, in IP_3R-TKO and control thymocytes. In agreement with previous studies linking pre-TCR Ca^{2+} signaling and thymocyte survival[188,217], we observed a significant increase in apoptosis at the ISP stage in IP_3R-TKO mice (Figure 3.8b). DP thymocyte apoptosis was also significantly increased. This decrease in survival was limited to stages late in the DN-to-DP transition as no significant difference in apoptosis was detected in DN3 and DN4 thymocytes. The lack of a survival defect in IP_3R-TKO DN4 thymocytes as compared to their immediate progeny, the ISP thymocyte, may be a consequence of a delay in down-regulation of the pro-survival signals thymocytes receive prior to successful pre-TCR signaling, or reflect a stage-specific requirement for pre-TCR signaling through IP_3R-Ca^{2+} in promoting the survival of pre-DP thymocytes. The mechanisms that control stage-specific thymocyte survival during the DN4-to-DP transition are relatively unknown and merit further investigation. Taken together, these data strongly suggest that the decrease in thymic cellularity observed in IP_3R-TKO mice is a combined result of both impaired thymocyte survival and proliferation, and that the accumulation of DN4 and ISP thymocytes is not a result of a survival advantage or uncontrolled proliferation. These data also demonstrate that IP_3R-Ca^{2+} is required for thymocyte survival at the ISP-to-DP transition and is essential for the proliferative burst associated with pre-TCR signaling in DN4 and ISP thymocytes.

As we did not observe an increase in proliferation or survival of IP_3R-TKO thymocytes that might explain the accumulation of cells at the DN4 and ISP stages, we next sought to determine whether the increased numbers of immature thymocytes was due to a developmental delay in the DN-to-DP transition that resulted in the accumulation of DN4 and ISP cells. In the late fetal thymus, the differentiation of one synchronous wave of thymocyte precursors can be observed[218]. We therefore examined T cell development in embryonic day (ED) 17.5 fetal thymi (Figure 3.9a). IP_3R- TKO thymocytes failed to develop to the DP stage, and accumulated at the DN4 and ISP stages, consistent with a delay in thymocyte differentiation through the post β-selection stage. Thus, in the absence of IP_3R-Ca^{2+}, post β-selection differentiation of thymocytes stalls. This results in an accumulation of cells at the DN4 and subsequent ISP stages as thymocytes inefficiently transition to the DP stage.

We next sought to address whether the developmental block observed *in vivo* was intrinsic to thymocytes lacking *Itpr*. Sorted DN3 thymocytes from IP_3R-TKO and littermate controls were cultured on OP9-DL1 cells for eight days, after which differentiation to the DP stage was assessed. In contrast to littermate controls, IP_3R-TKO thymocytes failed to progress to the DP stage and accumulated at DN4, recapitulating the delay in differentiation observed *in vivo* (Figure 3.9b). Collectively, these data identify a cell-autonomous requirement for IP_3R-Ca^{2+} at the β-selection checkpoint and in the pre-TCR-mediated differentiation, survival, and proliferation of αβ T cells.

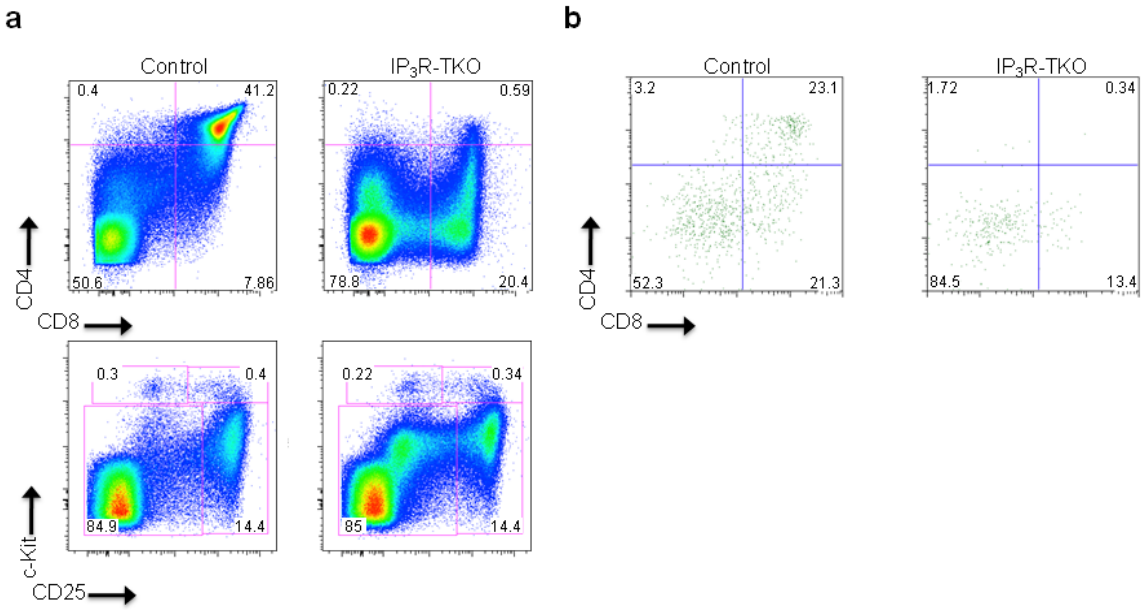
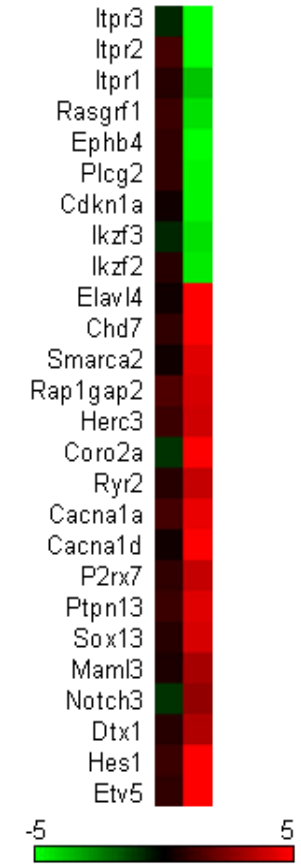


Figure 3.9 Thymocyte accumulation in IP_3R-TKO mice is due to inefficient transition of precursors through the post β-selection stages of development.

(**a**) Expression of surface CD4 and CD8 in ED 17.5 thymocytes (top) of control and IP_3R-TKO mice. Lineage$^-$CD4$^-$CD8$^-$ thymocytes were then subgated and examined for surface CD25 and c-Kit expression (bottom). (**b**) Sorted DN3 thymocytes from control and IP_3R-TKO mice were cultured on OP9-DL1 stromal cells for eight days and examined for expression of surface CD4 and CD8. Data are representative of two independent experiments.

3.3.4 IP_3R-Ca^{2+} reinforces an αβ T cell lineage gene regulatory program post β-selection

While our data demonstrated that pre-TCR Ca^{2+} was essential for the DN4-to-DP transition, genes and pathways regulated by Ca^{2+} at these stages have not been identified. To address this, we performed a global analysis of gene expression in ISP thymocytes isolated from IP_3R-TKO and control thymi (Figure 3.10a). We identified 954 differentially expressed genes at the ISP stage. KEGG pathway analysis of the differentially expressed genes revealed their involvement in focal adhesion, ECM-receptor interactions, tight junctions, endocytosis, and phosphatidylinositol-, and calcium signaling pathways (Figure 3.10b). Increased expression of *Sox13* and several direct Notch1 transcriptional targets such as *Hes1*, *Dtx1*, and *Notch3* were confirmed by quantitative RT-PCR (Figure 3.10c and data not shown). *Sox13* is a γδ-T cell lineage-restricted transcription factor that opposes the development of αβ T cells by antagonizing the high mobility group (HMG) box transcription factor *Tcf-1* (*Tcf7*)[86]. Tcf-1 is essential for differentiation and survival of thymocytes through the DN to DP transition, and its expression is rapidly increased upon pre-TCR signaling[74]. The phenotype of IP_3R-TKO mice bears a striking resemblance to Sox13 transgenic (*Sox13Tg*) and Tcf-$1^{-/-}$ mice. In all three models, thymocyte cellularity is significantly decreased as a consequence of impaired of αβ T cell development. Thymocytes of *Sox13Tg* and Tcf-$1^{-/-}$ display impaired DP thymocyte survival, and proliferation is impaired post β-selection. Importantly, a developmental arrest at the ISP stage is observed in Tcf-$1^{-/-}$ and IP_3R-TKO. Since the phenotypes of *Sox13*Tg and *Tcf-$1^{-/-}$*



b

KEGG Pathway Analysis of Ca^{2+}-regulated genes

Category	Count	Percent	p-Value
Focal adhesion	42	0.45	$7.53E^{-15}$
ECM-receptor interactions	25	0.27	$1.45E^{-12}$
Calcium signaling pathways	29	0.31	$2.98E^{-7}$
Tight junction	18	0.19	$5.94E^{-4}$
MAPK signaling pathway	26	0.28	$3.1E^{-3}$
Phosphatidylinositol signaling	10	0.11	$1.5E^{-2}$
Endocytosis	19	0.21	$1.6E^{-2}$

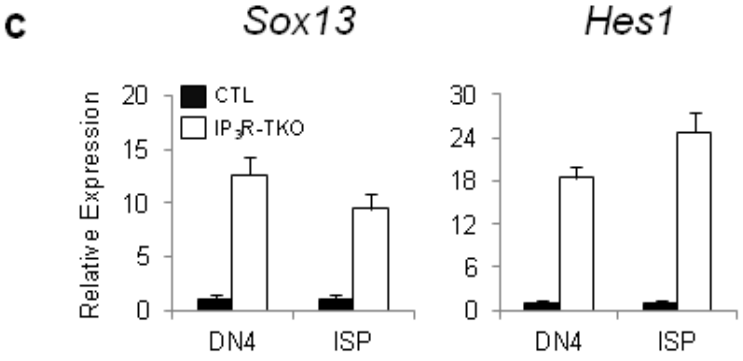


Figure 3.10 Deregulation of gene expression in the absence of IP_3R-Ca^{2+}. (**a**) Heat map for a subset of genes from microarray analysis of IP_3R-TKO and control ISP T cells. (**b**) KEGG analysis for functional pathways regulated by IP_3R-Ca^{2+} with a 3-fold threshold of differential expression. (**c**) Neonatal gene expression of *Sox13* and *Hes1* in IP_3R-TKO as measured by qRT-PCR in sorted DN4 and ISP thymocytes normalized to *18s* rRNA expression. Values represented are fold increase relative to Control (CTL). Data are representative of one (**a,b**) and three (**c**) independent experiments.

mice resemble that of our IP_3R-TKOs, we examined the expression of Sox13 and Tcf-1 (Tcf7) by western blot analysis. In agreement with gene expression data, Sox13 was significantly increased in ED 17.5 IP_3R-TKO thymi, the majority of which are transitioning to the DP stage, and Tcf-1 expression was nearly absent (Figure 3.11a). Importantly, expression of Tcf-1 and Sox13 proteins were similar in $Rag2^{-/-}IP_3R$-TKO and $Rag2^{-/-}$ thymocytes, demonstrating that IP_3R-Ca^{2+} influences their expression after TCRβ rearrangement (Figure 3.11b). In thymocytes, Tcf-1 functions as both a transcriptional activator and repressor through interactions with β–catenin. At the DN stage, Tcf-1 acts to repress Lef1 and Id2[76,77], and is essential for mediating DP thymocyte survival by increasing expression of the pro-survival protein Bcl-x_L[73]. Similar to *Tcf-1*$^{-/-}$, we observed elevated expression of Lef1 and Id2 in IP_3R-TKO thymocytes and decreased amounts of Bcl-x_L, consistent with diminished Tcf-1 function. Expression of Mc1-1 and Bcl-2, which are not regulated by Tcf-1 at the DP stage, was normal (Figure 3.14). Together with our IP_3R loss-of-function studies, these data indicate a requirement for IP_3R-mediated Ca^{2+} release downstream of the pre-TCR in establishing a gene regulatory program in post β-selected thymocytes, whereby Ca^{2+} signaling acts to reinforce the differentiation and survival of the αβ T cell lineage by repressing Sox13 thus enabling up-regulation of Tcf-1 post β-selection.

Notch signaling is required for T lineage specification and commitment[27,30,65]. Moreover, Notch and pre-TCR signals collaborate in mediating the differentiation, proliferation, and survival of T cells through the

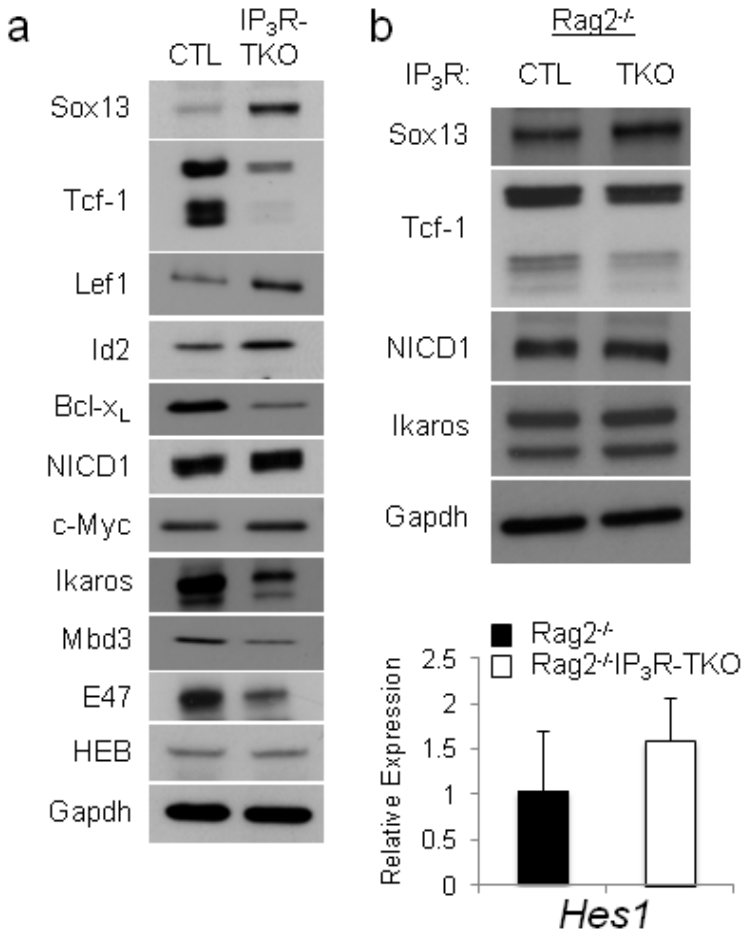


Figure 3.11 Loss of Tcf-1 and gain of Sox13 and Notch target gene expression in IP_3R-TKO mice

(**a**) Western blot analysis of pooled ED 17.5 thymi of control and IP_3R-TKO total thymocytes. Cleaved intracellular Notch1 (NICD1). (**b**) Western blot analysis (top) and qRT-PCR analysis of *Hes1* expression (bottom) of control and IP_3R-TKO total thymocytes on a $Rag2^{-/-}$ background normalized to *18s* rRNA expression. CTL, Control. Data are representative of three independent experiments.

β-selection checkpoint[41,219]. Of note, Notch signaling is repressed in T cells following β-selection by the Ikaros-NuRD chromatin remodeling complex[119,120]. Our microarray analysis of IP_3R-TKO ISP cells revealed up-regulated Notch targets in mutant ISP cells compared to controls, suggesting the dysregulation of Notch signaling. To investigate mechanisms responsible for this, we examined the expression of potential Notch signaling regulators by western blot analysis. Ikaros (Ikzf1) protein levels were severely reduced in ED 17.5 IP_3R-TKO thymocytes (Figure 3.11b). Mbd3, a central component of the NuRD chromatin remodeling complex required for NuRD-Hdac interaction[220], was also reduced in mutants relative to controls (Figure 3.11b). The change in Mbd3 expression was specific, as no differences were observed in other NuRD complex components or in the expression of another chromatin remodeling complex known to regulate Notch1, PRC2[123] (Figure 3.14). The E protein E47 positively regulates the expression of Notch1, and this function is antagonized by Id proteins at the β-selection checkpoint[105]. However, E47 (Tcf3) protein was reduced in ED 17.5 IP_3R-TKO thymocytes (Figure 3.11b). The E protein HEB is also required for the DN to DP transition, and its loss-of-function leads to a developmental block at the ISP stage[98]. In contrast to E47, HEB protein levels in IP_3R-TKO were similar to that in controls (Figure 3.11b). While HEB protein levels were not decreased, a disruption of HEB function due to increased Id2 expression cannot be excluded as a causative factor in the accumulation of ISP thymocytes and reduced DP thymocyte survival. Given the reduction of E2A observed in IP_3R-TKO thymocytes that would otherwise serve to buffer the availability of Id2 to disrupt

HEB function, an investigation into the contribution of E proteins to the developmental phenotype of IP_3R-TKO merits further investigation.

The expression of Ikaros, E47, Id2, total and cleaved intracellular Notch1 (NICD1) protein, and *Hes1* mRNA in $Rag2^{-/-}IP_3R$-TKO was similar to $Rag2^{-/-}$ thymocytes (Figure 3.11b), consistent with IP_3R-Ca^{2+} influencing Notch target gene expression after TCRβ recombination. Notch signaling can promote the degradation of E47 through activation of the Ras-Mapk pathway[221], but we did not observe a difference in Erk phosphorylation at this stage of development in mutant thymocytes relative to controls (Figure 3.11b). Notch receptor activity is controlled at multiple levels, including receptor maturation and processing, proteolytic cleavage, endocytic sorting, and degradation[18]. Therefore, the up-regulation of Notch target gene expression in IP_3R-TKO might also reflect changes in activity or availability of the Notch receptor. However, in agreement with gene expression in ISP thymocytes, no changes in the expression of total Notch1 or NICD1 were observed in ED 17.5 IP_3R-TKO thymocytes compared to controls (data not shown and Figure 3.11b). Taken together, these results indicate a requirement for IP_3R-mediated Ca^{2+} signaling in post β-selected thymocytes to repress Notch target gene expression, likely through positive regulation of the Ikaros-NuRD complex gene expression and activity.

3.3.5 Impaired survival and development of T-ALL in IP_3R-TKO mice

As early as 6 weeks of age, IP_3R-TKO and IP_3R-DFNKO mice developed thymic hyperplasia (data not shown), followed by the appearance of massive

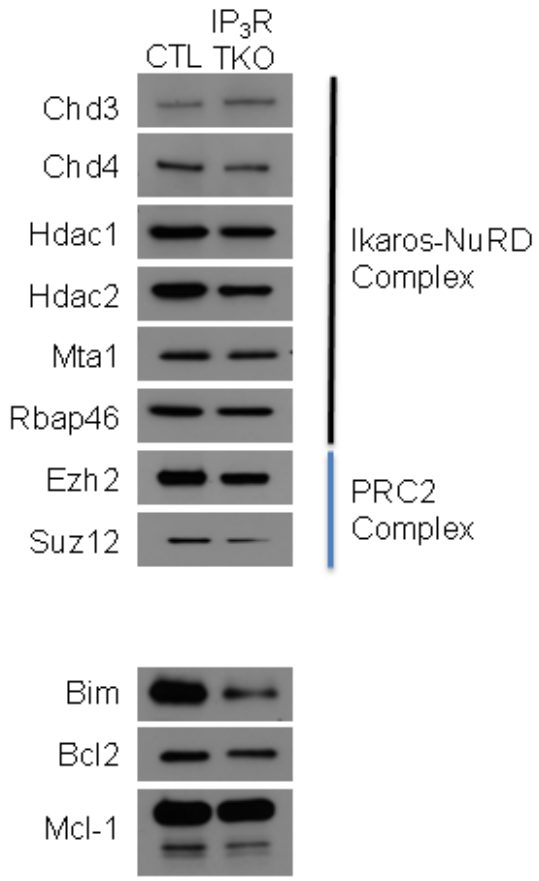


Figure 3.12 Normal expression of PRC2 and loss of Bim expression in IP_3R-TKO thymocytes.
Western blot analysis of pooled ED 17.5 thymi of Control and IP_3R-TKO total thymocytes. CTL, Control. Data are representative of three independent experiments.

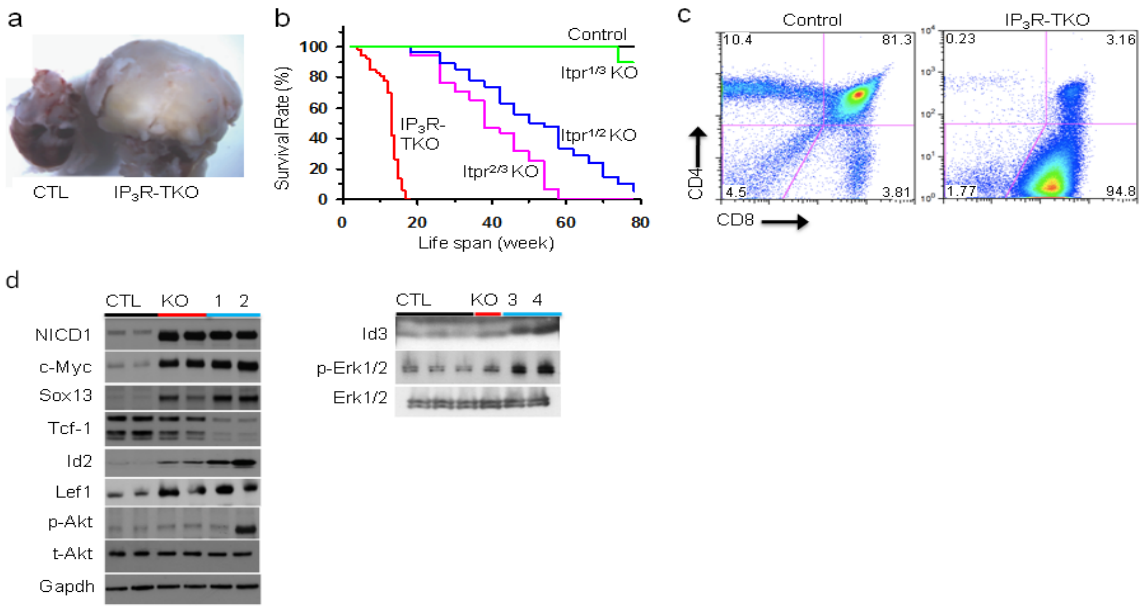


Figure 3.13 Development of T-ALL in the absence of IP_3R.
(**a**) Thymus of adult tumor-bearing IP_3R-TKO mouse compared to control thymus. (**b**) Kaplan-Meier survival curve of double and triple Itpr knockout lines (n>100, IP_3R-TKO). *Itpr1*$^{fl/-}$*Itpr3*$^{-/-}$*Tie2*Cre (Itpr$^{1/3}$ KO), *Itpr1*$^{fl/fl}$*Itpr2*$^{-/-}$*Tie2*Cre (Itpr$^{1/2}$ KO), or *Itpr2*$^{fl/-}$*Itpr3*$^{fl/fl}$*Tie2*Cre (Itpr$^{2/3}$ KO). (**c**) Surface CD4 and CD8 expression of control and T-ALL burdened thymi of IP_3R-TKO at 10 weeks of age. (**d**) Western blot of cleaved intracellular Notch1 (NICD1), c-Myc, Tcf-1, Sox13, Lef1, Id2, Id3, and total and phosphorylated Erk and Akt in adult control and IP_3R-TKO hyperplastic (KO), T-LBL (1,3), and T-ALL (2,4) burdened thymi. Data are representative of a minimum of ten (**a**, **c**), three (**d**), and one (**b**) independent experiments. CTL, Control.

thymic tumors by 12 weeks of age. These tumors resembled localized T cell lymphoblastic lymphoma[222] (T-LBL), since tumor invasion into adjacent tissues was observed, but disseminated disease was not (Figure 3.13a). By 16 weeks, severe morbidity and mortality were observed (n>100, IP_3R-TKO; Figure 3.13b), concomitant with significant lymphoblast infiltration of the peripheral blood, bone marrow, and spleen. The progression of advanced T-LBL to T cell acute lymphoblastic leukemia (T-ALL) is marked by a transition of bone marrow to include ≥25% immature lymphoblasts, which moribund IP_3R-TKO mice frequently possessed lymphoblasts. Post-mortem histological examination also revealed leukocyte infiltration into non-lymphoid organs including the lungs and liver (Figure 3.14). Lymphomas were transplantable, $CD8^+CD4^-CD3^-CD24^{hi}$, and expressed intracellular TCR-β consistent with cells arrested at the ISP cell stage (data not shown and Figure 3.13c). We also observed a progressive decrease in survival beginning at 6 months of age in *Itpr1*$^{fl/fl}$*Itpr2*$^{-/-}$*Tie2*Cre and *Itpr2*$^{fl/-}$ *Itpr3*$^{fl/fl}$*Tie2*Cre mice, but thymic tumors were not observed upon post-mortem examination. Together these findings suggest that the loss of all three *Itpr* genes leads to the development of an aggressive T-LBL corresponding to the ISP stage of development that rapidly disseminates and progresses to T-ALL.

3.3.6 Activation of Notch signaling and inhibition of Tcf1 function by Sox13 promote the development of T-ALL in IP_3R-TKO mice

Activating mutations of Notch1 have been implicated in the development of both T-LBL and T-ALL in mice and humans[223]. As post β-selected thymocytes in IP_3R-TKO mice fail to repress key Notch signaling targets, we examined

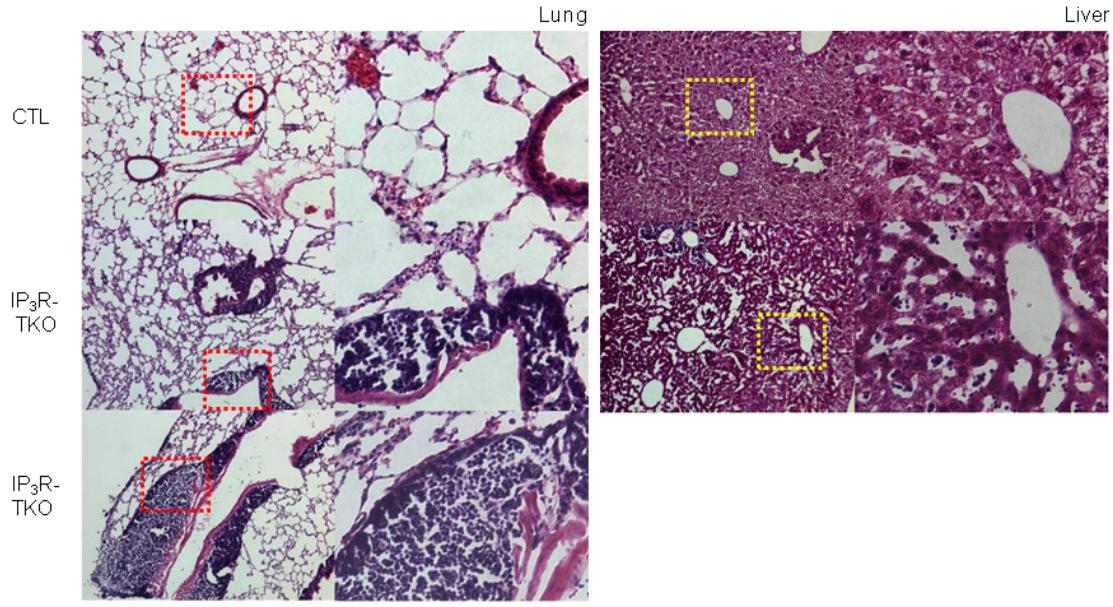


Figure 3.14 Leukocyte infiltration into lung and liver of moribund IP_3R-TKO mice.
H&E staining of lung (left) and liver (right) samples. Red dashed boxes represent image area magnified on right (lung), yellow dashed boxes represent image area magnified on right (liver).

Notch1 activity in tumor-burdened adult thymi. In contrast to fetal IP_3R-TKO thymocytes, NICD1 was significantly increased in adult IP_3R-TKO hyperplastic and tumor-burdened thymi compared to littermate controls (Figure 3.13d). The proto-oncogene c-Myc is an important transcriptional target of Notch1 that contributes to the development and growth of both T-LBL and T-ALL[222]. In IP_3R-TKO fetal thymocytes, c-Myc expression was similar to that in controls. However, in mutant adult thymi, c-Myc overexpression was coincident with hyperplasia, consistent with c-Myc activation being a critical early event in Notch1-mediated oncogenesis[224]. Oncogenic activation of Notch signaling in T-ALL is also associated with increased Ras-Mapk and PI3K-Akt activity[225]. Therefore, we examined the phosphorylation status of Erk and Akt. Erk phosphorylation was increased in the majority of tumors examined, but not at the onset of thymic hyperplasia in adult IP_3R-TKO (Figure 3.13d). In contrast, Akt phosphorylation was increased only in animals where disease had spread outside the thymus and into adjacent tissues and the bone marrow, in agreement with previous studies linking PI3K-Akt activation and the progression of T-LBL to T-ALL[222].

Deletions in TCF-1 have been reported in a subset of pediatric T-ALLs that resemble ETPs (ETP-ALL), and this subtype was associated with diminished expression of *TCF-1*[76,77]. Tcf-$1^{-/-}$ mice succumb to precursor T cell lymphoblastic lymphomas that resemble human T-ALL consistent with a role for Tcf-1 as a classical tumor suppressor. The development of T cell lymphomas in Tcf-$1^{-/-}$ mice was dependent upon high *Lef1* expression, as Lef1 deletion significantly delayed or prevented malignant transformation. Based upon the observation that IP_3R-

TKO fetal thymocytes display aberrant up-regulation of the Tcf-1 antagonist *Sox13* and lack significant *Tcf-1* expression, we investigated whether Tcf-1 function was impaired in adult IP_3R-TKO tumors. Tcf-1 protein was severely diminished in tumor-burdened but not hyperplastic adult IP_3R-TKO thymi. In contrast, Sox13 was significantly up-regulated in adult IP_3R-TKO thymocytes, and displayed a progressive increase in expression as thymi transitioned from marked hyperplasia to overt malignancy (Figure 3.13d). Consistent with diminished Tcf-1 function in IP_3R-TKO tumors, Lef1 was uniformly up-regulated in hyperplastic and tumor-burdened IP_3R-TKO thymi. Interestingly, Id2 displayed a stepwise increase in expression as thymi developed marked hyperplasia and transitioned from T-LBL to T-ALL mirroring the loss of Tcf-1 protein. Increased Id3 expression was also observed in both T-LBL and T-ALL thymic tumors but not in younger adult thymi with only moderate hyperplasia. As Id3 expression appeared normal in IP_3R-TKO fetal thymocytes, gain of Id3 expression is likely independent of Tcf-1or Sox13 (data not shown). We also noted the onset of malignancy in IP_3R-TKO mice occurs at a far earlier age than reported for *Tcf-1*$^{-/-}$ mice[76], which suggests the presence of additional Ca^{2+}-dependent modifiers that contribute to the acceleration of disease independent of Sox13 and Tcf-1. Collectively, these results demonstrate that loss of *Itpr* leads to the development of T-ALL that involves multiple collaborating genetic events, including the activation of Notch signaling, aberrant Sox13 expression, and the progressive loss of the tumor suppressor Tcf-1.

3.4 Discussion

In T lymphocytes, pre-TCR signaling induces the rapid cell cycle entry, survival, and differentiation of β-selected thymocytes to the DP stage. Ca^{2+} signaling mediated through the pre-TCR-induced activation of Plcγ1 has long been thought essential for this developmental transition. However, the contribution of IP_3R-mediated ER Ca^{2+} release to pre-TCR Ca^{2+} signaling and the influence of Ca^{2+} signaling on the network of transcription factors that drive T cells through the DN to DP transition have not previously been addressed. We have examined for the first time the role of the Ca^{2+} ion channel IP_3R in early thymocyte development. IP_3Rs appear to be functionally redundant in early thymocyte development, as disruption of the DN-to-DP transition requires the loss of all *Itpr* genes, consistent with an absolute requirement for IP_3R-Ca^{2+} signaling in the development of the αβ T lineage. A novel role for IP_3R-Ca^{2+} in γδ T lineage development was also uncovered, consistent with a recent report implicating calcineurin-Nfat in the development of $Vγ5^+Vδ1^+$ fetal thymocytes. However, the precise function of Ca^{2+} signaling downstream of the pre-TCR has not been fully resolved. It is possible to interpret the results presented here as consistent with a requirement for IP_3R independent of Ca^{2+} release, however no precedent exists concerning the ability of IP_3R, a transmembrane ion channel found at the ER and plasma membrane, to directly regulate developmental processes outside of its role as a Ca^{2+} ion channel. Furthermore, while our results are consistent with a defect in pre-TCR signaling, and Ca^{2+} release represents a significant downstream signaling event of the pre-TCR[176], it is

possible that pre-TCR independent IP_3R-Ca^{2+} signaling through other receptors are responsible for the developmental defects observed in IP_3R-TKO thymocytes. However we believe this to be unlikely given the severe disruption in early thymocyte development when Plcγ1 is uncoupled from LAT, a specific and essential pre-TCR component, which was attributed to a specific defect in Ca^{2+} mobilization[133]. The essential role of TCR-Plcγ1-IP_3R at later stages of thymocyte development and previous studies highlighting a role for Ca^{2+} mobilization in TCR and pre-TCR signaling reinforce this view[125,128,132].

The significant but incomplete inhibition of αβ T lineage development when IP_3R is ablated suggests that Ca^{2+} signaling is not absolutely required for thymocyte development through the DN-to-DP transition. However, our model is likely limiting in that residual IP_3R protein was detected in IP_3R-TKO thymocytes, suggesting the potential for a strong selective pressure to retain at least one intact Itpr allele that is sufficient to drive thymocyte development up to the ISP and DP stages, at which point survival becomes significantly impaired and development is arrested. As we used a Tie2Cre to ablate *Itpr*, which is active in HSCs throughout ontogeny, and Plcγ1 is absolutely required for hematopoietic development[131], it is possible that the selective pressure to retain intact *Itpr* alleles is present throughout hematopoietic development. Therefore, to more stringently test the requirement for IP_3R-Ca^{2+} in thymocyte development, the least amount of conditional alleles possible should be employed.

The phenotype of IP_3R-TKO mice represents the most severe disruption of early T cell development to date that can be unequivocally linked to Ca^{2+}

signaling. Although several Ca^{2+}-dependent signaling pathways have been shown to influence early T cell development, the developmental delays observed in these models can be best attributed to defective survival than differentiation, such as with classical Ca^{2+}-dependent PKCs[188,214]. Furthermore, the relevance of other prominent Ca^{2+}-regulated pathways such as calcineurin-Nfat to early T cell development remains controversial as no defects are observed in physiological settings[182,217]. Thus, prior to this work little was known about the downstream Ca^{2+}-dependent transcriptional targets and signaling pathways that are essential for thymocyte differentiation. The identification of Sox13 as a potential target of Ca^{2+} signaling in post β-selection thymocytes is the focus of on-going research in the lab, as we are attempting to separate the effects of Sox13 from that of IP_3R-Ca^{2+} potentially through the use of siRNA knockdown techniques and conventional genetic methods. In addition, we are attempting to define the mechanism of Ca^{2+}-dependent Sox13 regulation through examination of the Sox13 promoter and enhancer regions. Important differences exist between the phenotypes of IP_3R-TKO and *Sox13Tg* mice, and this will also be the focus of future studies.

Loss of IP_3R leads to a disruption in the DN-to-DP transition, and a specific block at the ISP stage. The E protein HEB has been implicated as essential in the ISP-to-DP transition[98], as well as Tcf-1[71]. The severe block at the ISP stage in IP_3R-TKO resemble $HEB^{-/-}$ more so than Tcf-$1^{-/-}$, and the balance of E and ID proteins is significantly altered in IP_3R-TKO thymocytes. It will be important to determine whether HEB function is disrupted in IP_3R-TKO mice. This

may be accomplished by gene expression studies examining HEB transcriptional targets as well as through modulating Id2 or HEB expression in IP_3R-TKO thymocytes. The specific impairment in the survival of post β-selected thymocytes is consistent with the known role of Ca^{2+} signaling, however the restriction of this defect to stages late in the DN-to-DP transition was unexpected, given that thymocytes are sensitive to disruptions in IP_3R-Ca^{2+} as early as the DN2 stage and pre-TCR Ca^{2+} signals appear to specifically up-regulate pro-survival factors[188]. The difference in timing for the requirement of IP_3R-Ca^{2+} between this work and others may be due to a number of factors, including the limitations of examining thymocyte development *in vitro* using immortalized cell lines of defined developmental stage, the use of chemical inhibitors with varying degrees of toxicity, and the possibility of a strong selective pressure for thymocytes to retain even low amounts of IP_3R expression. Future work investigating the stage-specific requirements for pre-TCR signaling in thymocyte survival throughout the DN4-to-DP transition will be required to further address this issue.

The data we have presented here suggest a model in which pre-TCR Ca^{2+} signaling through IP_3R establishes a gene regulatory program in post β-selected thymocytes that reinforces the differentiation and survival of the αβ T cell lineage (Figure 3.15a). In the absence of pre-TCR Ca^{2+} signaling, T cell differentiation is arrested at the DN4 and ISP stages, and the survival and proliferation of ISP thymocytes is compromised. Loss of IP_3R leads to the de-repression of the Tcf-1 antagonist Sox13, as well as up-regulation of Notch1 transcriptional target genes

such as *Hes1*, tying pre-TCR Ca^{2+} signaling to the repression of Notch signaling subsequent to β-selection. Loss of IP_3R function also leads to the development of highly aggressive T-ALL, suggesting a previously unappreciated role for pre-TCR Ca^{2+} signaling in suppressing the development of leukemia (Figure 3.15b). Future studies to investigate the relationship of Sox13 to leukemia are warranted, as deletions in the tumor suppressor TCF-1 have only been identified in a small minority of ETP T-ALL cases while TCF-1 down-regulation is strongly associated with ETP-ALL[76].

The IP_3R-TKO phenotype presented here is notably less severe than homozygous LAT^{Y136F} knock-in mice, which had been reported to selectively impair PLCγ1-mediated Ca^{2+} mobilization, and raises the possibility that pre-TCR Ca^{2+} signaling is not mediated solely through PLCγ1-IP_3R Ca^{2+} release. Interestingly, the defects observed at later stages of thymocyte development in IP_3R-TKO mirror those of Plcγ1-deficient thymocytes. Specifically, the $CD4^+$ SP T cell lineage was virtually absent and unexpectedly, given the substantial decrease in DP thymocytes, normal numbers of $CD8^+$ SP T cells were observed. As TCR-Ca^{2+} mobilization was found to be severely impaired in thymocytes, we would suggest that in DP thymocytes undergoing TCR-mediated selection the activation of Ca^{2+}-dependent signaling pathways is virtually non-existent. Thus, DP thymocytes in IP_3R-TKO mice would be undergoing positive and negative selection in an environment deficient for calcineurin-Nfat (positive selection)[155] and PKC-Bim activation (negative selection)[156,181]. In these conditions, thymocytes that were prevented from dying are “positively” selected in response

to stronger signals that would normally induce cell death, as is the case in Plcγ1-deficient and Cnb1/Bim double-deficient mice. In support of this interpretation, we observed significantly reduced Bim expression in IP_3R-TKO thymocytes (Figure 3.12).

It has been proposed that a differential requirement for Ca^{2+} signaling exists throughout thymocyte development, where strong and sustained increases in Ca^{2+} are required for late thymocyte development, while small localized and transient Ca^{2+} release as mediated by the IP_3R is sufficient to promote early thymocyte development. This model was put forward to explain the lack of requirement for SOCE and other ion channels that modulate Ca^{2+} signaling in DP thymocyte selection, and that to date only one ion channel has been found to be required for thymocyte development, the magnesium channel Trpm7[125,128,159]. Our data are consistent with this hypothesis, and highlight the central role of Ca^{2+} signal throughout thymocyte development. Additional functions for Ca^{2+} in early T cell development remain to be explored, including the contribution of SOCE-independent Ca^{2+} and ion channels to pre-TCR Ca^{2+} signaling and development.

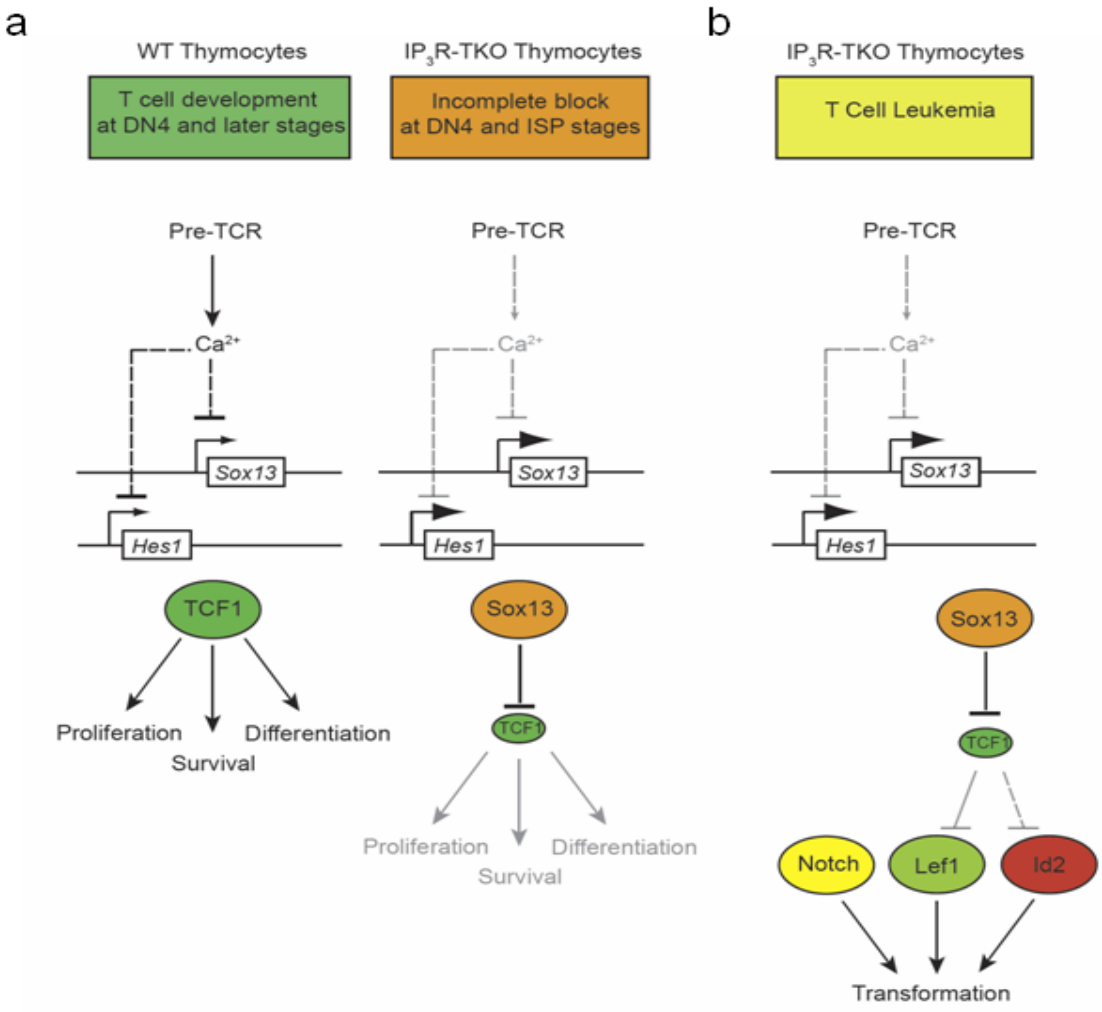


Figure 3.15 A model for Ca^{2+} signaling in early thymocyte development and malignancy.

(**a**) In wild-type thymocytes, signaling through the Pre-TCR initiates the release of Ca^{2+} from intracellular stores via IP_3R's. Ca^{2+}-dependent signaling pathways act to repress transcription of *Sox13* and the Notch transcriptional targets such as *Hes1*. In the absence of IP_3R and thus Ca^{2+} signaling downstream of the Pre-TCR, both *Sox13* and *Hes1* are derepressed. Aberrant expression of Sox13 impairs T cell development through antagonizing TCF-1 function. (**b**) IP_3R-deficient mice develop an aggressive T cell malignancy resembling human T cell acute lymphoblastic leukemia (T-ALL) involving multiple genetic events, including activation of Notch signaling and aberrant up-regulation of Sox13 thereby antagonizing the tumor suppressor function of Tcf-1.

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