The Role of IL-7/IL-7R Signalling in Thymic Dysfunction in HIV-1 infection

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Abstract

Immune reconstitution following T-cell depletion consists of expansion of circulating T-cells or *de novo* synthesis of T-cells from the thymus. The IL-7/IL-7 receptor signalling pathway is critical for the maturation and differentiation of thymocytes before they leave the thymus as mature T-cells. Viral infections including HIV have been shown to decrease IL-7Rα (CD127) expression on circulating $CD4^+$ and $CD8^+$ T-cells. However little is known about the effects of HIV infection on CD127 expression and activity in thymocytes despite existing evidence of HIV infection of the thymus. Thymic function is altered in HIV infection leading to a dysregulation of the thymic epithelial network and reduced thymic output which may contribute, in part, to impaired immune reconstitution in progressive HIV disease. *In vitro* studies demonstrate that HIV infection interrupts thymopoiesis resulting in a developmental block in thymopoiesis similar to that seen in models of IL-7/IL-7R deficiencies suggesting a role for altered IL-7 signalling in HIV associated thymic dysfunction. Therefore we hypothesize that thymic dysfunction which occurs in HIV infection is due to reduced IL-7R and/or altered IL-7 signalling in thymocytes resulting in impaired de novo T-cell synthesis.

In order to address this hypothesis an *in vitro* system for the functional study of human thymocytes has been optimized. The research conducted as part of this thesis assessed if *in vitro* HIV infection or if cytokines that are upregulated in the

course of HIV infection altered CD127 expression on maturing thymocytes. It also evaluated if *in vitro* HIV infection disrupts thymocyte function at different stages of maturation and whether this disruption in function is due to impaired IL-7/IL-7R signalling.

The host factors IL-7, TNF-α and IL-4, which are upregulated in HIV infection, are found to downregulate CD127 expression on thymocytes. IL-4 pre-treatment of thymocytes reduced the ability of IL-7 to induce STAT-5 phosphorylation. Furthermore following in *vitro* HIV infection of thymocytes, CD127 expression of single positive CD8 thymocytes was decreased. *In vitro* HIV infection altered IL-7 activity as demonstrated by lower levels of Bcl-2 and phospho-STAT-5 expression in thymocytes following IL-7 stimulation. These accumulated results suggests that HIV may play a role in impaired thymic function by altering IL-7 responsiveness.

Understanding the mechanisms of thymic dysfunction in HIV infection may provide some insight into therapies leading to immune reconstitution through increased thymic output.

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
AKT	Phosphorylation of protein kinase B
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumine
Bcl-2	B cell lymphoma 2
C	Celsius
CAEV	Caprine arthritis-encephalitis virus
CD	Cluster of differentiation
CDC	Centers for Disease Control
CHEO	Children's Hospital of Eastern Ontario
CFSE	Carboxyfluorescein succinimidyl ester
CT	Computed tomography
CTL	Cytotoxic T cells
CLP	Common lymphoid precursor
D	Diversity
DC	Dendritic cell
DL-1	Delta like 1
DMEM	Dulbecco's Modified Eagle Medium: Nutrient Mixture
DMSO	Dimethyl Sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid

DP	Double Positive
ECD	Phycoerythrin-Texas red
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EIAV	Equine infectious anemia virus
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluoroscein isothiocyanate
FIV	Feline immunodeficiency virus
FOXO	Forkhead box O1
FTOC	Fetal thymic organ culture
GM-CSF	Granulocyte-macrophage colony stimulating factor
HAART	Highly active antiretroviral therapy
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSC	Hemopoietic stem cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISP4	Immature single positive CD4
J	Joining
Jak	Janus Kinase
Kbp	Kilo base pair

kDa	Kilo Dalton
LN_2	Liquid nitrogen
MACS	Magnetic Affinity Cell Sorting
MEM	Minimal essential media
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NIH	National Institute of Health
NK	Natural killer cell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC5	PE-cyanin 5
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethyleneglycol
PHA	Phytohemaglutinin
PI	Propidium Iodide
PI3K	Phosphatidylionositol-3 kinase
PVDF	Polyvinylidene fluoride
PWM	Pokeweed mitogen
RAG	Recombination activation gene
RNA	Ribonucleic acid
SAPK	Stress-activated protein kinase
SCF	Stem cell factor
SCID	Severe combined immunodeficiency

SH2	Src homology 2
SIV	Simian immunodeficiency virus
SOCS	Suppressor of cytokine signal
SP	Single positive
STAT	Signal transducer and activator of transcription
SV40T	Simian vacuolating virus large T antigen
TAT	Transactivating regulatory protein
TCR	T-cell receptor
$TCID_{50}$	Tissue culture infectivity dose
TEC	Thymic epithelial cells
TGF	Transforming growth factor
TN	Triple negative
TNF	Tumour necrosis factor
TREC	TCR–rearrangement excision cercles
TSLP	Thymic stromal lymphopoietin
UNAIDS	United Nations AIDS
V	Variable

1. Introduction

The immune system is a highly specialized system that relies on the interplay of a large network of factors such as lymphoid organs, cells and molecular messengers (eg. chemokines and cytokines). Disrupting just one factor can result in an altered immune network leading to immunodeficiency, autoimmunity or impaired T-cell development.

1.1 Thymopoiesis

1.1.1 T-cell development in the thymus

The thymus is a glandular lymphoid organ located just above the heart. The thymus is the main site of T-cell development, although there is a small subset of cells that develop extrathymically in secondary lymphoid organs such as lymph nodes, spleen, Peyer's patches and the liver [1-4]. Extrathymic development does not follow the same developmental pathways as T-cell development in the thymus and gives rise to a unique repertoire of cells with distinct functions [1].

Hematopoietic stem cells (HSC) originate in the bone marrow and migrate into the thymus where they undergo stepwise maturation into mature T-cells. Initially the HSC are pluripotent and can give rise to many types of cells in the hematopoietic lineage [5-7]. Commitment to the T-cell lineage only occurs once the cells have entered the thymus. Specific cell surface markers can phenotypically distinguish each differentiation stage. All hematopoietic precursors express CD34 on the cell

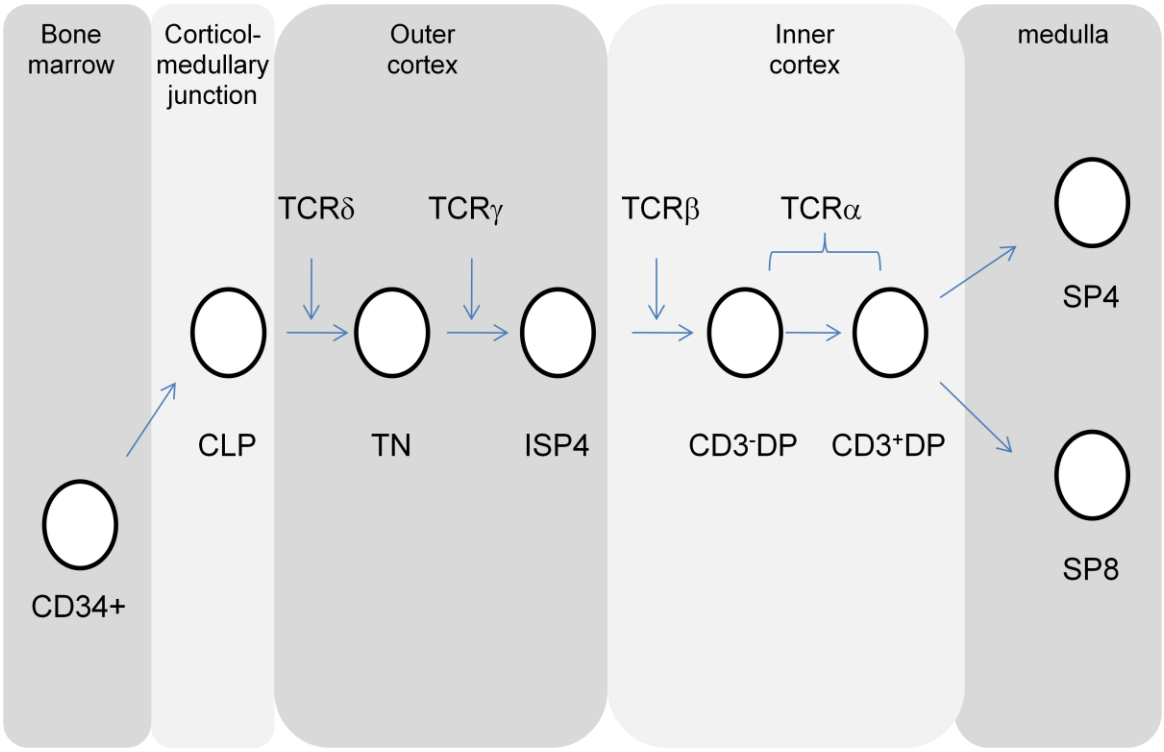
surface (reviewed in [8]). The earliest phenotype of the hematopoietic progenitor in the bone marrow, also known as the common lymphoid precursor (CLP), is $CD34^{+}CD38^{-}$, while the more differentiated committed progenitors are $CD34^{+}CD38^{+}$[9]. Complete T-cell lineage commitment only occurs following the up regulation of CD1a giving rise to cells that are $CD34^{+}CD38^{+}$ $CD5^{+}CD1a^{+}$ [10,11]. These early thymic precursors, known as pre-T1, encompass the immature subset, also known as triple negatives (TN) since they do not express the receptors CD3,CD4 or CD8 (Figure 1).

1.1.2 TCR gene rearrangement

A critical step in the maturation of thymocytes is T-cell receptor (TCR) gene rearrangement which involves the cutting and joining of variable (V), diversity (D) and joining (J) elements to create the rearranged TCR [12]. This rearrangement process occurs in order to maximize the repertoire of antigens recognized by the receptor. V(D)J rearrangement depends on the recombination activation genes (RAG)-1 and RAG-2 whose expression is highly regulated during T-cell development[13]. There are two different loci ($\alpha\beta$ or $\gamma\delta$) for gene rearrangement which give rise to two different T-cells differing in phenotype and function. $\alpha\beta$ T-cells makeup 95% of the T-cell pool, they express CD4 and CD8 receptors and have helper and cytotoxic functions. $\gamma\delta$ T-cells consist of 5% of the T-cell population, they do not express CD4 or CD8 molecules on their surface and their antigen stimulation is MHC independant[14].

Figure 1 T-cell development pathway

Progenitor cells that are CD34+ enter the thymus at the corticol-medullary junction. Once in the thymus, the precursor cells receive signals to commit to a T-cell lineage as the common lymphoid progenitor (CLP). The cells then migrate through the thymus undergoing stage specific maturation and differentiation. The CLP that enter the outer cortex are initially negative for CD3,CD4 and CD8 receptors (TN). Thymocytes undergo TCR-rearrangement in an ordered fashion TCRδ >TCRγ > TCRβ >TCRα. During the stages of TCR-rearrangement the cells upregulate the CD4 receptor giving rise to an intermediate step, the immature single positive CD4 (ISP4). Subsequently the thymocytes upregulate the CD8 receptor giving rise to the $CD3^{-/+}$DP cells. Once the cells enter the inner cortex they undergo negative selection by interacting with corticol thymic epithelial cells. The thymocytes then migrate to the medulla where they are positively selected and become either SP4 and SP8. These cells then leave the thymus to enter the circulation to form the mature T-cell pool.


Bone marrow
Corticol-medullary junction
Outer cortex
Inner cortex
medulla
TCRδ
TCRγ
TCRβ
TCRα
SP4
CLP
TN
ISP4
CD3-DP
CD3+DP
SP8
CD34+

The order of gene rearrangement in development is TCRδ, TCRγ, TCRβ followed by TCRα. TCRδ locus rearrangement is initiated in the $CD34^{+}CD1a^{-}$ thymic subset and the majority of the δ rearrangement occurs in the transition from $CD34^{+}CD1a^{-}$ to $CD34^{+}CD1a^{+}$. CD4 receptors are upregulated resulting in the immature single positive stage (ISP4) $CD3^{-}CD4^{+}CD8^{-}$. The bulk of the TCRγ locus rearrangement occurs between the TN to ISP4 transition. Next, thymocytes upregulate CD8 receptors, giving rise to the double positive (DP) $CD3^{-}CD4^{+}CD8^{+}$ thymocytes. The majority of the β locus rearrangement occurs in the transition from ISP4 to $CD3^{-}$DP stage [15]. This stage in the maturation of thymocytes is known as β-selection and is a critical checkpoint in the development pathway. Thymocytes that fail to rearrange the TCRβ locus will die by apoptosis.[16] Thymocytes that have successfully rearranged the TCRβ locus will continue the pathway forming the pre-TCR complex which comprises: β-chain, pre-TCRα locus and CD3 molecules [17]. Successful rearrangement is verified by specialized positive and negative selection processes.

1.1.3 Positive and negative selection

Positive and negative selection occur in order to form a repertoire of cells that recognize self- major histocompatibility complex (MHC) proteins but not self-peptide. The pre-TCR complex on the surface of thymocytes interacts with MHC molecules on the surface of cortical thymic epithelial cells (TEC)[13]. Selection occurs through direct interaction of cell surface ligands rather than soluble factors since chemically-fixed TEC are still able to support selection[18]. There are two critical events in the

selection process. Positive selection selects cells that are able to recognise and interact with self-MHC, while other cells die by apoptosis[19]. Negative selection targets cells that are able to recognise self-peptide, and signals them to die by apoptosis. These processes eliminate the development of a potentially hazardous auto-reactive T-cell[20]. A differential avidity model has been proposed for the mechanisms of T-cell selection. Thymocytes that recognize self-MHC/peptide with low avidity will receive positive signals and will survive. Thymocytes that recognize self-MHC/peptide with high avidity will receive signals for deletion and will die[21].

Following selection, cells migrate into the thymic medulla where they mature into either single positive CD4 (SP4) or single positive CD8 (SP8) thymocytes. Following positive selection, the continuous TCR signalling of DP cells lead to the activation of the zinc-finger transcription factors Th-Pok and GATA-3, which drive the lineage decisions toward $CD4^+$T-cells [22-24]. The termination of the TCR signal following positive selection allows DP thymocytes to respond to γ-chain cytokines and activate the transcription factor Runx3 and drive lineage commitment towards $CD8^+$ T-cells [25-28]. Only 1-3% of thymocytes will successfully complete development and leave the thymus to form the mature T-cell pool.

1.1.4 Thymic Microenvironment

Thymocyte development strongly relies on the thymic environment. The architecture of the thymus is such that thymocytes receive different signals from the thymic stroma as they migrate through the thymus. The stroma delivers environmental

cues which T-cells require in order to survive, proliferate and differentiate [29,30]. Chemokines as well as the flow of interstitial fluid regulate the trafficking of developing thymocytes[30]. Progenitor cells enter the thymus at the cortico-medullary junction. Positive selection takes place within the cortex and negative selection occurs in the medulla with interaction between thymocytes and TEC. Next, the selected thymocytes migrate to the medulla where they receive signals from medullary TEC to differentiate into either an SP4 or SP8 thymocyte [30]. The major subsets of stromal cells within the thymus are the TECs [13,31,32]. Thymic epithelial cells stimulate thymocytes by direct binding as well as by the release of soluble factors required for thymocyte differentiation and proliferation [33]. Cell-to-cell contact between thymocytes and TEC occurs via CD2 on thymocytes and CD58 on TEC [34]. Activated thymocytes interact with TEC via LFA-1 (CD11a) on thymocytes and ICAM-1 on TEC [35]. These interactions result in the secretion of soluble factors such as interleukin (IL) IL-7, tumour necrosis factor-α (TNF-α), IL-6, IL-1, granulocyte-macrophage colony stimulating factor (GM-CSF) and thymulin which are required for thymocyte differentiation and proliferation [33,36-41]. Thymocyte-TEC interaction is required in the organization and development of the thymic stroma. Defects in thymocyte development that minimize the cross-talk between thymocytes and TEC result in the disruption of the development of cortical and medullary TEC and the organization of stromal cells into a three dimensional structure[42,43].

1.2 *In vitro* models for the study of thymocytes

The study of thymocyte development and function requires an appropriate human *in vitro* culture system. Several culture systems have been developed in order to study thymocyte development, most of which have been conducted in murine models or using murine TEC or equivalent matrices. Each experimental approach has its own advantages and disadvantages. The foetal thymic organ culture (FTOC) consists of murine thymic lobes that are cultured on membranous filters and are able to support thymic development *in vitro* [13,33,44,45]. This system has demonstrated the capability of producing functional T-cells *in vitro* and is an excellent tool for the study of T-cell development. The advantage of such a system is that it maintains the architecture of the thymus which has been demonstrated to be important for *in vitro* T-cell development [44,46,47]. The limitation of this system is the technical difficulties of establishing organ cultures, the requirement for mouse thymic lobes (although these cited reports utilize human thymocytes on this platform), as well as the high cost and manipulation associated with such studies.

Another system which has been developed for the study of thymocytes is the dispersed cell culture system [48-51]. This system consists of culturing human thymocytes *ex-vivo* along with TEC in a suspension culture. The advantage of this system is the exclusive use of human TEC, eliminating any potential xenogeneic artefacts that may influence experimental outcomes. A disadvantage of this system is that certain genes are turned off when cells are not cultured in a three dimensional

architecture and an arrest in T-cell development may occur [29,47,52]. The three dimensional structure of the thymus is required to maintain Notch signalling which is a crucial pathway in the commitment of progenitor cells to a T-cell fate [52,53]. Nonetheless the dispersed culture system facilitates studies of the effects of exogenous stimuli or viral infections on individual thymic subsets, something that is quite difficult with FTOC.

A cell suspension model, developed by Zúñiga-Pflücker *et al*, involving the OP9-DL1 culture system has been shown to allow the development of hemopoietic stem cells (HSC) into mature T-cells [54]. OP9 cells are a murine bone marrow-derived stromal cell line, which express the notch ligand Delta like 1 (DL1). The advantage of this system is its simplicity and versatility. Although the system is based on a mouse cell line, the use of this co-culture system has been adapted for use with human thymocytes and has been successful in supporting the development of T-cells *in vitro* [55,56]. Choosing the appropriate study system is critical in the experimental design and data interpretation of any thymocyte functional studies.

1.3 IL-7/IL-7R system

1.3.1 Expression and signalling of IL-7 and it's receptor CD127

IL-7 is a 25 kDa glycoprotein belonging to the hematopoietin cytokine family [57]. IL-7 is produced by MHC II-expressing cortical thymic epithelial cells, bone marrow stromal cells, T-zone fibroblastic reticular cells, follicular dendritic cells, vascular

endothelial cells, keratinocytes and epithelial cells in the intestine [33,39,57-61]. IL-7 signals through the IL-7 receptor complex (IL-7R), which is composed of two subunits: the IL-7Rα chain (CD127) and the IL-2Rγ chain which is shared by a number of other cytokines including IL-2, IL-4, IL-9, IL-15 and IL-21 [62,63].

CD127 is expressed on lymphoid cells such as thymocytes, developing B-cells, mature T-cells, monocytes, macrophages, dendritic cells and epithelial cells. The human CD127 gene is 19 kbp and encodes a 65-75 kDa transmembrane glycoprotein composed of a 219 amino acid extracellular region, a 25 amino acid transmembrane region and a 195 intracellular region [64]. CD127 has 6 potential N-glycosylation sites, which have been associated with increased IL-7 binding by increasing its "on rate". There are two potential hypotheses to account for the increase in binding affinity. The first hypothesis suggests that glycosylation of CD127 may change the overall charge of the receptor affecting CD127/IL-7 binding. The second hypothesis suggests that glycosylation of CD127 may alter the conformation of unbound CD127, changing the balance between unbound CD127 and CD127 poised to bind IL-7 [65].

1.3.2 Regulation of CD127 expression

CD127 expression is highly regulated in both T-cell development and during immune responses. Bone marrow progenitor cells highly express CD127, but once developing cells have committed to a specific lineage the receptor is downregulated [66]. Immature B-cells express high levels of CD127 until the pro-B stage and CD127

expression is then lost by the mature B-cell stage. During thymopoiesis, CD127 is highly expressed on double negative (DN) immature cells and is then completely downregulated by the DP stage. This is hypothesized to occur in order to allow appropriate negative selection by avoiding the delivery of additional pro-survival signals, thereby avoiding the potential retention of auto-reactive T-cells. CD127 is again re-expressed following lineage selection on SP4 or SP8 cells [62,63,67]. In the periphery, naive T-cells express CD127. Once the cells encounter antigen and become effector T-cells, CD127 expression is greatly reduced, perhaps contributing to their propensity to die in the contraction phase (95% of cells die by apoptosis). In following a linear model of T-cell activation a subset of antigen-specific effector T-cells retain CD127 expression, survive the contraction phase and develop into memory T-cells[62,63].

1.3.2.1 γc cytokines affect CD127 expression on T-cells

Multiple cytokines have regulatory effect on the expression of CD127. In mice IL-2, IL-4, IL-7 and IL-15 stimulation suppressed IL-7Rα transcripts [68]. In humans IL-2, IL-4 or IL-7 stimulation results in decreased CD127 expression on the surface of both thymocytes and mature $CD4^+$ and $CD8^+$ T-cells [49,69-72]. Decreased CD127 expression may occur through activation of T-cells. Alternatively, an altruistic model has been proposed to explain the regulation of IL-7 on its own receptor CD127. In this model, IL-7 is constitutively transcribed and is therefore available in a limited amount to T-cells. T-cells that have encountered an IL-7 signal downregulate their

CD127 receptor [68,73]. In this manner, the available IL-7 is not being consumed by cells that have already received signal, but rather is available to cells that may still require IL-7 stimulation.

1.3.2Antigens affect CD127 expression

Several viral infections have been shown to affect CD127 expression such as CMV, EBV, HCV and HIV [74-79]. These results generated the hypothesis that chronic infections leading to viral persistence results in a decrease in CD127 expression on $CD4^+$ T-cells and $CD8^+$ T-cells[74,76,79,80]. The exact mechanisms of the down regulation of CD127 following antigen encounter have not been fully elucidated, however TCR-mediated activation has been implicated [81]. The regulation of CD127 by exogenous stimuli is cell-specific since HIV-1 TAT viral protein can upregulate CD127 on macrophages [82] and downregulate CD127 on $CD8^+$T-cells [83].

1.3.3 IL-7R signalling pathways

1.3.3.1 JAK/STAT pathway

IL-7 binding to CD127 induces the formation of a heterodimer with the IL-2Rγ chain. The Janus Kinases (Jak) Jak3 and Jak1, physically associate with the IL-2Rγ and CD127 chains respectively, then cross-phosphorylate each other, thereby creating docking sites for src homology 2 (SH2) domain signalling molecules[63]. The main residue phosphorylated is tyrosine 449 (Y449) in the cytoplasmic tail of CD127. The signal transducer and activator of transcription (STAT)-5, which contains an SH2 domain, docks at this residue[84]. After STAT-5 molecules dimerize, and become

phosphorylated molecules translocate to the nucleus to bind DNA sequences and regulate IL-7-dependent transcription of genes such as those for TCRγ rearrangement, B cell leukemia (Bcl)-2, and Pim 1 [62,85-87].

1.3.3.2 PI3K pathway

IL-7 activates the phosphatidylionositol-3 kinase (PI3K) pathway. PI3K is recruited to the Y449 residue of the CD127 chain[88]. The p85 subunit of PI3K is then activated and leads to the phosphorylation of protein kinase B (AKT). This subsequently activates downstream targets such as GSK-3, FOXO1 and FOXO3a. PI3K signalling has been implicated to play a role in IL-7-induced survival, proliferation and glucose metabolism[62,84,89].

1.3.3.3 Other signalling pathways

IL-7 also activates and recruits other signalling molecules such as scr family kinases $p59^{fyn}$ and $p53^{lck}$, PYK2 , and Map Kinases[90-92]. In B-cells IL-7 signaling leads to the activation of the ERK pathway. In the presence of low levels of IL-7 stimulation, ERK signaling has been shown to be absolutely required for IL-7 mediated proliferation [93]. This pathway may not be as indispensable in T-cells since IL-7 mediated proliferation of T-cells can still occur in the absence of ERK activation[94]. In T-cells, IL-7 signaling activates members of the MAP kinase family ,SAPK/JNK and p38Map Kinase. The exact role of SAPK/JNK in IL-7 function has not yet been

elucidated. With the use of specific inhibitors Crawley *et al* demonstrated a direct role for p38Map kinase in IL-7 mediated proliferation [92].

1.4 IL-7- mediated function in T-cells.

1.4.1 IL-7 signalling is a pre-requisite for a functional immune system

Disrupting IL-7 signalling can result in impaired immunity as seen in patients with $T^-B^+NK^+$ Severe combined immunodeficiency (SCID), a genetic defect that results in a mutation in the IL-7R gene, and thereby inactivation of the IL-7Rα signalling pathway. SCID patients have severely impaired T-cell development, which in turn contributes to a deficient peripheral immune response [95]. The first studies on IL-7 demonstrated an important role for IL-7 in B-cell development in mice. However, human B-cell development is not dependent on IL-7 since humans with SCID mutations in the IL-7R gene have a functional B-cell repertoire.

1.4.2 Role of IL-7 in immune function is elucidated with knockout mice

IL-$7^{-/-}$ mice have a 20 fold decrease in thymic cellularity and an increase in TN cells, indicative of a developmental block at the TN stage [96]. The phenotype with IL-$7R^{-/-}$ knockout mice is much more severe with a 90-99.99 % decrease in thymic cellularity[97]. Similar results were seen in experiments involving the blocking of IL-7 with antibodies, which resulted in 99% reduced cellularity when compared to matched litter controls [98]. The most severe phenotype of IL-$7R^{-/-}$ knockout mice is

thought to be due to the additional loss of thymic stromal lymphopoietin (TSLP) signalling, as TSLP also shares the CD127 receptor[99].

1.4.3 Role of IL-7 in VDJ rearrangement

V(D)J gene rearrangement is a critical step in T-cell development, since a block in the recombination events result in a block in overall T-cell development[100]. V(D)J gene rearrangement is primarily controlled by the expression of RAG-1 and RAG-2, since these proteins are responsible for cleavage of the TCR loci. In both IL-$7^{-/-}$ and IL-$7R^{-/-}$ mice, V(D)J gene rearrangement is impaired, resulting in an accumulation of TN cells and a loss of $\gamma\delta$ T-cells[101]. IL-7 signals control RAG-mediated cleavage of TCR -γ locus. The mechanisms by which IL-7 does this is by inducing histone acetylation which enables chromatin accessibility for the initiation of TCRγ V(D)J recombination [12,102].

1.4.4 IL-7 increases thymocyte survival and proliferation

IL-7 also plays a role in thymocyte proliferation and survival. Immature thymocytes proliferate in response to IL-7 in order to increase the number of cells undergoing TCR rearrangement and allow for the diversification of the TCR repertoire [63]. Early experiments in FTOC indicated that IL-7 was able to promote the survival of thymocytes independent from an increase in proliferation [63,103,104]. IL-7 promotes the survival of thymocytes by up-regulating Bcl-2 and by inhibiting the translocation of pro-apoptotic proteins such as Bax and Bad from the cytosol to the mitochondria [105-108]. Evidence for the regulation of Bcl-2 by IL-7 includes the observation that the

expression profile of Bcl-2 and CD127 in thymocytes is paralleled in development. Both molecules are expressed in TN cells, expression is minimal in DP cells and they are re-expressed in SP4 and SP8 cells [62]. Over expression of Bcl-2 in IL-$7^{-/-}$ knockout mice rescues T-cell development of $\alpha\beta$ T-cells, confirming the role for Bcl-2 in thymocyte survival [109].

1.4.5 The role of IL-7 in lineage choice of thymocytes

The role of IL-7 in lineage fate determination during T-cell development has been recently established. TCR and IL-7 signals coordinate in order to specify lineage choice of thymocytes[26]. Thymocytes that interact with self-antigen presented by MHC I molecules are more likely to become $CD8^+$ T-cells. Those cells which encounter self-antigen presented by MHC II molecules have more potential to develop into $CD4^+$ T-cells [110]. The transcription factors Th-POK and Runx3 play key roles in thymocyte lineage determination. Th-POK is the key regulator of CD4 lineage determination and Runx 3 activation results in a CD8 lineage specification[23,28,111,112]. Deficiencies in Th-POK activation can change the lineage fate of a MHC-II restricted thymocyte to become a $CD8^+$ T-cell. As well over expression of TH-POK can alter the lineage of MHC-I restricted thymocytes to become $CD4^+$ T-cells [23,111]. Following positive selection, IL-7 signalling activates Runx 3 which in turn activates the transcriptional enhancer *Cd8a.* Runx 3 also forms a complex which negatively regulates the expression of Th-POK by activating a silencing complex[113] [26,28,112]. Thereby IL-7 signalling of TCR primed DP thymocytes leads to a specific CD8 lineage [26].

1.4.6 The role of IL-7 in peripheral immune response

The impact of IL-7 on the immune system is multifaceted. This cytokine is required in thymopoieisis in order to enhance the diversity and breadth of the existing T-cell pool. In the periphery, IL-7 maintains the existing T-cell pool through a process known as T-cell homeostasis and also increases CTL activity[63,114,115]. IL-7 also plays a role in the establishment and maintenance of the memory T-cell pool and recall responses[116,117]. Since this cytokine regulates many aspects of the immune system, dysfunction in IL-7 signalling could have a severe impact on an immune response.

1.5 HIV-1 Immunopathogenesis

1.5.1 Brief history of HIV-1/AIDS

In 1981, in the cities of San Francisco and New York, USA, there were outbreaks of pneumocystis carinii pneumonia and increased cases of Karposi Sarcoma in homosexual men [118,119]. The Centers for Disease Control (CDC) investigated the cases and concluded that they were due to an infectious agent resulting in an acquired immunodeficiency syndrome (AIDS). In 1983, two independent groups (Dr. R. Gallo from the National Institutes of Health (NIH) in the United States and Dr. L. Montagnier from the Institut Pasteur in France, identified the human immunodeficiency virus (HIV-1) as the etiological agent of AIDS [120,121]. According to United Nations AIDS (UNAIDS) in 2008, 33.4 million people are living with HIV-1 and 2 million people died from causes related to HIV-1. The devastating loss of life

as well as socio-economical ramifications from this pandemic makes it one of the greatest humanitarian challenges of the 21st century.

1.5.2 Clinical stages of HIV-1 infection

HIV-1 infection can be divided into 3 clinical stages: 1) acute infection 2) clinical latency and 3) clinical AIDS [122]. Acute infection is defined as the phase shortly following infection. It results in an initial spike in viremia followed by a sharp decrease due to the initial immune response [123,124]. There is a decline in $CD4^+$ T-cells and initially the CTL response is robust [122,125]. The clinical latency phase results in a viral set point. Although classically latency refers to a period with no viral replication, this is not the case in HIV-1, since there is ongoing viral replication within the lymphoid tissues and a gradual decline in $CD4^+$ T-cells resulting in an inversion of the CD4/CD8 ratio [125]. While $CD8^+$ T-cell numbers are maintained in the periphery, CTL function is impaired [126]. The clinical AIDS phase is characterized by the development of opportunistic infections or malignancies eventually resulting in death [127].

1.5.3 HIV-1 life cycle

Human Immunodeficiency virus is classified as part of the Retroviridae family and belongs to the lentivirus genus. Other members of the lentivirus genus include simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), caprine arthritis-encephalitis virus (CAEV) and equine infectious anemia virus (EIAV) [127]. HIV-1 is an enveloped virus of about 100 nm in diameter with a reverse

transcriptase and 2 copies of single stranded RNA. Its genome is comprised of 9 genes subdivided into 3 categories: 1) structural genes: env, gag and pol; 2) accessory genes: vpu, vpr, vif and nef; and 3) regulatory genes: tat and rev [128-133]. The major targets of HIV-1 are immune cells that express the CD4 protein, in particular $CD4^+$ T-cells. However other cells have also been shown to be infected by HIV-1 such as thymocytes, monocytes, macrophages, glial cells and astrocytes[122,123,134-136]. HIV-1 binds via an envelope protein (gp120) to CD4 on the host cell surface, as well as to a cellular co-receptor such as CCR5 or CXCR4. The virion then fuses to the cell and the viral core is released into the cytoplasm. Once the viral core is uncoated, it releases the RNA genome which is then reverse transcribed by a viral reverse transcriptase. Viral DNA is subsequently translocated to the nucleus where it integrates into the host genome as provirus. Once all the viral proteins are transcribed, virus assembly and budding occurs at the cellular plasma membrane [127]. Highly active antiretroviral therapy (HAART) in HIV-1 infection aims to block different steps in the virus life cycle and ultimately reduce viral loads below 50 copies/ml of blood. Reducing the viral load in a host may allow immune recovery through expansion of the existing T-cell pool or *de novo* synthesis of T-cells from the thymus [31].

1. 6 HIV-1 infection and the Thymus

1.6.1 Clinical evidence of thymic dysfunction

The thymus is a target organ of HIV-1 infection. Examination of the thymus of HIV^+ pediatric patients reveals a loss of lymphoid cells, disruption of the cortico-medullary

boundary and a general destruction of thymic architecture [137,138]. Following HIV-1 infection, the distribution of thymocyte subsets within the thymus is altered when compared to a healthy pediatric thymus. Specifically there is a relative decrease in $CD3^+$DP and SP4 cells and an increase in TN and SP8 cells[139]. This is indicative of a direct loss of infected $CD4^+$ T-cells as well as a block in early thymopoiesis due to the disruption of the thymic microenvironment. This selective thymocyte depletion and disruption of the thymic microenvironment is thought to contribute to a faster progression to AIDS [137,138]. Thymic function can be quantified by measuring the excisional DNA products of TCR-gene rearrangements in peripheral blood mononuclear cells (PBMC). These TCR–rearrangement excision circles (TREC) are not duplicated during mitosis, resulting in their dilution with cellular proliferation. Douek *et al,* demonstrated that a reduction in TREC is observed in HIV-1 infection, as in aging, and correlates with a decline in thymic function. The decrease in TREC in HIV-1 infection likely reflects HIV-1 induced impairment of thymic function. Patients on antiretroviral treatment have an increased prevalence of TREC, indicating at least partial recovery of thymic function [140]. Another measure of thymic function is thymic size as measured by computed tomography (CT). Thymic size is directly related to naive CD4 count in both $HIV\text{-}1^+$ and $HIV\text{-}1^-$ individuals. Thymic volume is altered in HIV-1 infection and inversely correlates with viral load [141]. Immune recovery following HAART has been associated with an increase in naive CD4 count, TREC levels and thymic volume [142]. This confirms the requirement for thymus function in immune recovery following T-cell depletion.

1.6.2 Experimental evidence of thymic dysfunction

Evidence for the direct infection of thymocytes with HIV-1 has been provided with the use of FTOC, and in thymocyte/TEC suspension cultures. Thymocytes in these cultures are able to support high levels of HIV replication[143-146]. In HIV-1-infected SCID-hu Thy/Liv mouse models, there is a depletion of intrathymic progenitor T-cells, which precedes the loss of infected DP thymocytes, suggesting that HIV-1 infection interrupts thymocyte development at an early stage [76]. Further experimental evidence of thymocyte dysfunction was demonstrated in animal models of macaques infected with SIV. Infected animals have reduced levels of circulating TREC when compared to healthy controls, indicative of thymic dysfunction[147]. This is also observed in FIV models, where infected felines have reduced $CD4^+$ T-cells and reduced thymic size[148].

1.6.3 Mechanism(s) of HIV-1 induced thymic dysfunction

The exact mechanisms of HIV-1-induced thymic dysfunction have yet to be fully elucidated. Targets of HIV-1 within the thymus are primarily CD4-expressing mature thymocytes, however other cells such as macrophages, dendritic cells, and epithelial cells can also be infected [51,76,137,149,150]. Reduced thymic output following HIV-1 infection can be due to increased cell death of infected thymocytes or a decrease in immature thymocyte proliferation following HIV-1 infection[11,151]. Thymic function may also be disrupted at the positive/negative selection stages in development. Thymic dendritic cells (DC) play an important role in thymopoieisis. These cells

express MHC and are key players in positive and negative selection shaping the T-cell repertoire. Thymic DC are permissive to HIV-1 infection, resulting in cell death, thereby decreasing the number of available thymic DC for selection, which in turn contributes to altered T-cell development[152]. Immature thymocytes can also be infected and progress through development giving rise to infected maturing SP4 and SP8 thymocytes which in turn leave the thymus and contribute to the pool of infected cells in the periphery[153,154].

Another contributing factor to HIV-induced thymic dysfunction is the disruption of the thymic microenvironment. Cells of the thymic stroma are targets of HIV-1 infection. *In vitro* studies, as well as studies with SCID-hu mouse models have demonstrated the ability of HIV-1 to directly infect and destroy stromal cells [155]. This destruction leads to an altered thymic architecture and contributes to altered thymopoieisis. Trans-activator protein (TAT), an HIV accessory protein, modulates the expression of fibronectin on the cell surface of TEC. This results in decreased interactions between thymocytes and TEC and hence disrupts the maturation of thymocytes from ISP thymocytes to DP thymocytes[156] .

Thymocytes express the co-receptors CXCR4 and CCR5 that facilitate HIV-1 infection of cells. CXCR4 is highly expressed on ISP4 cells, is decreased at the DP stage and re-expressed at the SP stage. Expression of CCR5 is only moderate on

the surface of all thymocytes [149]. Different tropic strains of HIV-1 have diverse abilities to infect thymocytes and or TEC and therefore use distinct mechanisms resulting in various cytopathic effects on thymic function[149,157]. HIV-1 infection of the thymus favours the emergence of X4 tropic virus particles that potentially contribute to disease progression[158]. Infection with CXCR4 strains seems to be much more prevalent in the thymus and in paediatric patients with an active thymus[154] .

1.7. The role of IL-7/IL-7R in HIV-1 infection

1.7.1 CD127 expression in HIV-1 infection

CD127 expression on T-cells is highly regulated and has been shown to be altered in chronic infections [74,76,79,80]. Carini *et al* first demonstrated a decrease in CD127 expression on $CD8^+$ T-cells of HIV^+ individuals[159]. Several studies have since confirmed the link between HIV infection and decreased CD127 expression on $CD8^+$ T-cells [75,76,78,160-163]. Our research group and others have demonstrated that HAART-treated HIV^+ individuals had higher levels of CD127 expression than infected individuals indicating a link between HIV-1 viral replication and CD127 expression[74,75,78,162,164]. The effect of HIV-1 on CD127 expression is not limited to $CD8^+$ T-cells since the same phenomena occurs on $CD4^+$ T-cells of $HIV\text{-}1^+$ individuals[76,165]. In fact, the regulation of CD127 by HIV-1 may play a role in the immunopathogenesis of the virus since the expression of CD127 has been correlated with measures of disease progression such as decreased CD4 count, increased viral load and increased immune activation[78,164,166,167].

1.7.2 IL-7 and HIV-1 infection

IL-7 has been termed the master regulator of T-cell homeostasis. This is in part substantiated by the inverse relationship between circulating IL-7 concentrations and the number of circulating CD4 T-cells[62,63,160,168-170]. Napolitano *et al* showed that the plasma IL-7 levels of HIV-1 infected individuals was elevated compared to healthy controls (0.3-50 pg/ml vs 0.3-8 pg/ml respectively). Despite elevated IL-7 levels, there is a decline in T-cell homeostasis potentially contributing to the decline in $CD4^+$ T-cells in the periphery.[144-146,155,171]. Muthukumar *et al* demonstrated similar patterns of failed homeostasis despite elevated plasma IL-7 levels during SIV infection of rhesus macaques[172]. Studies have demonstrated that $CD4^+$ and $CD8^+$ T-cells from HIV-1^+ individuals are less responsive to IL-7 [161-163,173]. The mechanism of decreased IL-7 responsiveness has been hypothesized to be due to a decreased CD127 expression on the surface of peripheral $CD4^+$ and $CD8^+$ T-cells in HIV-1^+ individuals. However, Vranjkovic *et al (unpublished)* demonstrate that the reduced IL-7 responsiveness in HIV-1 infection was partially due to a defect in IL-7 signalling. IL-7 responsiveness, as measured by STAT-5 phosphorylation and proliferation, was lower in isolated $CD8^+CD127^+$ T-cells from HIV-1^+ individuals when compared to $CD8^+CD127^+$ T-cells from healthy individuals. These results demonstrate impaired IL-7 responsiveness that is independent of CD127 expression. This indicates that HIV-1 can affect both CD127 expression and IL-7 signalling independently of CD127 expression.

1.8 Hypothesis

The overall hypothesis of this thesis is that thymic dysfunction that is observed in HIV-1 infection is due to the down regulation of CD127 on the thymocyte subsets and/or impaired IL-7 signalling resulting in reduced or dysfunctional *de novo* T-cell synthesis.

1.9 Rationale

The proposed project is aimed at understanding thymic dysfunction in HIV-1 infection. Douek *et a*l demonstrated a reduction in thymic function in HIV-1 infection that is partially restored with effective antiviral HIV-1 therapy [140]. Since IL-7 production is not impaired in HIV-1 infection and IL-7 signalling is crucial for several stages of T-cell development, a block in this pathway due to HIV-1 infection would result in the disruption of T-cell development. Recently, there have been several clinical trials of IL-7 aiming at reconstituting a depleted immune system in viral infections such as HIV-1, cancers following chemotherapy and bone marrow transplantation [174,175]. However there is evidence that IL-7 signalling may be impaired in HIV-1 infection (Vranjkovic et al., unpublished; [161,176]). The focus of this thesis research has been to study the effect of HIV-1 infection on thymocytes and in particular to address the effects of HIV-1 infection on IL-7- mediated activity in thymocytes.

1.10 Specific Aims

Aim 1: To determine the optimal conditions for *in vitro* study of human thymocytes.

Aim 2: To determine the factors that downregulate CD127 expression on thymocytes.

Aim 3: To determine if *in vitro* HIV-1 infection of thymocytes disrupts IL-7- mediated signalling and cellular activity.

2: Materials and methods

2.1 Cell preparation and culture

2.1.1 Thymocyte isolation

Thymic tissue was obtained during elective cardiac surgery at the Children's Hospital of Eastern Ontario (CHEO) with informed consent obtained prior to surgery. This study was approved by the CHEO Research Ethics Board. Thymic tissue was washed twice with warmed phosphate buffered solution (PBS) (Invitrogen, Burlington, On). The thymus was submerged in PBS. The connective tissue surrounding the thymic organ was removed. Thymic tissue was cut into 1-3 mm^3 pieces with a scalpel and dispersed with the plunger of a 60 cc syringe. Thymocytes were isolated by layering the resulting supernatant on a Ficoll-Paque^{TM} PLUS *(*Amersham Pharmacia, Piscataway, NJ*)* density gradient following the manufacturer's protocol. The cellular supernatant from the dispersed tissue was subsequently layered at a ratio of 2:1 on Ficoll-Paque^{TM} PLUS and centrifuged at 1600 rpm for 30 minutes with no brake. The buffy coat layer was collected and washed twice with cold PBS. The isolated cells were resuspended in McCoys 5A selective medium (Invitrogen) supplemented with 2mM glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich Inc., Oakville Ont) and 10% fetal bovine serum (FBS) (Cansera, Rexdale, Ont.) (complete medium). Cells were stored at 4°C prior to use.

2.1.2 Thymocyte Purity

The recovered thymocytes were purified from non-thymocyte contaminating cells by depleting with Magnetic Affinity Cell Sorting (MACS) microbeads *(*Miltenyi Biotec, Auburn, CA*)* according to the manufacturer's protocol. Thymocytes were resuspended in 80 μl of MACS buffer (PBS, 0.5% bovine serum albumin (BSA) (Sigma-Aldrich), 2mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) and 20 μl of MACS microbeads per 10^7 total cells, and incubated for 15 minutes at 4°C. A cocktail of microbeads that had been conjugated to antibodies specific for relevant immune cells was used and included: anti-CD56 (NK cells), anti-CD19 (B-cells), anti CD14 (monocytes) and anti-CD11c (dendritic cells). Cells were then washed in 10-fold excess MACS buffer, and magnetically separated using magnets and columns from Miltenyi Biotec. The negatively-selected cells were resuspended in complete medium. Cell purity was assessed by flow cytometry. Thymocytes ($1x10^5$/100 μl) were stained with 5 μl of CD2-Phycoerythrin (PE) (Clone 39c1.5), 5 μl of CD56- PE-cyanin 5 (PC5) (clone N901), 5 μl of CD19-PE (clone J3-119), 5 μl of CD14-Fluoroscein isothiocyanate (FITC) (clone RM052) and 5 μl of CD11c-PE (clone Bu15) (all from Beckman Coulter, Missisauga, On ,Canada). Due to an inconsequential number of contaminating cells (non-thymocytes) in thymic isolation the purification step was foregone for all functional studies.

2.1.3 Thymocyte phenotype

Thymocyte phenotypes were assessed by 4 colour flow cytometry. Thymocytes were stained with the following fluorochrome-conjugated monoclonal antibodies: CD3-(PE-Texas red (ECD) (clone UCHT1), CD4-FITC (clone 13B8.2), CD8-PC5 (clone B9.11), CD127-PE (clone R34.34) (all from Beckman Coulter). The distribution of the following developmental stages of T-cell maturation was evaluated: (TN) $CD3^-CD4^-CD8^-$, (immature single positive CD4 $ISP4^+$) $CD3^-CD4^+CD8^-$, (DP) $CD3^{+/-}CD4^+CD8^+$ and (SP) $CD4^+$ or $CD8^+$ cells. The gating strategy for phenotype analysis is depicted in figure 1. Total thymocytes (gate 1) were identified based on the forward scatter/ side scatter profiles of live cells. The cells were then gated on either $CD3^-$ (gate 2) or $CD3^+$ (gate 3) in a single parameter histogram. The cells in gate 2 and gate 3 were then analysed by a dual parameter histogram for CD4 and CD8 expression. The expression of CD127-PE (Beckman Coulter) was measured on the various subsets.

2.1.4 Thymic epithelial cell (TEC) isolation

TEC were isolated following previously described methods [50]. The dispersed thymic tissue was washed twice in PBS. The tissue was then digested in collagenase (2.5 mg/ml) and DNase (150 units/ml) for 90 minutes (Invitrogen) at 37ºC, the tissue was shaken every 15 minutes. The resulting fragments were then washed twice by resuspending the tissue slurry in PBS and spinning at 1800 RPM for 10 minutes. The tissue was split into 2 T-75 falcon tissue culture flask (VWR, Mississauga, Ontario) with 7 ml of serum-free medium (D-MEM/F12 selective medium

supplemented with 100u/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml epidermal growth factor, 10^{-9} M cholera toxin, insulin 3 μg/ml and transferrin 10 μg/ml) (all from Sigma-Aldrich). Serum-free medium was utilised to culture TEC in order to minimize fibroblast contamination of the cultures. After a 24-hour incubation, medium was replaced and the cellular suspension was further divided into 2 T-75 tissue culture flasks. After 4-5 days, the flasks were washed with warm PBS and any non-adherent cells were removed. The adherent cells were subsequently fed every 3-4 days by replacing the medium with fresh serum-free medium. After 7-10 days of culture, the epithelial-like cell morphology and purity was assessed using intracellular cytokeratin-FITC (clone J1B3)(Beckman Coulter).

2.1.5 Peripheral Blood Mononuclear Cells (PBMC)

All research conducted using blood from human subjects was approved by the Ottawa Hospital Research Ethics Board. Blood from HIV-1-seronegative donors was collected into heparin-containing tubes. PBMC were isolated by Ficoll-Paque™ PLUS (Pharmacia Fine Chemicals, Oiscataway NJ, USA) gradient separation following the manufacturer's instructions. Blood was layered at a ratio of 2:1 on Ficoll-Paque™ PLUS and centrifuged at 1600 rpm for 30 minutes with no brake. The buffy coat layer was collected and washed twice with cold PBS. The isolated cells were resuspended in complete RPMI Medium (Invitrogen) supplemented with 100 IU/ml each of penicillin and streptomycin and 10% FBS.

2.1.6 OP9-DL1 cells

OP9-DL1 cells were provided by Dr. Zúñiga-Pflücker (University of Toronto). Cells were maintained in minimal essential medium-α (MEM-α) (Invitrogen) supplemented with 100u/ml penicillin, 100 μg/ml streptomycin with 20 % FBS. Cells were maintained in a single monolayer and then trypsinized and reseeded at a density of 0.2 x 10^6 cells/ml every 2-3 days.

2.2 Thymocyte Co-culture optimization

2.2.1 Storage of whole thymic tissue

Whole thymic tissue was suspended in McCoys medium + 10% FBS and stored at 37°C or at 4°C in a T-25 flask. Alternatively, thymic tissue was cut into 1-3 mm^3 pieces and resuspended in FBS+ 10% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) prior to cryopreservation at -80°C or in liquid nitrogen (LN_2). All tissue was stored for 10 days. Following storage, thymocytes were isolated from the recovered tissue as described above. Thymocyte viability was assessed by trypan blue exclusion and thymocyte cellular distribution was determined by flow cytometry using the phenotype strategy described above.

2.2.2 Storage of isolated thymocytes

Freshly isolated thymocytes were resuspended in McCoys medium at 5x10^6 cells/ml and stored at either 37°C or 4°C in a T-25 flask. Alternatively, thymocyte suspensions were cryopreserved in FBS +10% DMSO at 5x10^6 cells/ml at -80°C or

in LN_2 for 10 days. Following storage, cell viability was determined by trypan blue exclusion and thymocyte subset distribution was determined by flow cytometry.

2.2.3 Comparing the effect on thymocytes of allogeneic vs. autologous TEC co-culture

Thymocytes stored at 4°C for 7-10 days were co-cultured with autologous TEC, or allogeneic TEC. Fresh thymocytes were co-cultured with allogeneic TEC. Thymocytes were co-cultured at a 1/25 ratio with TEC, $2x10^6$ thymocytes/ 8 $x10^4$ TEC were cultured in 2 ml of McCoys 5A complete medium in a 12 well tissue culture plate for 96 hours at 37°C and 5 % CO_2. Thymocyte subset distribution was determined by flow cytometry.

2.2.4 Comparing the effect on thymocytes of TEC co-culture vs. OP9-DL1 co-culture

Thymocytes were either cultured alone or co-cultured with allogeneic TEC or OP9-DL1 cells. Thymocytes ($2x10^6$)/ TEC (8 $x10^4$) or OP9-DL1 (8 $x10^4$) were cultured in 2 ml of McCoys 5A complete medium in a 12 well plate for 24-96 hours at 37ºC and 5 % CO_2. Thymocyte viability was measured by trypan blue exclusion. Thymocyte subset distribution and CD127 expression was determined by flow cytometry.

2.2.5 Measurement of apoptosis

Apoptosis was measured by Vybrant® Apoptosis Assay Kit from molecular probes according to manufacturer protocol. Thymocytes were co-cultured for 96 hours with

either OP9-DL1 or TEC as previously described. Subsequently, 1 $X10^5$ thymocytes were resuspended in 100 μl annexin V binding buffer and stained with 5 μl Alexa Fluor® annexin V and propidium iodide (PI)(1 μg/ml)(Invitrogen) in the dark at room temperature for 15 minutes. The cells were then diluted with 400 μl of binding buffer and kept on ice prior to analysis by flow cytometry. As a positive control for apoptosis, thymocytes were incubated with apoptosis-inducing camptothecin (0.2 μg/ml) (Sigma-Aldrich) for 5 hours.

2.3 IL-7 responsiveness of thymocytes

2.3.1 IL-7 induced Intracellular STAT-5 phosphorylation of total thymocytes

Thymocytes (1 $X10^5$/100 μl) were incubated in a 96 well microtiter plate with medium or IL-7 (1 ng/ml) (Sigma-Aldrich) for 15 minutes at 37ºC and 5 % CO_2 and washed in PBS. The cells were prepared for intracellular staining by Caltag "fix and Perm" (Invitrogen) reagents following the manufacturer's protocol. The cells were resuspended in 100 μl of reagent A (Fix) incubated in the dark at room temperature for 15 minutes and washed in PBS. Cells were resuspended in ice cold methanol, in order to optimize the FITC signal, for 10 minutes at 4ºC and washed in PBS. Cells were resuspended in 100 μl of reagent B (Perm) with Alexa Fluor® 488 mouse antihuman STAT5 pY694 (BD Biosciences, San Jose, CA, USA) for 20 minutes at room temperature in the dark and washed in PBS. Cells were resuspended in 200 μl of PBS and samples were analysed by flow cytometry. The percentage of $pSTAT5^+$ cells was measured from an unstained control. For pSTAT-5 expression in thymic subsets, surface staining for CD3-ECD was performed prior to fixation and

staining for pSTAT-5, CD4-PC7 and CD8-PC5 was performed during the permeabilization stage.

2.3.2 The effect of co-culture on pSTAT-5 following IL-7 stimulation

To assess the effect of co-culture on the ability of thymocytes to respond to IL-7, thymocytes were co-cultured as previously described with either TEC or OP9-DL1 cell for 96 hours. IL-7-induced STAT-5 phosphorylation was then assessed by flow cytometry.

2.3.3 Bcl-2 expression in total thymocytes following IL-7 stimulation

Thymocytes (1 X10^5) were incubated in a 96 well microtiter plate with IL-7 (1 ng/ml) for 48 hours at 37ºC and 5 % CO_2. The cells were then prepared for intracellular staining by Caltag "fix and Perm" reagents following the manufacturer's protocol. Thymocytes were fixed with 100 μl of reagent A for 15 minutes in the dark at room temperature and washed in PBS. Cells were then resuspended in 100 μl permeabilization reagent B with Bcl-2-FITC (BD Biosciences), and incubated in the dark at room temperature for 20 minutes. Lastly, cells were washed and analysed by flow cytometry. The percentage expression of Bcl-2 was measured from a cut-off set using an isotype match control IgG1. For Bcl-2 expression of thymic subsets, surface staining for CD3-ECD was performed prior to fixation and staining for Bcl-2-FITC, CD4-PC7 and CD8-PC5 was performed during the permeabilization stage.

2.3.4 The effect of co-culture on Bcl-2 expression in thymocytes following IL-7 stimulation

In order to determine the effect of co-culture on thymocyte function, IL-7-induced Bcl-2 expression was measured following co-culture. Thymocytes were either cultured alone or co-cultured with TEC or OP9-DL1 cells. Thymocytes ($2x10^6$)/ TEC ($8 x10^4$) or OP9-DL1 ($8 x10^4$) were cultured for 96 hours at 37ºC and 5 % CO_2. Following co-culture, thymocytes ($1 X10^5$) were incubated IL-7 (1 ng/ml) for 48 hours at 37ºC and 5 % CO_2. The cells were then prepared for intracellular Bcl-2 expression as previously described in section 2.3.3.

2.3.5 IL-7 induced proliferation of thymocytes

IL-7-induced proliferation of thymocytes was measured by Carboxyfluorescein succinimidyl ester (CFSE) dilution. Thymocytes were labelled with CFSE (CellTrace™ CFSE Cell Proliferation Kit) (Invitrogen) as per the manufacturer's protocol. Thymocytes were resuspended in CFSE 8 μm/1 $X10^7$ cells and incubated at 37°C, in the dark for 10 minutes. Cell were incubated with 15 volumes of cold complete RPMI on ice, in the dark for 5 minutes and then washed and resuspended in complete RPMI ($10x10^6$ cells / ml). Thymocytes were cultured at 1-10 X 10^6 cells/ml in 100 μl in a 96 well microtiter plate with medium alone, Phytohemaglutinin (PHA) (0.5 μg/ml, Sigma-Aldrich), IL-7 (1, 10, 25 ng/ml), PHA (0.5 μg/ml) + IL-7 (10 ng/ml), pokeweed mitogen (PWM) (1 μg/ml, Sigma-Aldrich) or colchicine (Sigma-Aldrich) (6 μg/ml) as a negative control, since colchicine blocks cell division by

depolarizing microtubules[177]. Thymocytes were cultured for 7 days at 37ºC and 5 % CO_2 and then cell division was assessed by flow cytometry.

2.3.6 IL-7 induced glucose uptake of thymocytes

Thymocytes were either cultured alone or co-cultured with TEC or OP9-DL1 cells. Thymocytes ($2x10^6$)/ TEC ($8 x10^4$) or OP9-DL1 ($8 x10^4$) were cultured with medium or IL-7 (10 ng/ml) for 96 hours at 37ºC and 5 % CO_2. Thymocytes ($1x10^5$) were washed and resuspended in 100 μl of Krebs/ringer buffer and incubated at 37°C for 30 minutes. Thymocytes were incubated with 2 μci of ^{3}H-D-glucose (GE Healthcare, Piscataway, NJ) for 45 minutes at 37°C. Glucose uptake was terminated by the addition of ice cold Krebs/ringer buffer and washed 3 times to remove any residual glucose. The cells were solubilized in 0.1 % SDS and radioactivity was measured by liquid scintillation counting on a Wallac MicroBeta TriLux (Perkin Elmer,Waltham,MA).

2.3.7 IL-7 induced PI3K signalling pathway

Thymocytes ($1 X10^6$) were initially incubated in serum-free medium for 1 hour to suppress endogenous signals prior to stimulus with IL-7 (R & D, Minneapolis, MN) (1-10 ng/ml) for 30 minutes at 37ºC and 5 % CO_2. Thymocytes were then washed in PBS and pelleted at 1600 rpm for 5 minutes. Cell lysates were prepared by incubating cell pellets with 40 μl of lysis buffer on ice for 20 minutes followed by centrigugation at 14,000 rpm for 20 minutes. Total protein present in supernatants was quantified using the Pierce BCA assay kit following manufacturer's instructions

(Pierce Biotechnologies, Rockford, IL, USA). In brief, 25 μl of each sample or appropriate diluted BSA standard (25- 2000 μg/ml) were added to one well of a 96-well UV plate. To each well, 200 μl of a 50:1 mixture of BSA reagent A and BSA reagent B were added and incubated for 30 minutes at 37ºC. The plate was read using a SpectroMAX 190 microplate reader at 562 nm wavelengths. Calculation of protein content in each sample against the BSA standard curve was performed using the SoftMax Pro software. Total protein (5 μg/30μl) was combined with 2 x loading buffer and heated at 90ºC for 5 minutes. Samples were then loaded onto an 8% SDS–PAGE gel. Following 1 hour of electrophoresis at 100 V, proteins were blotted onto Polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Milipore, Billeria, MA). Transfers were conducted for 1 h at 100 V, after which the membranes were blocked in 5% commercial blocking solution/TBST (Pierce Biotechnologies) for 1 hour with shaking at room temperature. Membranes were probed with antibodies against phospho-AKT, total AKT, phospho-FOXO1a/FOXO3 and β-actin (Cell Signalling, Danvers, MA) overnight at 4°C with shaking. Membranes were washed four times for 15 minutes in TBST at room temperature with shaking. Membranes were then probed with secondary antibody anti-rabbit IgG conjugated to horseradish peroxidase (Cell signalling) for 1 hour at room temperature followed by four 15 minute washes in TBST. Proteins were detected by ECL chemiluminescence (Pierce Biotechnologies).

2.4 Host factor regulation of CD127 expression on thymocytes

Thymocytes were cytokine-treated prior to CD127 expression analysis. Thymocytes/ TEC ($2x10^6$/ $8 x10^4$) were cultured in 2 ml of complete medium in a 12 well plate. Thymocytes were stimulated at the onset of co-culture with IL-7 (1-5000pg/ml), TNF-α (1-100 ng/ml), GM-CSF (1-200 μg/ml), IFN-α (1-100 ng/ml) or IL-4 (1-100 ng/ml) (R & D). Cultures were incubated at 37ºC and 5 % CO_2 and CD127 expression on thymic subsets was measured every 24 hours for a 96 hour period by flow cytometry.

2.4.1 The effect of IL-4 on IL-7 induced pSTAT-5 in thymocyte

Thymocytes/TEC co-cultures were stimulated with IL-4 (100 ng/ml) for 48 hours at 37ºC and 5 % CO_2. IL-7-induced STAT-5 phosphorylation was measured by incubating 1 $X10^5$ IL-4-stimulated thymocytes with IL-7 (1 ng/ml) for 15 minutes at 37ºC and 5 % CO_2. The cells were prepared for intracellular staining as previously described and pSTAT-5 expression was measured by flow cytometry. Control cultures were not pre-treated with IL-4.

2.4.2 The effect of IL-4 on IL-7 induced thymocyte proliferation

Thymocytes/TEC co-cultures were stimulated with IL-4 (100 ng/ml) for 48 hours at 37ºC and 5 % CO_2. Thymocytes were washed and stained with CFSE as previously described. Thymocytes were cultured in a 96 well microtiter plates at 10 $x10^6$ cells/

ml with IL-7 (10 ng/ml) for 7 days. Proliferation was assessed by flow cytometry. Control cultures were not pre-treated with IL-4.

2.5 Viral Stocks

The viral strains used in this study were an X4 tropic strain HIV-1_{IIIB}, an R5 tropic strain HIV-1_{ADA}, and a dual tropic strain HIV-1_{cs204}. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HTLV-III_B/H9 from Dr. Robert Gallo [131]; HIV-1-1 Ada-M from Dr Howard Gendelman[178]. The dual tropic clinical isolate HIV-1 cs204 was a gift from Dr F. Diaz-Mitoma (CHEO/University of Ottawa, Ottawa, ON, Canada).

2.5.1 Propagation of Viral stocks

All viral stocks were propagated in 1-3 day PHA/IL-2 blasts. PBMC (30 x 10^6) were cultured at 1 x 10^6 cells/ml in RPMI +10% FBS + PHA (2.5 μg/ml) and IL-2 (20 u/ml) at 37ºC and 5 % CO_2. On day 3 of stimulation, 10 x 10^6 PBMC were resuspended and cultured in 1 ml of virus supernatant or 1 ml of RPMI (for Mock HIV-1) for 4 hours at 37°C and 5% CO_2, after which time, cells were resuspended in RPMI + 10% FBS + PHA (2.5 μg/ml) and IL-2 (20 u/ml) in tissue culture flasks at 1x 10^6 cells/ml. Every 3-4 days of culture, fresh PBMC blasts +10 ml of fresh RPMI + 10% FBS + PHA (2.5 mg/ml) and IL-2 (20 u/ml) was added to the viral cultures. When culture supernatants contained 20-30 ng/ml of HIV p24 protein (approximately 14-21 days), cells were centrifuged at 2700 rpm for 10 minutes and cell free supernatants

were collected and stored at -80ºC before subsequent concentration of the viral stocks. Mock cultures were incubated in parallel and treated identically as viral stocks with the exception of the addition of virus.

2.5.2 Determination of p24 protein levels in viral stocks

Concentration of p24 in viral stocks was determined using an HIV-1 p24 Capture enzyme linked immunosorbent assay (ELISA)(SAIC-Frederick, Frederick, MD) in accordance with the manufacturer's instructions. To lyse any virus, viral supernatants were incubated with 1 % Triton-X (Sigma-Aldrich) for 60 minutes at 37°C. Viral stock samples, as well as appropriate diluted standards (75-40, pg/ml), were aliquoted into one well of the ELISA plate coated with p24 capture antibody and incubated for 2 hours at room temperature. The plate was washed 3 times with commercial wash buffer (KPL, Gaithersburg, MD). The plate was incubated with 100 μl of primary p24 antibody and washed 3 times with commercial wash buffer. The plate was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 60 minutes at room temperature and washed 3 times. The plate was subsequently incubated with 100 μl of detector reagent, TMB Peroxidase Substrate System (KPL) for up to 30 minutes and the reaction was stopped by the addition of hydrochloric acid (HCL, 1N). Plates were read at 450 nm on a SpectraMax 190 microplate reader (MDS Analytical Technologies). Calculation of protein content in each sample was extrapolated from a standard curve using the SoftMax Pro software.

2.5.3 Concentrating viral stocks by polyethelene glycol (PEG) precipitation

Viral supernatants (500 μl) were incubated overnight at 4 ºC with equal volumes of 20% polyethykeneglycol (PEG) 20,000 solution (Sigma-Aldrich) (500 μl) in 1.5 ml conical bottom eppendorf tubes. The solution was then ultra-centrifuged at 17, 860 X g for 20 minutes at 4 ºC. Resulting pellets were resuspended in 50 μl fresh McCoy medium. All stocks are combined and re-aliquoted prior to freezing at -150 ºC. Viral infectivity was measured by $TCID_{50}$ determination. Mock cultures were treated the same on a per volume basis.

2.5.4 $TCID_{50}$ Determination of Viral Stocks

The 50% tissue-culture infectious dose ($TCID_{50}$) of each viral stock was determined in PBMC. PBMC ($1x10^6$ cells/ml) were cultured in RPMI + 10% FBS + PHA (2.5 μg/ml) + IL-2 (20 U/ml) at 37°C and 5% CO_2. On day 3 of PBMC stimulation, a viral stock was diluted 1:12 in RPMI + 10% FBS + PHA (2.5 μg/ml) + IL-2 (20 U/ml) and 200 μl was aliquoted in triplicate into one row of a 96 well plate. To the next 6 rows, in triplicate, 150 μl of RPMI + 10% FBS + PHA (2.5 μg/ml) + IL-2 (20 U/ml) was added. Next, 50 μl of diluted virus from the first row was transferred to the second row and so on, producing a series of 4-fold dilutions of virus across the 6 rows. Finally, to every well in the plate, 50 μl (4×10^6 cells/ml) of stimulated PBMC were added, and the plate was incubated at 37°C and 5% CO_2. On day 4 of culture, 150 μl of medium was discarded from each well and replaced with fresh medium (RPMI + 10% FBS + PHA (2.5 μg/ml) + IL-2 (20 U/ml)). On day 7 of culture, supernatants

from each well were harvested, centrifuged and assayed for HIV-1-1 p24 protein expression by ELISA. Wells in which the p24 content exceeded 50 ng/ml were scored as positive, and the $TCID_{50}$ for the viral stock was calculated using the Spearman-Karber Method [179] as follows:

$$M = xk + d[0.5 – (1/n)(\Sigma r)]$$

Where, xk = the dose of highest dilution; r = the number of negative wells; d = the spacing between dilution; n = the number of wells in each dilution. Therefore, the 50% endpoint equals 4^{-M}. To convert the value to the 50% titer (10^{x}), $x = M \bullet \log 4$. Finally, the $TCID_{50}$/ml is determined by multiplying by 5, to correct for the original dilution (i.e. 1000 ul / 200 ul).

2.6 Detection of HIV-1 in infected thymocytes by Nested PCR

2.6.1 DNA Isolation

DNA was isolated from 1 $x10^6$ infected thymocytes using the QIAGEN DNeasy blood and tissue kit (Qiagen, Mississauga, ON, Canada). Thymocytes were pelleted at 1600 rpm for 5 minutes. Pellets were resuspended in 200 μl of PBS with 20 μl of proteinase K and 200 μl of buffer AL and incubated at 56ºC for 10 minutes. Ethanol (100%, 200 μl) was added to the sample, then the mixture was added to a DNeasy spin column and centrifuged at 8000 rpm for 1 minute. The column was washed with 500 μl of buffer AW1 and centrifuged at 8000 rpm for 1 minute. The column was washed again with 500 μl of buffer AW2 and centrifuged at 14,000 rpm for 3

minutes. DNA was eluted with 100 μl of buffer AE by centrifuging column at 8000 rpm for 1 minute.

2.6.2 PCR for the detection of HIV-1-1

Integrated HIV-1-1 was detected by ALU-LTR nested PCR [180]. In the first round of PCR, integrated HIV-1 sequences were amplified with 2 outward-facing Alu primers (Alu1 and ALu2) which anneal to conserved regions of the Alu repeat element and an HIV-1 LTR (LM-667) specific primer with a lambda phage-specific heel sequence at the 5' end (Table 1). DNA (1/10) was amplified in a 50 μl reaction as follows: 25 μl of Qiagen Master mix, LM667 primers (100 nm), Alu1 primers (300nm) and Alu2 primers (300 nm). First round PCR amplification conditions were as follows: DNA denaturation 2 minutes at 95°C and then 30 cycles of amplification (94°C for 60 s, 55°C for 60s and 72°C for 60s) and finally an elongation step for 7 minutes at 72°C . The second round of PCR only amplifies specific sequences from the first round utilising a lambda T primer and an LTR primer (AA55M) (Table 1). The second round PCR was amplified in a 50 μl reaction as follows: 1/10 of the product from the first round of PCR, Lambda T primers (300nm) and AA55M (300 nm). The second round of PCR amplification conditions were the same as in round 1. PCR products were resolved by electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized using a UV transluminator.

Table1 Primers for Alu -LTR nested PCR for the detection of HIV-1-1

PRIMERS	SEQUENCE (5'-3')
LM-667	ATGCCACGTAAGCGAAACTCTGGCTAACTAGGGAACCCACTG
Alu 1	TCCCAGCTACTGGGGAGGCTGAGG
Alu 2	GCCTCCCAAAGTGCTGGGATTACAG
Lambda T	ATGCCACGTAAGCGAAACT
AA55M	GCTAGAGATTTTCCACACTGACTAA

2.7 The effect of *in vitro* HIV-1 infection on CD127 expression on thymocytes

2.7.1 In vitro HIV-1 infection of thymocytes

Prior to infection, thymocytes were resuspended to a concentration of $2x10^6$ cells/ml in polybrene (Sigma-Aldrich) (3 μg/ml) for 1 hour at 37ºC. Thymocytes ($6x10^6$) were then washed twice with PBS and the subsequent pellet was infected with cell-free HIV-1 supernatants at a multiplicity of infection (M.O.I) of 0.01 for 2 hours at 37ºC with shaking every 30 minutes. As a control, cells were also mock-infected in parallel cultures. Thymocytes were then washed twice in PBS and resuspended in McCoys 5A complete medium (Invitrogen). Infected thymocytes ($1x10^6$ /ml) were co-cultured with TEC ($4 x10^4$/ml) in 5 ml of McCoys 5A complete medium in a 6 well tissue

culture plate for 96 hours at 37ºC. CD127 expression on thymic subsets was measured by flow cytometry.

2.8 The Effect of in vitro HIV-1 infection on IL-7 responsiveness of thymocytes

2.8.1 The effect of in vitro HIV-1 infection on IL-7 induced pSTAT-5 of thymocytes

In order to assess the ability of infected thymocytes to respond to IL-7, the phosphorylation of STAT-5 was measured. Thymocyte co-cultures were infected with HIV-1 as previously described in section 2.7.1. Following 96 hours of culture, thymocytes were isolated and washed. Thymocytes ($1x10^5$) were aliquoted into 100 μl into a 96 well microtiter plate with IL-7 (1-10 ng/ml) and incubated for 15 minutes at 37ºC and 5 % CO_2. The cells were prepared for intracellular staining by Caltag "fix and Perm" reagents as previously described in section 2.3.1. Briefly, the cells were fixed with reagent A for 15 minutes followed by a 10 minute incubation in ice cold methanol. The cells were washed and resuspended in reagent B with anti-pSTAT-5 for 20 minutes.

2.8.2 The effect of in vitro HIV-1 infection on IL-7 induced Bcl-2 expression in thymocytes

To determine the effect of HIV-1 infection on the induction of anti-apoptotic pathways by IL-7, Bcl-2 expression was measured. Thymocyte co-cultures were infected with HIV-1 as previously described in section 2.7.1. Following 96 hours of culture, thymocytes were isolated, washed, aliquoted ($1x10^5$) into 100 μl into a 96

well microtiter plate with IL-7 (1-10 ng/ml) and incubated for 48 hours at 37ºC and 5 % CO_2. The cells were prepared for intracellular staining by Caltag “fix and Perm” reagents as previously described. Briefly the cells were fixed with reagent A for 15 minutes were washed and resuspended in reagent B with Bcl-2-FITC for 20 minutes. For Bcl-2 expression of thymic subsets, surface staining for CD3-ECD was performed prior to fixation and staining for CD4-PC7 and CD8-PC5 was performed during the permeabilization stage.

2.8.3 The effect of in vitro HIV-1 infection on IL-7 signalling via the PI3K pathway

To determine the effect of HIV-1 infection on the induction of the PI3K pathway by IL-7, AKT phosphorylation and FOXO1/FOXO3a phosphorylation was measured by Western blot. Thymocyte co-cultures were infected with HIV-1 as previously described in section 2.7.1. Following 96 hours of culture, thymocytes were isolated and washed. Protein was isolated from infected thymocytes ($1x10^6$) as previously described in section 2.3.7. Cell lysates were prepared and protein was resolved on an 8% SDS–PAGE, blotted onto PVDF membrane and probed with antibodies against phospho-AKT, total AKT, phospho-FOXO1a/FOXO3 and β-actin (all from cell signalling technologies) followed by the secondary antibody anti-rabbit IgG conjugated to horseradish peroxidase. Proteins were detected by ECL chemiluminescence (Perkin-Elmer).

2.9 Statistical analysis

All statistical analyses and graphing was performed using GraphPad Prism 5.0 Software (San Diego, CA, USA). Statistical significance was measured by either Students *t*-test for paired samples or analysis of variance (ANOVA) with the Dunnett post-test pairwise comparison. All flow cytometry data were analysed using the FCS Express 2.0 software (De Novo Software, Thornhill, ON ,Canada).

3. Results

3.1 Optimization of methods for the culture of thymocytes

3.1.1 Thymocyte Phenotype

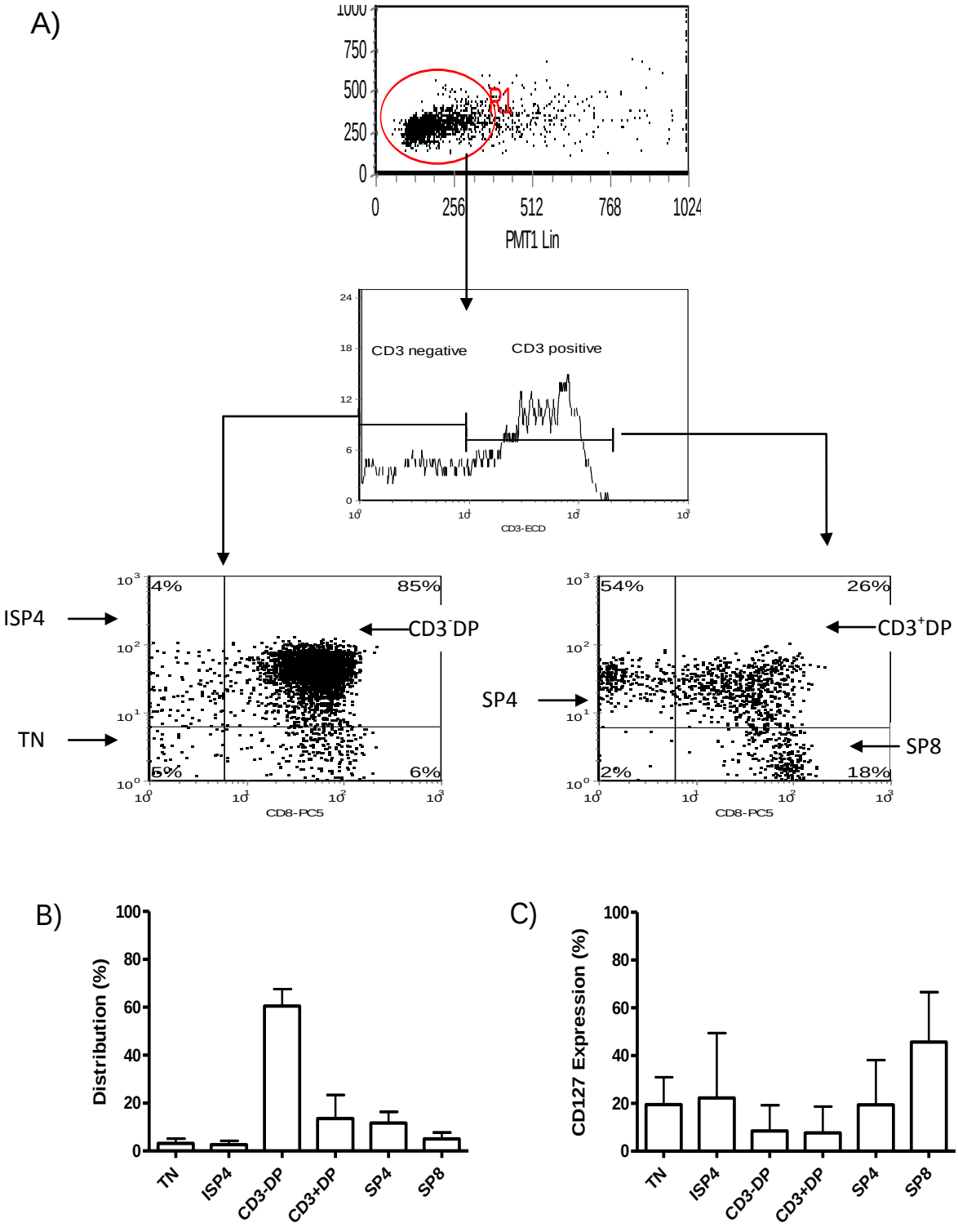
To first determine the purity of negatively-selected cells from thymic tissue, isolated cells were stained with a panel of antibodies: CD14 (monocytes), CD11b (DC), CD56 (NK cells), CD19 (B-cells), and CD2 (thymocytes). All of the non-thymocyte cells were present at less than 1% of the isolated cells (Table 2). The isolated cells stained over 95% positive for CD2, indicating that the population of isolated thymocytes was relatively pure. To determine subset distribution of freshly isolated thymocytes, cells were stained with antibodies to CD3, CD8 and CD4. The phenotype of thymocytes was assessed by flow cytometry and analysed according to established subsets: [181,182] $CD3^-CD4^-CD8^-$ (TN), $CD3^-CD4^+CD8^-$ (immature single positive CD4 $ISP4^+$), $CD3^-CD4^+CD8^+$ ($CD3^-$DP), $CD3^+CD4^+CD8^+$ ($CD3^+$DP), $CD3^+CD4^+CD8^-$ (SP4) and $CD3^+CD4^-CD8^+$ (SP8). The gating strategy consisted of gating on live thymocytes based on forward scatter and side scatter patterns (gate 1). Thymocytes were further gated based on CD3 expression; CD3 negative (gate 2) and CD3 positive (gate 3). These gates were then subdivided into a dual parameter histogram based on CD4 and CD8 expression (Figure 2A).

The most immature subsets account for the smallest proportion of cells, where 3 % ± 2 % of the cells are TN and 3 % ± 2 % of the cells are $ISP4^+$ (Figure 2B). The majority of the cells are DP cells, where 61 % ± 7 % are $CD3^-$DP and 14 ± 10% are

Table 2 Phenotypic characterization of thymocyte-enriched populations

Markers	% of positive cells
CD2	97
CD14	< 1.0
CD56	< 1.0
CD19	< 1.0
CD11b	< 1.0

Figure 2. Thymocyte distribution A) Flow cytometry gating strategy for thymocyte distribution. Thymocytes were gated on live cells based on forward scatter/ side scatter patterns. The live thymocytes were then gated on CD3 expression. The distribution of CD4 and CD8 cells within the CD3 gates were then analysed B) Distribution of thymic subsets within the thymocyte pool. Freshly isolated thymocytes were stained with fluorochrome labelled antibodies to CD3, CD4 and CD8 and the proportion of cells within each subset (TN, ISP4, CD3-DP, CD3+DP, SP4 and SP8) was measured. Results are expressed as means ± standard deviation (n=23). C) CD127 expression on freshly isolated thymocyte subsets (n=14).


A)
R1
PMT1 Lin
CD3 negative
CD3 positive
CD3-ECD
ISP4
TN
CD3⁻DP
4%
85%
6%
6%
CD8-PC5
SP4
CD3⁺DP
SP8
54%
26%
2%
18%
CD8-PC5
B)
Distribution (%)
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8
C)
CD127 Expression (%)
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8

$CD3^{+}$DP. The mature subsets SP4 and SP8 comprise the remainder of the thymic pool at 12 % ± 5 % and 5 % ± 3 % of the population respectively. This is consistent with other published reports [182-184].

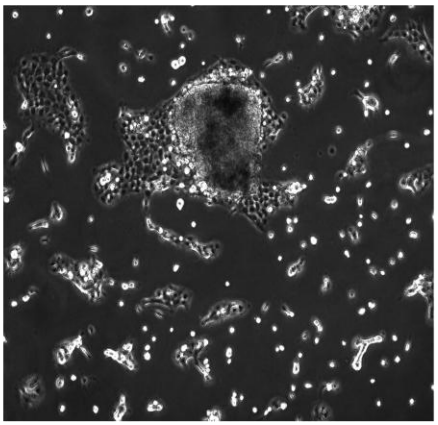
Since IL-7 signalling via IL-7R plays a critical role in thymocyte development, the expression of CD127 on the various thymocyte subsets was evaluated. All subsets of freshly isolated thymocytes express CD127 however this occurs to varying degrees (Figure 2C). The $CD3^{+/-}$ DP cells express the least amount of CD127 (< 10 % of cells were CD127+) whereas the SP8 cells have the highest CD127 expression with CD127 expressed on 60% ± 16% of cells consistent with published reports [183-185]

3.1.2 Thymic Epithelial Cell isolation and Phenotype

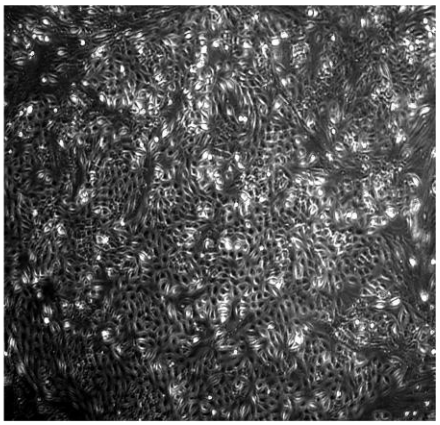
Thymic epithelial cells grow out from digested thymic tissue (Figure 3A). TECs are typically recognized by their mosaic-like cobblestone appearance [186] (Figure 3B). Serum-free medium was used in order to minimize fibroblast contamination in TEC cultures. Typically, fibroblasts are long and fibrous and grow in a scattered manner throughout the course of culture. Cultures that had visual evidence of fibroblasts were discarded. The purity of the TEC cultures was also assessed by cytokeratin staining. Cytokeratin is an intermediate filament protein found within cells of epithelial origin. Cells isolated from thymic tissue were cytokeratin positive (Figure 3C).

Figure 3. Thymic epithelial cells. A) Thymic epithelial cells growing out from a piece of thymic tissue. B) Mosaic-Like coblestone pattern of human thymic epithelial cells. C) Cytokeratin staining of TEC cells following 10 days of culture.

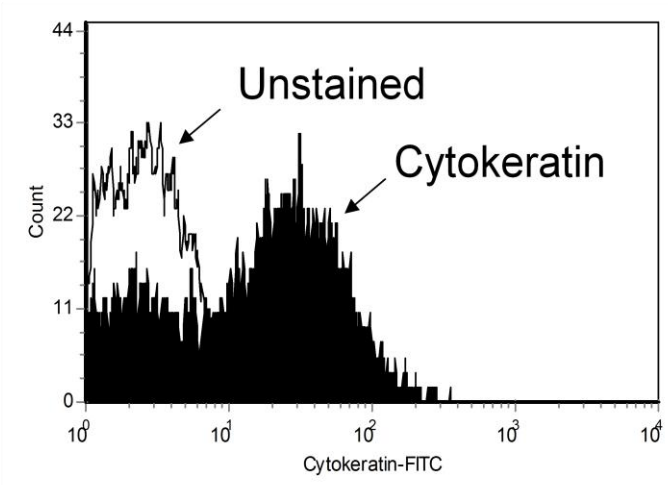
A)



B)



C)


44
33
22
11
0
Count
Unstained
Cytokeratin
10^0
10^1
10^2
10^3
10^4
Cytokeratin-FITC

3.2 Optimizing thymocyte isolation and storage conditions

Thymocyte functional studies require that isolated thymocytes be co-cultured with TEC. Thymocytes can be isolated from thymic tissue the same day as sample acquisition; however TEC isolation requires 7-10 days for cells to grow out from digested tissue. This lag time necessitates storage of the isolated thymocytes that may adversely affect thymocyte cell populations. The optimal storage conditions should ensure the highest recovery of cells with the highest viability and conservation of the original phenotype. Whole thymic tissue could potentially be cryopreserved in order to later isolate thymocytes when the TEC have been isolated and are available for co-culture. Alternatively, freshly isolated thymocytes could be stored or cultured until the TEC are isolated. To eliminate the need for these storage conditions that may be detrimental to the thymocytes, the use of allogeneic TEC or a TEC line may be viable alternatives. Since primary TEC can be cultured for up to 5 weeks and a TEC line can theoretically be maintained indefinitely, this would allow for immediate use of the isolated thymocytes, eliminating any detrimental effects of thymocyte storage. Although methods to isolate thymocytes and methods for the co-culture of thymocytes with TEC are published [50,51,187,188], there is little to suggest how or if these conditions can be optimized.

3.2.1 Effect of storage of whole thymic tissue

To determine the effect of storing tissue on thymocyte viability and subset distribution, tissues were stored at 4°C, 37°C, -80°C or in LN_2 for 10 days before thymocyte isolation. In this way, thymocytes could be isolated at a time when TEC

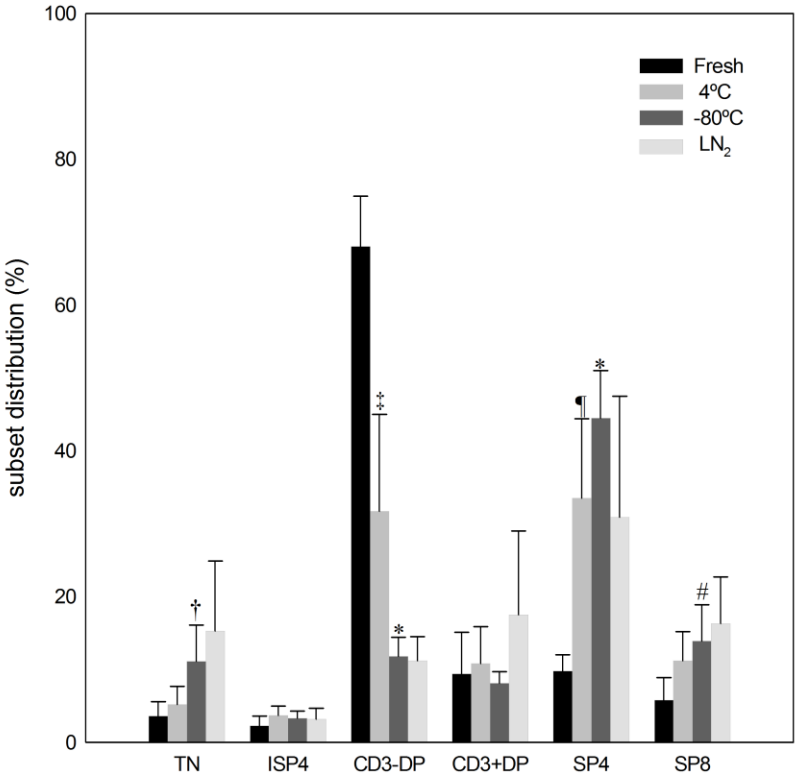
are ready for co-culture. The subsets were analysed by flow cytometry and viability was verified by trypan blue exclusion and flow cytometry forward scatter and side scatter profiles. Thymocytes were recovered from tissue stored at 4°C, -80°C and LN_2; however no thymocyte-containing buffy coat was visible in a Ficoll-gradient with tissue preparations stored at 37°C. Viability of the cells recovered from tissues stored at 4°C was 77% ± 26%. Cryopreserved tissues stored at -80°C or in LN_2 had slightly lower viabilities of 70% ± 12% and 71% ± 10% respectively. The distribution of thymocytes within the T-cell pool was altered by storage conditions of whole tissue. The proportion of isolated $CD3^-$DP cells significantly decreased under all storage conditions when compared to freshly isolated thymocytes. The proportion of mature SP4 cells increased when whole tissue was stored at 4°C or cryopreserved at -80°C or in LN_2 (Figure 4A). The changes in thymocyte distribution after storage at 4°C or cryopreservation at -80°C or LN_2 suggest that these are not ideal storage conditions for studies of function of the various thymocyte subsets.

3.2.2 Effect of storage on freshly isolated thymocytes.

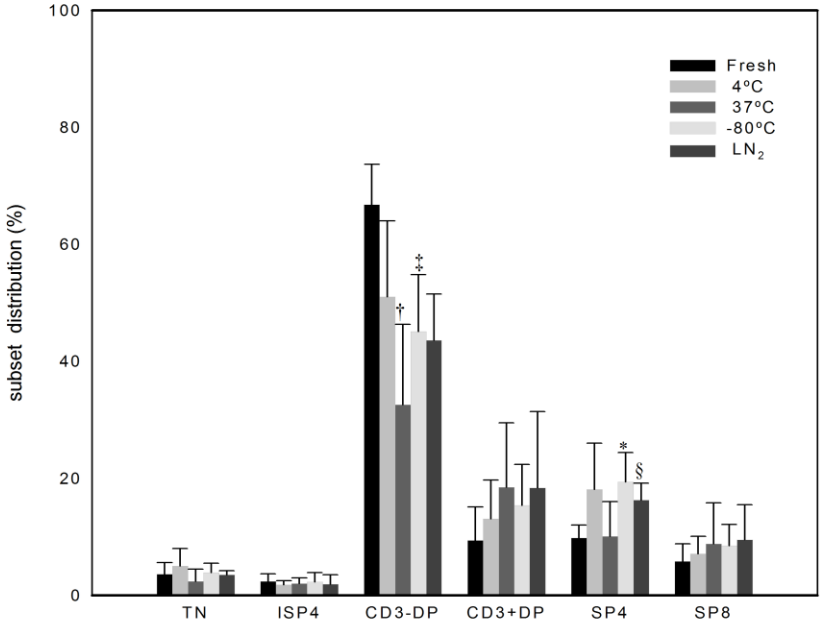
To determine the effect of storage on the cell viability and phenotype of freshly isolated thymocytes, cells were stored at 37°C, 4°C and cryopreserved at -80°C or in LN_2 for 10 days prior to analysis by flow cytometry. The storage of isolated thymocytes at 4°C resulted in the highest viability at 92% ± 4%. Thymocytes that were stored at 37°C were 78% ± 1% viable while cells cryopreserved at -80°C or in

Figure 4. The effect of storage on thymocyte distribution. A) Storage of whole tissue results in altered thymic subset distribution. Whole thymic tissue was freshly processed, or stored for 10 days at 4°C, -80°C or in LN_2. Thymocytes were isolated after tissue storage and stained with fluorochrome labelled antibodies to CD3, CD4 and CD8 and the proportion of cells within each subset (TN, ISP4, CD3-DP, CD3+DP, SP4 and SP8) was measured. Results are expressed as means ± standard deviation (n=4). † p=0.011, ‡ p=0.005, * p≤ 0.001, § p =0.002 , ¶ p =0.006, and # p=0.038, each versus freshly isolated thymocytes. B) Effect of storage of isolated thymocytes on subset distribution. Thymocytes were freshly isolated and either immediately analysed or stored for 10 days at 4°C, 37°C, -80°C or in LN_2 and stained with fluorochrome labelled antibodies to CD3, CD4 and CD8 and the proportion of cells within each subset (TN, ISP4, CD3-DP, CD3+DP, SP4 and SP8) was measured. Results are expressed as means ± standard deviation (n=4). † p=0.003, ‡ p=0.026, * p= 0.028, § p =0.037, each versus freshly isolated thymocytes.

A)


100
80
60
40
20
0
subset distribution (%)
Fresh
4ºC
-80ºC
LN2
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8

B)


100
80
60
40
20
0
subset distribution (%)
Fresh
4°C
37°C
-80°C
LN2
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8

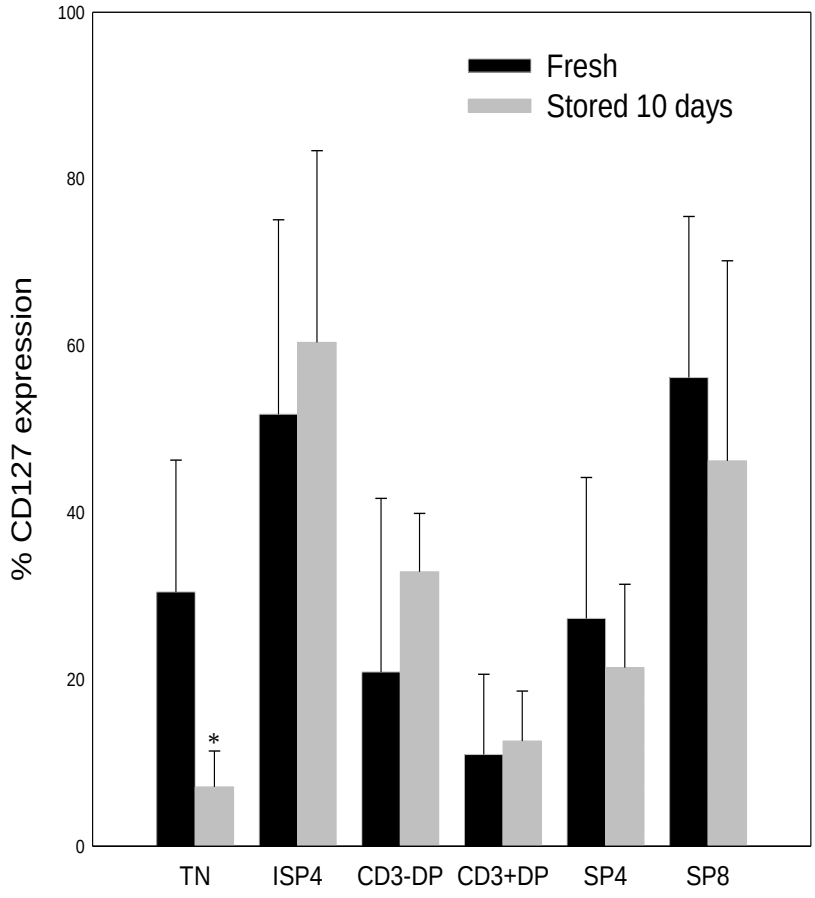
LN_2 had a reduced cell viability of 65% ± 4% and 48 ± 10% respectively. The distribution of thymocyte subsets was altered by storage at 37°C, -80°C or in LN_2 (Figure 4B). The proportion of cells in the $CD3^-$DP subset was significantly decreased when cells were stored at 37°C, -80°C or in LN_2 and the number of cells in the $CD3^+$DP subset was significantly increased when cells were stored at 37°C or cryopreserved in LN_2. Cells stored at 4°C had the highest viability and retained phenotypes most similar to freshly isolated cells. Hence, if storage of freshly isolated thymocytes is required, 4°C appears to be the optimal condition prior to co-culture with TECs. This is in accordance with published procedures that store thymocytes at 4°C prior to co-culture with TEC [51].

Storing thymocytes at 4ºC for 7-10 days was associated with no statistically significant changes in CD127 expression on ISP4, $CD3^-$DP, $CD3^+$DP, SP4 or SP8 subsets (Figure 5). A significant decrease in CD127 expression on TN cells was seen with storage, although it must be recognized that the TN subset comprises only 3% of the total thymocyte population.

3.3 Optimizing thymocyte co-culture systems.

To eliminate the requirement for thymocyte storage that may be detrimental to the thymocytes, the use of allogeneic TEC or a cell line such as OP9-DL1 may also be viable alternatives. This would allow immediate use of the isolated thymocytes, eliminating any detrimental effects of thymocyte storage.

Figure 5 The effect of storage on CD127 expression on thymocyte. Isolated thymocytes were either stained on isolation day or stored at 4°C for 10 days prior to staining with antibodies to CD3, CD4, CD8 and CD127 to determine the percentage of each subset that expressed CD127. Storage at 4°C does not affect CD127 expression on thymocyte subsets. (fresh n=12; stored 10 days n=4, *p=0.003 by t-test)


100
80
60
40
20
0
% CD127 expression
Fresh
Stored 10 days
*
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8

3.3.1 Co-culture of thymocytes with autologous or allogeneic TEC.

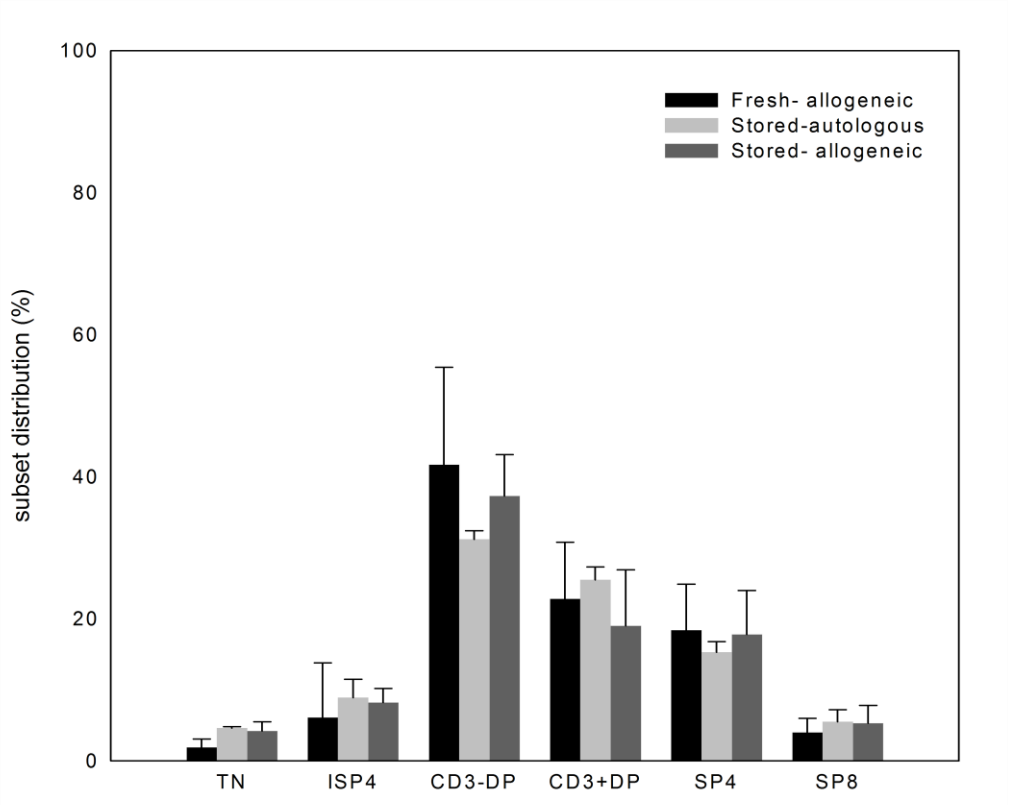
To determine the effect of the co-culture of autologous versus allogeneic TEC on thymocyte subset distribution and viability, co-cultures of 1) fresh thymocytes with allogeneic TEC, 2) stored thymocytes with allogeneic TEC or 3) stored thymocytes with autologous TEC were evaluated. Co-cultures of stored thymocytes (4°C for 7 to 10 days) for 96 hours with TEC resulted in a thymocyte distribution similar to that seen on freshly isolated thymocytes co-cultured with allogeneic TEC for 96 hours (Figure 6A). This was the case whether thymocytes stored at 4°C were co-cultured with autologous or allogeneic TEC, indicating that co-culture with either sources of TEC may be a feasible option for thymocyte *in vitro* suspension cultures. Viability of thymocytes was not adversely affected by the different co-culture systems (data not shown).

3.3.2 CD127 expression levels on thymocyte subsets following co-culture.

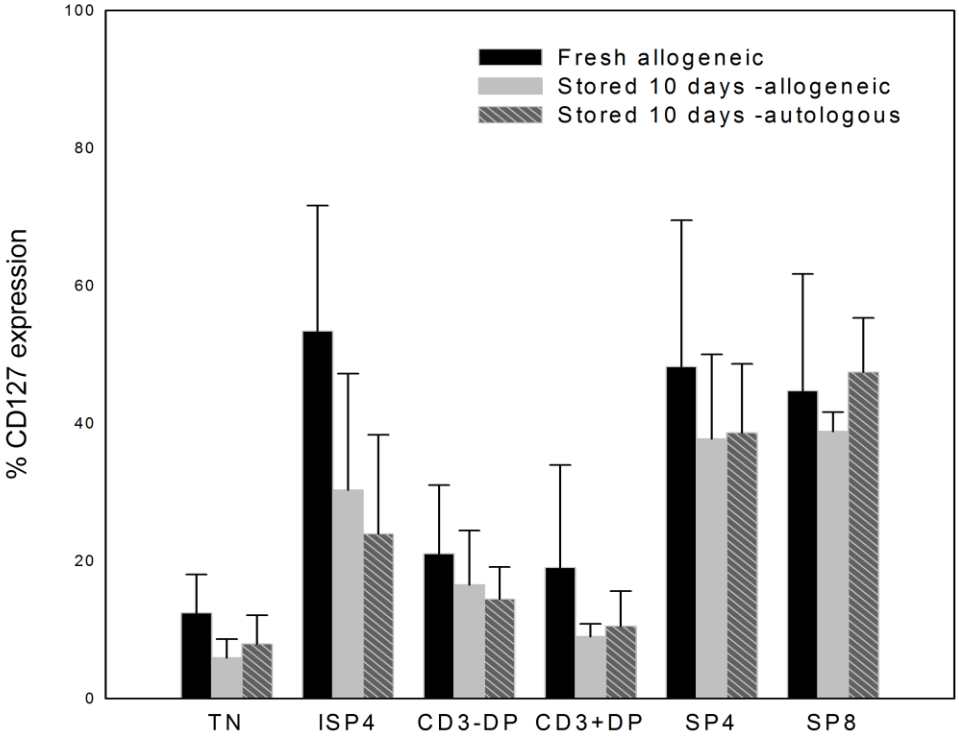
To determine the effect of co-culturing thymocytes with allogeneic or autologous TEC on CD127 expression, the expression of this molecule on the individual thymocyte subsets was evaluated over time in culture. Culturing stored thymocytes with either autologous or allogeneic TEC for 96 hours resulted in a similar distribution of cells expressing CD127 indicating that this form of TEC co-culture does not affect CD127 expression (Figure 6B).

Figure 6. The effect of co-culture on thymocyte distribution and CD127 expression Varied co-culture systems have no significant effect on subset distribution. Fresh thymocytes were co-cultured with allogeneic TEC, or freshly isolated thymocytes stored at 4°C for 10 days prior to co-culture with either autologous, or allogeneic TEC for 96 hours. Thymocytes were stained with fluorochrome labelled antibodies to CD3, CD4 and CD8 and the proportion of cells within each subset (TN, ISP4, CD3-DP, CD3+DP, SP4 and SP8) was measured. Results are expressed as means ± standard deviation (n=4). B) CD127 expression was evaluated on fresh thymocytes co-cultured with allogeneic TEC for 96 hours, or thymocytes stored at 4°C for 10 days prior to co-culture with autologous TEC for 96 hours or allogeneic TEC for 96 hours. Results are expressed as means ± standard deviation.

A)


100
80
60
40
20
0
subset distribution (%)
Fresh- allogeneic
Stored-autologous
Stored- allogeneic
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8

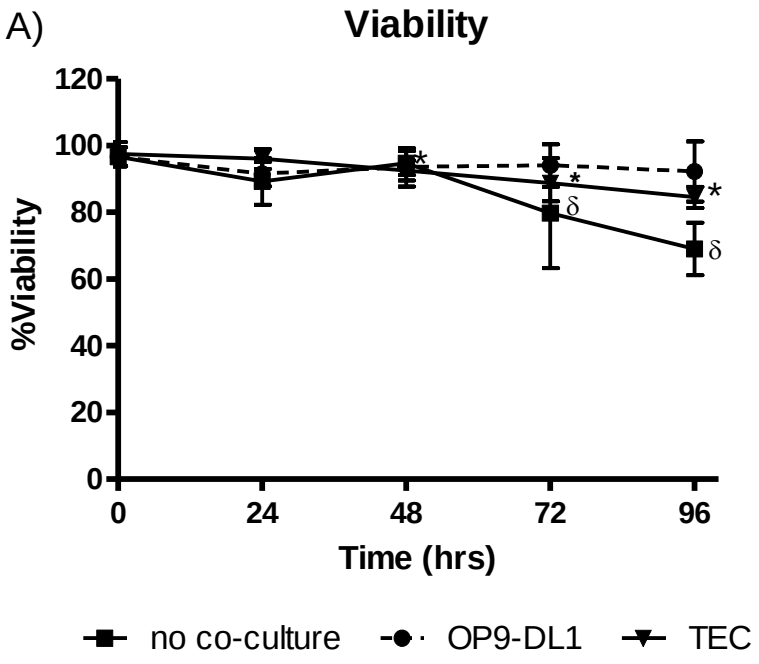
B)


100
80
60
40
20
0
% CD127 expression
Fresh allogeneic
Stored 10 days -allogeneic
Stored 10 days -autologous
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8

3.3.3 OP9-DL1 co-culture maintains thymocyte viability but not cell number.

Within a co-culture system, contact with epithelial cells results in the release of soluble factors that are required for the viability of thymocytes. Thymocytes were cultured alone or co-cultured with either human TEC or OP9-DL1 cells for 96 hours and cell number and viability were measured by trypan blue exclusion. Cell viability and cell number decreased over time in thymocytes that were cultured alone (Figure 7) indicating that their survival is dependent on TEC-delivered signals. The viability of thymocytes co-cultured with TEC only slightly decreased over time with 84 % ± 3% of cells viable after 96 hours. Thymocytes co-cultured with OP9-DL1 cells also fared well, with a viability of 92 % ± 9% after 96 hours of co-culture, indicating that both culture systems are able to provide the survival signals required for thymocytes in a short term culture. Although co-culture with OP9-DL1 cells resulted in a high viability of thymocytes, the cell number was significantly reduced with only 34 ± 9% of input cells remaining in culture after 96 hours. To confirm that the co-culture systems provided adequate survival signal, apoptosis was assessed. The level of spontaneous apoptosis, as measured by annexin V^+/ PI^-, in thymocytes after 96 hours was 56 ± 27 % when cells were cultured alone and 57 ± 14 % when thymocytes were co-cultured with TEC (Figure 8). However, co-culture with OP9-DL1 cells resulted in protection from apoptosis with only 13 ± 5% of thymocytes staining annexin V^+PI^-, significantly lower than co-cultures with TEC ($p= 0.009$; Figure 8). When the proportion of total annexin V^+ cells was evaluated (annexin V^+/ PI^+ and annexin V^+/ PI^-), similar results were obtained.

Figure 7. The effect of co-culture on cellular viability and cell number. Thymocytes were co-cultured with either TEC or OP9-DL1 cells at a ratio of 1/25 (thymocytes/TEC or OP9-DL1) or cultured alone as a control for 96 hours. A) Viability of the thymocytes was assessed by trypan blue exclusion every 24 hours. Thymocytes cultured alone resulted in the largest decrease in cell viability over time, whereas in co-culture with OP9-DL1 cells, thymocyte viability over 96 hours in culture was maintained. n=4, *p≤ 0.0001 and δ p=0.001 by analysis of variance and p< 0.05 by Dunnett's simultaneous test versus time 0. B) The number of thymocytes in culture was determined every 24 hours with cells co-cultured alone, with TEC or with OP9-DL1. Thymocytes cultured with OP9-DL1 had a statistically significant decrease in cell number after 72 and hours in culture. n=4, ϕ p=0.002 by analysis of variance and p< 0.05 by Dunnett's simultaneous test versus time 0


A)
Viability
%Viability
120
100
80
60
40
20
0
0
24
48
72
96
Time (hrs)
*
*
*
δ
δ
no co-culture
OP9-DL1
TEC

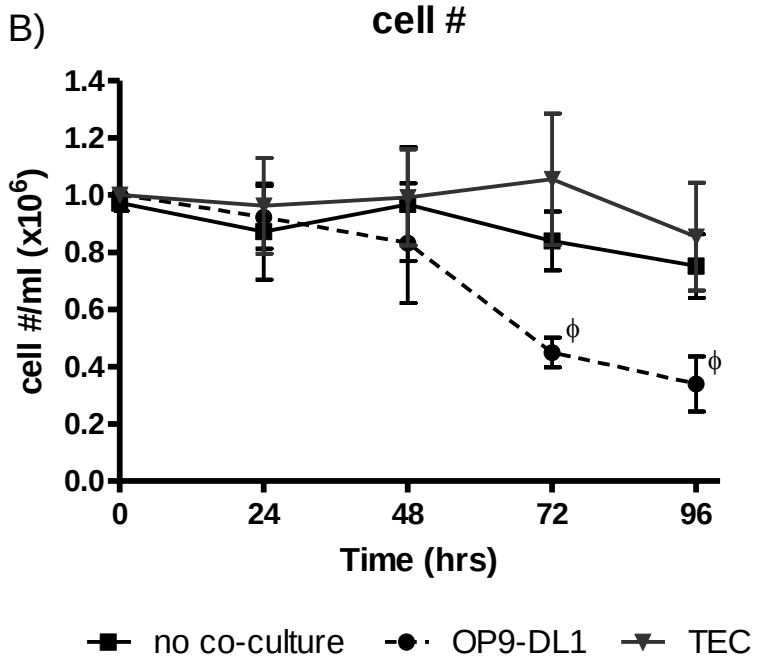
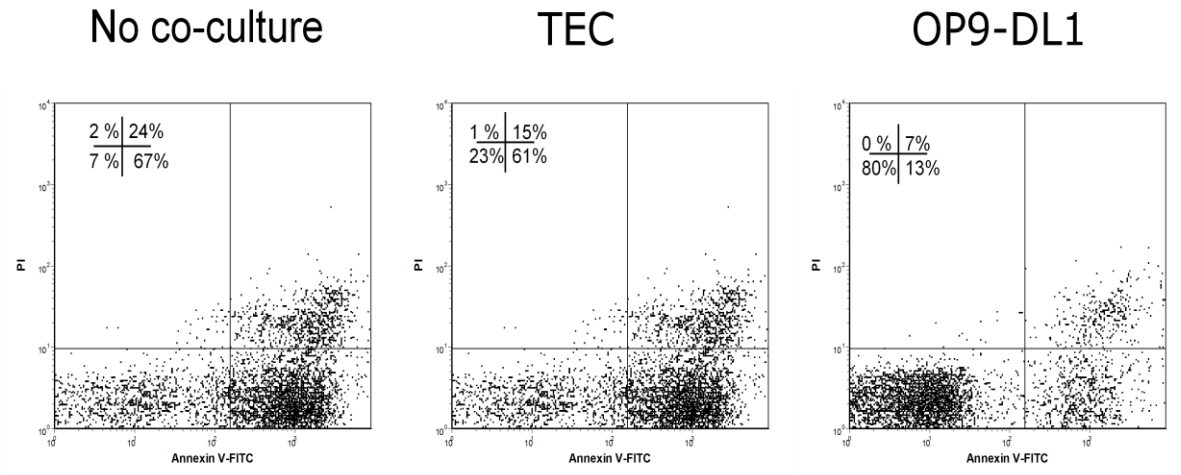
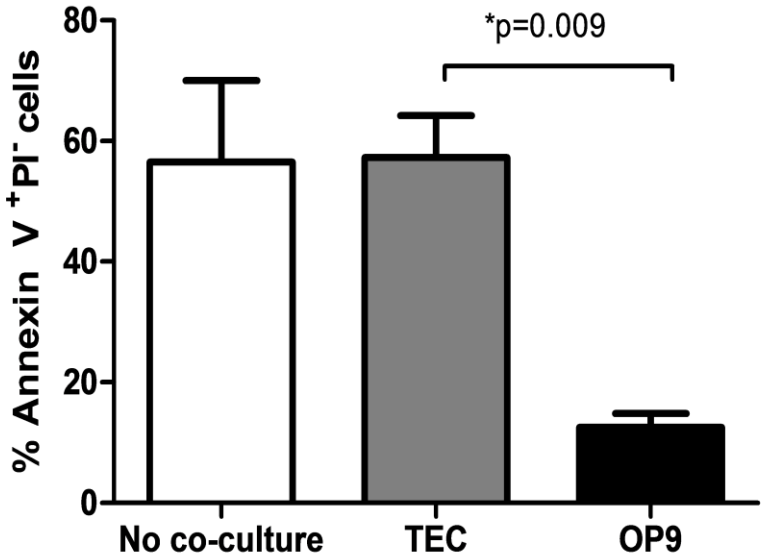

B)
cell #
cell #/ml (x10^6)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
Time (hrs)
ϕ
ϕ
no co-culture
OP9-DL1
TEC

Figure 8. Apoptosis of thymocytes in co-culture . Thymocytes were cultured alone as a control or co-cultured with TEC or OP9-DL1 for 96 hours. A) Representative flow histograms where 10,000 events were acquired and apoptosis of the cells was determined by annexin V/PI staining and. B) Summary data (mean ± SD) the percentage of apoptotic ($Annexin^{+}PI^{-}$) thymocytes that were cultured alone, or with TEC or OP9-DL1 cells (n=4, p=0.009 by paired student T-test)

A)


No co-culture
TEC
OP9-DL1
2 % 24%
7 % 67%
1 % 15%
23% 61%
0 % 7%
80% 13%
PI
Annexin V-FITC

B)


*p=0.009
% Annexin V +PI- cells
80
60
40
20
0
No co-culture
TEC
OP9

3.3.4 Thymocyte phenotype changes with co-culture

In order to determine if the subset distribution of total human thymocytes is altered in short term co-cultures, the phenotype of thymocytes co-cultured with either OP9-DL1 or TEC over 96 hours was evaluated. Short term co-culture with OP9-DL1 cells resulted in a lower proportion of cells in the $CD3^{+}$DP subsets compared to TEC co-cultures, although this did not reach statistical significance. There was also a higher proportion of SP4 cells compared to TEC co-cultures (29 % *vs.* 12 % respectively p=0.04; Figure 9). Since IL-7 is critical for thymocyte function and the expression of its receptor is highly regulated on thymocytes[183,189], the effect of co-culture on the expression of the IL-7 receptor (CD127) was investigated. Co-culturing thymocytes with OP9-DL1 cells resulted in lower CD127 expression on SP4 cells when compare to TEC co-cultures (p=0.05) (29.8 ± 14.5 % vs. 51.8 ±1.3 %, respectively; Figure 9C). Culture conditions had no significant effect on CD127 expression on the other thymic subsets.

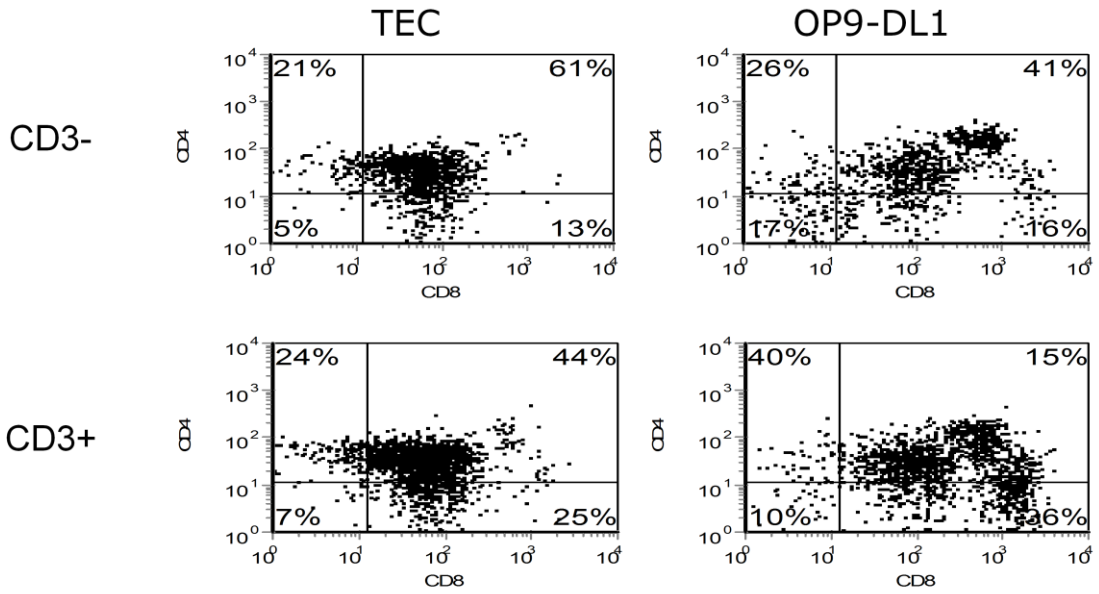
3.4 IL-7 mediated function and signalling in thymocytes

3.4.1 IL-7 stimulation of thymocytes up regulates Bcl-2 expression

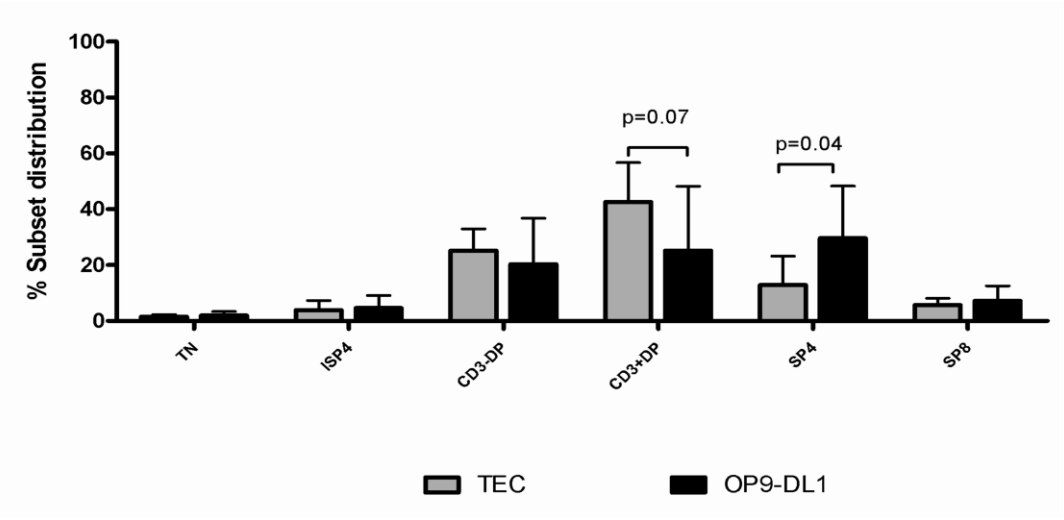
IL-7 signalling leads to enhance survival in TN, ISP4, SP4 and SP8 thymocytes. This aspect of its function has been attributed to the ability of IL-7 to induce expression of the anti-apoptotic molecule, Bcl-2[109,190,191]. The objective of this study was to confirm the ability of IL-7 to increase Bcl-2 expression in unfractionated thymocytes, since this assay will be used in further functional studies following *in vitro* HIV infection. To determine the effect of IL-7 stimulation on Bcl-2 expression,

Figure 9. Effect of co-culture on thymocyte phenotype. Thymocytes were co-cultured with TEC or OP9-DL1 for 96 hours. A) Representative figure of subset distribution of bulk thymocytes where 10,000 events were acquired. B) OP9-DL1 co-culture resulted in a trend towards a lower proportion of $CD3^{+}DP$ ($p=0.07$) compared to TEC and a statistically significant higher proportion in SP4 cells ($p=0.04$) when compared to TEC cultures. $n=4$ $p=0.04$ by student t-test C) CD127 expression on individual subsets was determined by staining bulk thymocytes and gating on individual subsets. Thymocytes co-cultured with OP9-DL1 cells had a lower level of CD127 expression on SP4 cells when compared to TEC co-cultures. ($n=4$ $p=0.05$ Student t-test)

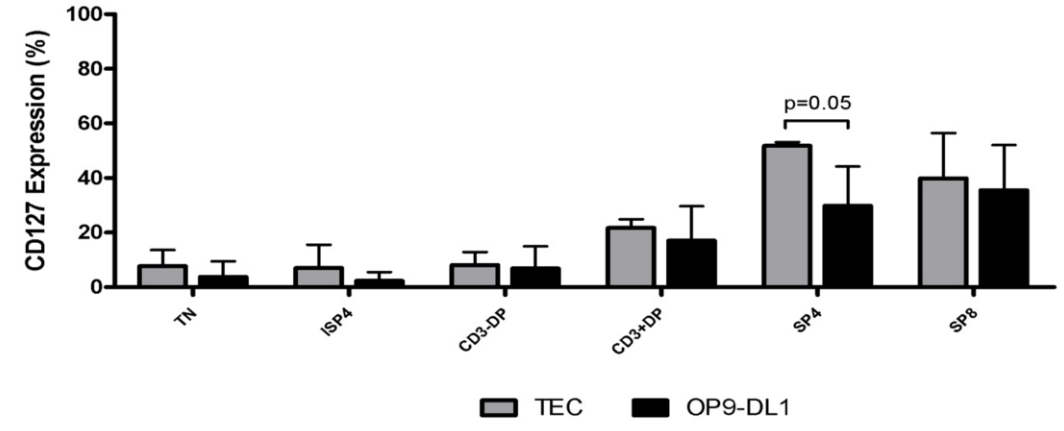
A)


TEC
OP9-DL1
CD3-
CD3+
CD4
CD8
21%
61%
5%
13%
26%
41%
17%
16%
24%
44%
7%
25%
40%
15%
10%
36%

B)


% Subset distribution
p=0.07
p=0.04
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8
TEC
OP9-DL1

C)


CD127 Expression (%)
p=0.05
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8
TEC
OP9-DL1

thymocytes were stimulated with IL-7 (1 ng/ml) for 48 hours (based on the optimized concentration for mature T-cells[192]) and Bcl-2 expression was measured by intracellular flow cytometry. Thymocytes co-cultured with TEC for 48 hours express a basal level of Bcl-2. IL-7 stimulation of bulk thymocytes resulted in an increase in Bcl-2 expression above the basal level (Figure 10 A) which is consistent with other published reports[185,190,193]. To determine if Bcl-2 expression varies amongst thymic subsets, bulk thymocytes were stained with antibodies to CD3, CD4 and CD8 (Figure 10 B). The expression of Bcl-2 in TN cells was not detectable, possibly due to the small proportion of cells within that subset. ISP4 cells had very high levels of basal Bcl-2 expression; however this subset of thymocytes did not further upregulate Bcl-2 expression following IL-7 stimulation. The expression of Bcl-2 was similar in $CD3^-$DP and $CD3^+$DP, with some basal expression that was further increased following IL-7 stimulation. Almost 100 % of SP4 cells have basal Bcl-2 expression and IL-7 stimulation resulted in an increase in the amount of Bcl-2 expression within this subset. SP8 cells also have basal expression of Bcl-2 which was increased following IL-7 stimulation.

3.4.2 IL-7 induced Bcl-2 expression is enhanced in thymocytes co-cultured with OP9-DL1 cells.

Since co-culture conditions may affect the capacity of thymocytes to respond to IL-7, the ability of thymocytes to upregulate Bcl-2 in response to IL-7 following culture alone or co-culture with either autologous TEC or OP9-DL1 cells was measured. IL-7 had little effect on Bcl-2 expression in thymocytes cultured alone (Figure 11).

Figure 10. Induction of Bcl-2 in thymic subsets following IL-7 stimulation. IL-7 induced Bcl-2 expression was measured on thymic subsets by stimulating unfractionated thymocytes with IL-7 (1 ng/ml) for 48 hours. The induction of Bcl-2 with respect to media control was measured by intracellular flow cytometry by gating on thymic subsets based on CD3, CD4 and CD8 expression. Interleukin-7 induced Bcl-2 expression was evaluated in: A) bulk thymocytes and B) TN, ISP4, $CD3^{+/-}$DP, SP4 and SP8 cells. Data are representative of 5 experiments. .

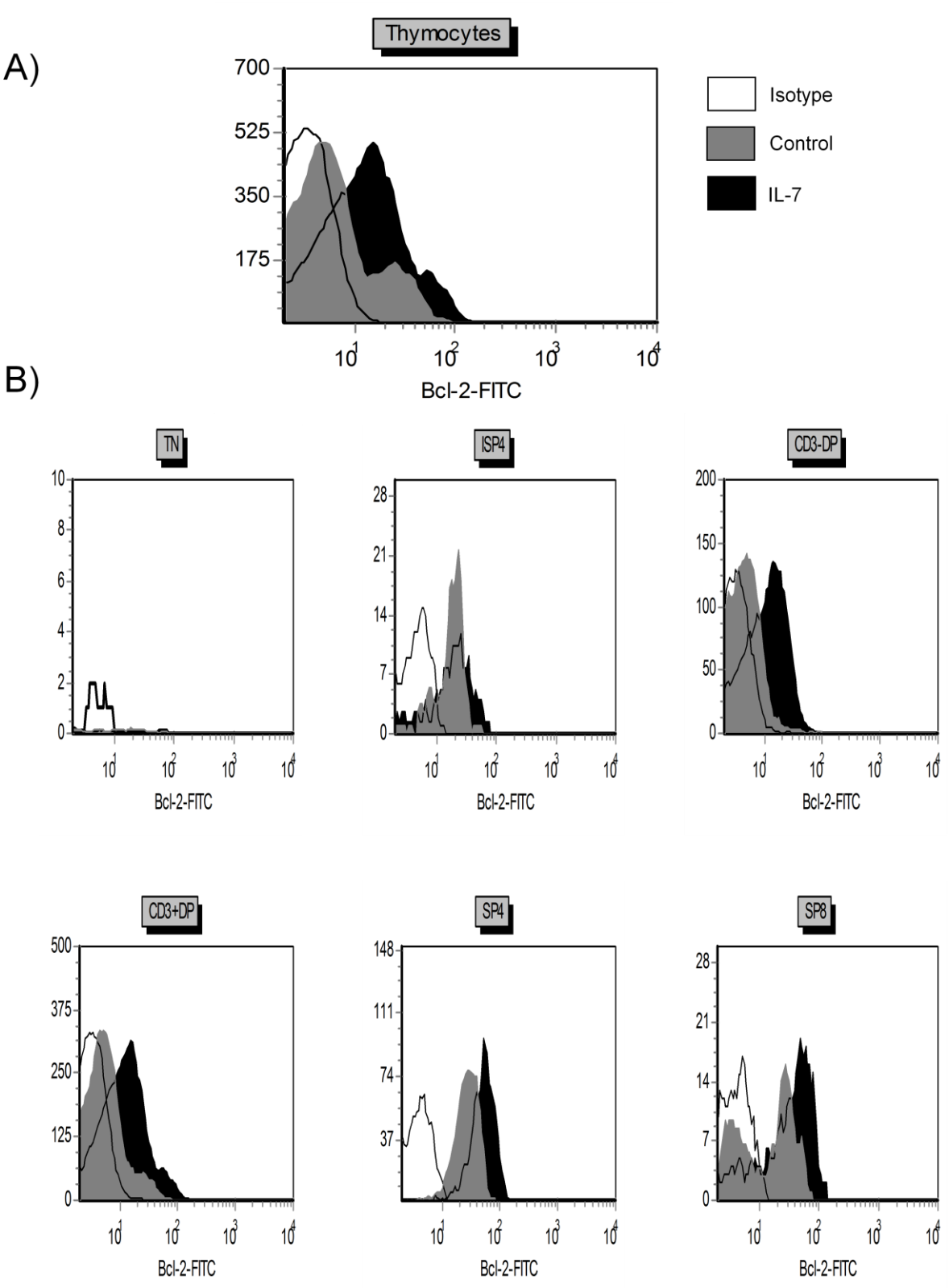
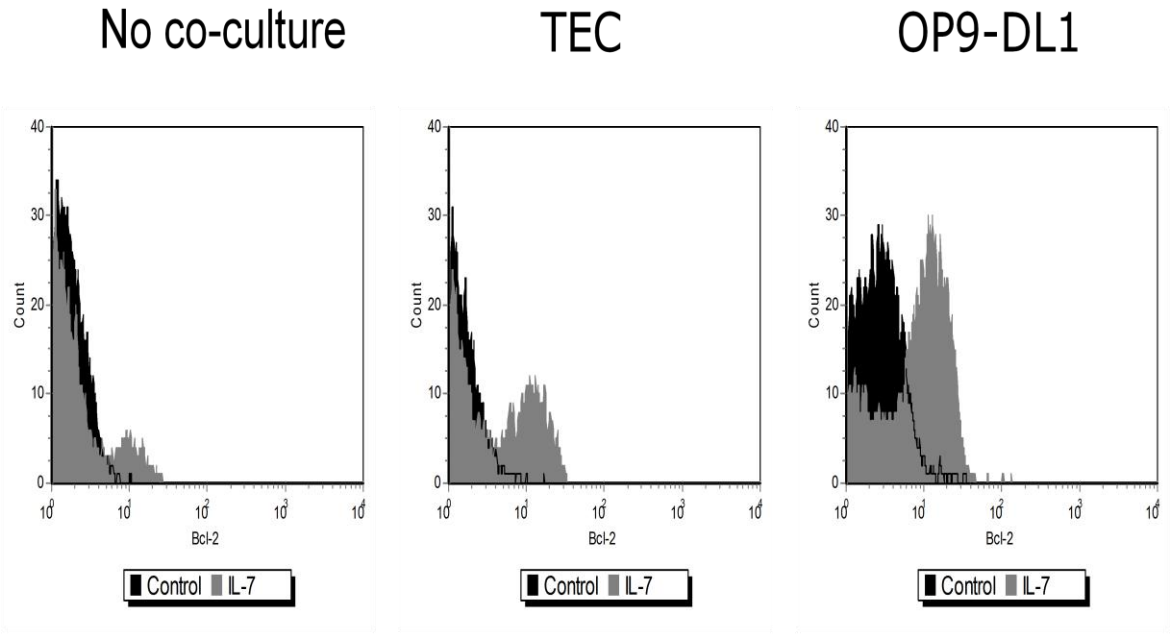
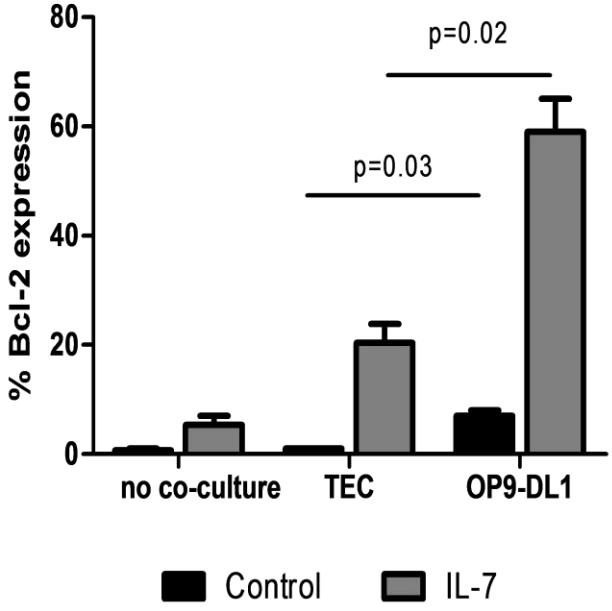

A)
Thymocytes
700
525
350
175
Isotype
Control
IL-7
Bcl-2-FITC
B)
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8
Bcl-2-FITC

Figure 11. Interleukin-7 induced Bcl-2 expression of co-cultured thymocytes. Thymocytes were cultured alone or co-cultured with either OP9-DL1 or TEC cells for 96 hours prior to stimulus with IL-7 (1 ng/ml) for 48 hours. Bcl-2 expression was measured by intracellular staining. A) A representative histogram of Bcl-2 expression in co-culture. B) Graphical summary of the mean ± SD assessing the percentage of Bcl-2 expression following IL-7 stimulation in thymocytes cultured alone, with TEC or OP9-DL1 cells. Thymocytes co-cultured with OP9-DL1 had a significantly higher level of Bcl-2 expression in response to IL-7 than thymocytes cultured with TEC. n=4 p=0.02 by paired student t-test.


No co-culture
TEC
OP9-DL1
Count
Bcl-2
Control
IL-7


% Bcl-2 expression
p=0.02
p=0.03
no co-culture
TEC
OP9-DL1
Control
IL-7

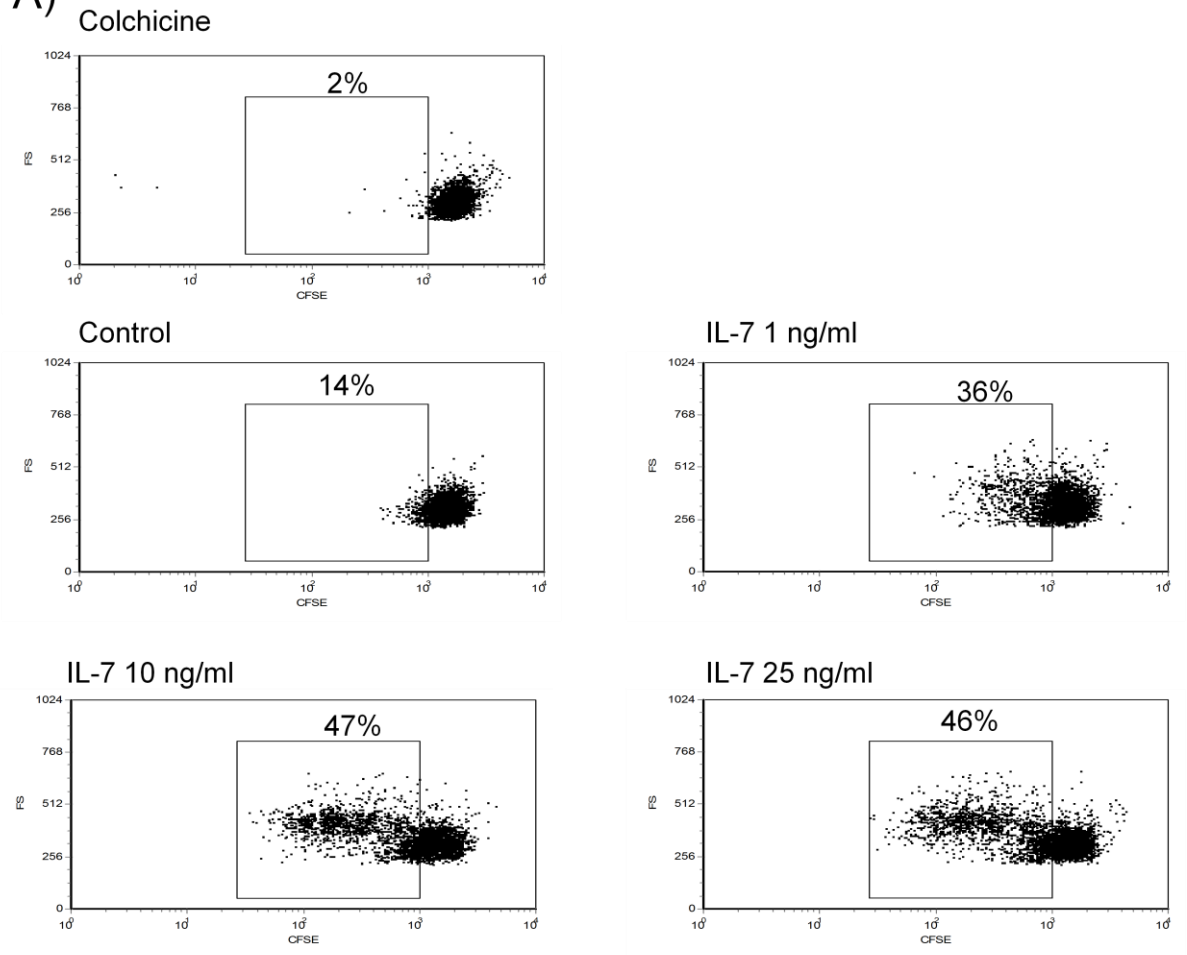
There was a higher level of basal Bcl-2 expression in bulk thymocytes following 96 hours of culture in OP9-DL1 co-cultures compared to TEC co-cultures as measured by the % of cells expressing Bcl-2 (OP9-DL1: 7.0 ± 1.7 % vs. TEC 1.0 ± 0.0 % (p=0.03)). IL-7 stimulation resulted in a higher % of cells expressing Bcl-2 in thymocytes co-cultured with OP9-DL1 cells compared to thymocytes co-cultured with TEC (59.0 ± 10.4% vs. 20.3 ± 6.1 %; Figure 6 p=0.024) which is consistent with previous reports showing that Notch signalling can enhance IL-7 signalling in thymocyte precursors [194]. The absolute increase in Bcl-2 expression following IL-7 stimulation of thymocytes was also significantly higher in thymocytes co-cultured with OP9-DL1 cells when compared with thymocytes co-cultured with TEC (52.0 % ± 9.8 % vs. 19.3 % ± 6.1 %; p=0.012).

3.4.3 Thymocytes proliferate in response to IL-7

To determine the proliferative capacity of thymocytes, bulk thymocytes were stained with CFSE and incubated for 7 days with various stimuli such as PWM 3μg/ml or IL-7 (0-25 ng/ml) (Figure 12 A). Unstimulated thymocytes did not proliferate after 7 days. IL-7 (1-10 ng/ml) induced proliferation of thymocytes in a dose dependent manner. There was no further induction of proliferation when cells were incubated with a high concentration of IL-7 (25 ng/ml). Thymocytes were also stimulated with sub-optimal concentrations of the T-cell mitogen PHA with or without IL-7 (Figure 13). Thymocytes did not proliferate to PHA alone and PHA did not increase the proliferative response of thymocytes to IL-7. To determine if individual thymic subsets had differential responses to IL-7, bulk thymocytes were CFSE-labelled

Figure 12. Interleukin-7 induced proliferation of thymocytes. A) Thymocytes were stained with CFSE and cultured for 7 days with increasing concentrations of IL-7. Control gates were set based on CFSE staining profile with colchicine treatment. Representative data B) Thymocytes proliferated in a dose dependant manner to IL-7 stimulus n=5, * $p=0.01$, ** $p<0.0001$ by paired student t-test compared to unstimulated control.

A)


Colchicine
2%
Control
14%
IL-7 1 ng/ml
36%
IL-7 10 ng/ml
47%
IL-7 25 ng/ml
46%
FS
CFSE

B)

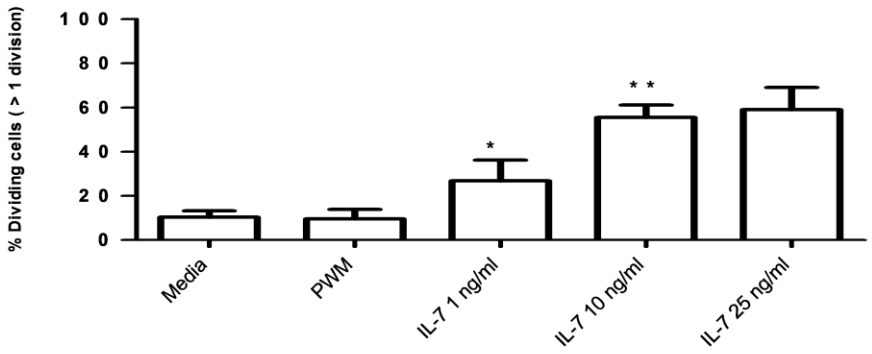
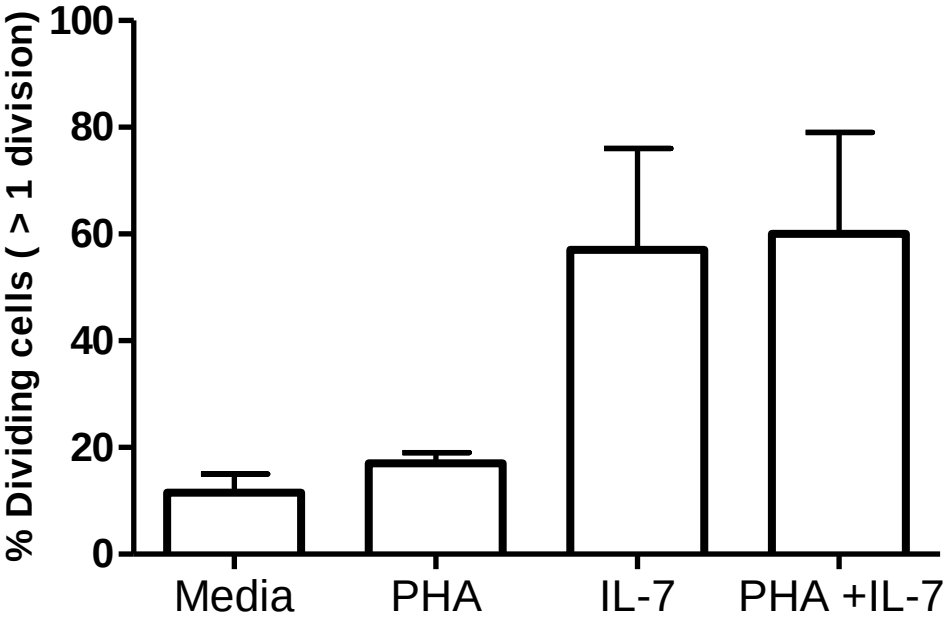

% Dividing cells (> 1 division)
100
80
60
40
20
0
*
* *
Media
PWM
IL-7 1 ng/ml
IL-7 10 ng/ml
IL-7 25 ng/ml

Figure 13. PHA has no effect of on thymocyte proliferation. Thymocytes were stained with CFSE then stimulated with suboptimal concentrations of PHA (2.5 μg/ml) or IL-7 (10ng/ml) or both PHA and IL-7 and cultured for 7 days. The percentage of thymocytes that have undergone more than one division (mean ± SD) are presented . Thymocytes did not proliferate to PHA alone. PHA stimulation did not increase IL-7 induced proliferation of thymocytes. n=2

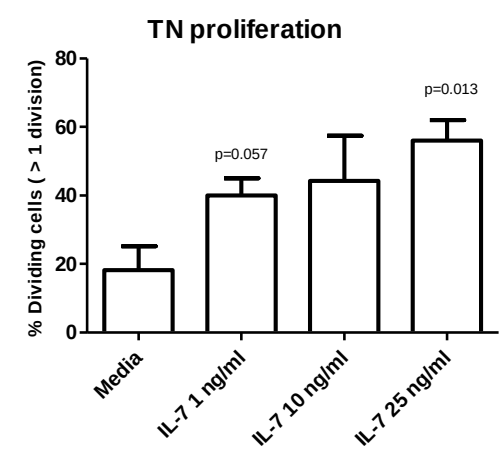

% Dividing cells (> 1 division)
100
80
60
40
20
0
Media
PHA
IL-7
PHA +IL-7

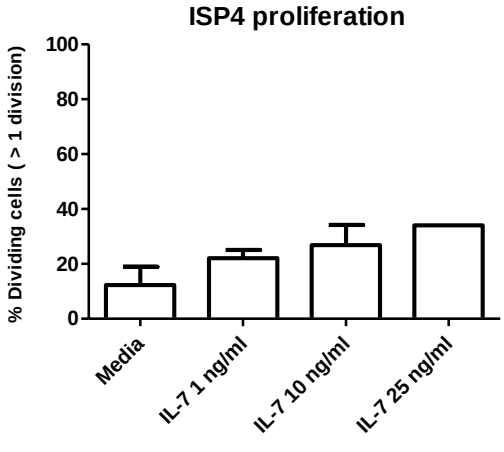
prior to IL-7 stimulation and then stained with CD3, CD4 and CD8 antibodies following 7 days of incubation (Figure 14). TN cells which express moderate levels of CD127 proliferated in response to the higher concentrations of IL-7 ($p=0.013$). Although ISP4 express similar levels of CD127 to TN, they did not proliferate to IL-7. $CD3^-$DP which have low expression of CD127 did not respond to IL-7 stimulation as measured by proliferation. The more mature subsets, which express the highest level of CD127 did respond to IL-7, with $CD3^+$DP having 65.8 % ± 17.5 %, SP4 62.6% ± 21.5% and SP8 71.6 % ± 14.3 % of cells going through more than one division and this was significantly different compared to media controls.

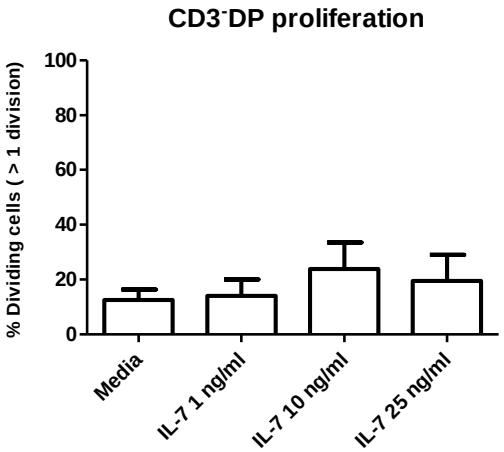
3.4.4 Thymocytes uptake glucose in response to IL-7

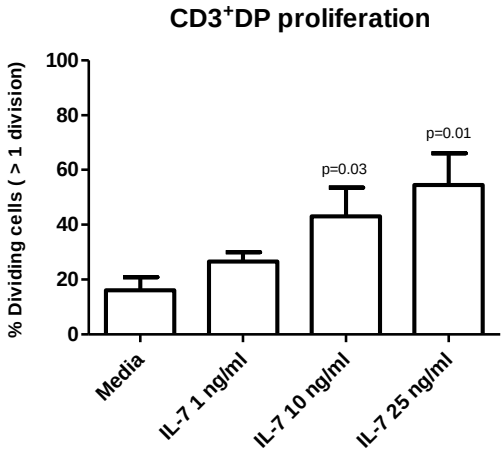
To determine the effect of IL-7 on metabolic processes, IL-7-induced glucose uptake in thymocytes was measured. IL-7 has been demonstrated to induce glucose uptake in recent thymic emigrants and circulating T-cells [195,196]. Thymocytes were either cultured alone or co-cultured with TEC or OP9-DL1 cells and stimulated with IL-7 (10 ng/ml) for 96 hours (Figure 15). The cultures were pulsed with 1 μCi of ^{3}H-D-glucose for 45 minutes and glucose uptake was measured using a microbetta plate reader. Thymocytes that were cultured alone or co-cultured with TEC had a small increase in the amount of glucose uptake following IL-7 stimulation, but this did not reach statistical significance. Thymocytes co-cultured with OP9-DL1 cells had a statistically significant increase in IL-7 induced glucose uptake ($p=0.02$).

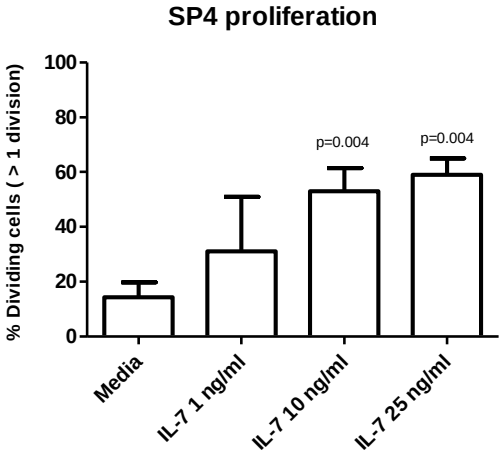
Figure 14. Interleukin-7 induced proliferation of thymocyte subsets. A) IL-7 induced proliferation was measured in thymic subsets. Ufractionated thymocytes were stained with CFSE and stimulated with IL-7. Following 7 days of culture cells were stained with antibodies to CD3, CD4 and CD8 and proliferation of each subset was determined by flow cytometry. Data are presented as mean ± SD, n= 4. Data were analyzed by paired student t-test.


TN proliferation
% Dividing cells (> 1 division)
0
20
40
60
80
p=0.057
p=0.013
Media
IL-7 1 ng/ml
IL-7 10 ng/ml
IL-7 25 ng/ml


ISP4 proliferation
% Dividing cells (> 1 division)
0
20
40
60
80
100
Media
IL-7 1 ng/ml
IL-7 10 ng/ml
IL-7 25 ng/ml


CD3-DP proliferation
% Dividing cells (> 1 division)
0
20
40
60
80
100
Media
IL-7 1 ng/ml
IL-7 10 ng/ml
IL-7 25 ng/ml


CD3+DP proliferation
% Dividing cells (> 1 division)
0
20
40
60
80
100
p=0.03
p=0.01
Media
IL-7 1 ng/ml
IL-7 10 ng/ml
IL-7 25 ng/ml


SP4 proliferation
% Dividing cells (> 1 division)
0
20
40
60
80
100
p=0.004
p=0.004
Media
IL-7 1 ng/ml
IL-7 10 ng/ml
IL-7 25 ng/ml

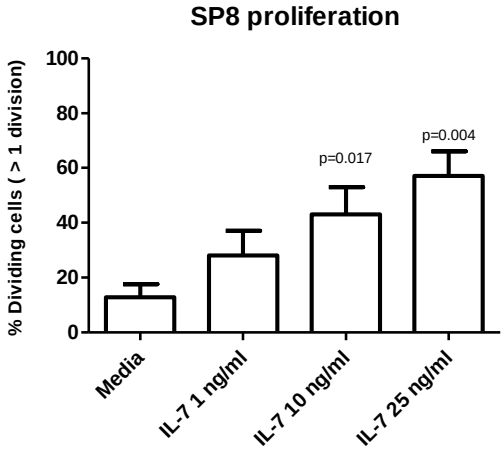
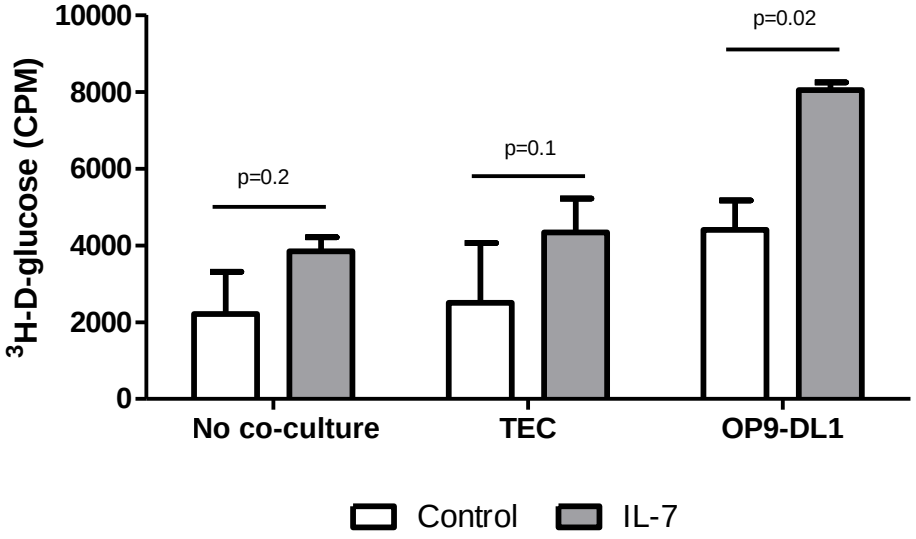
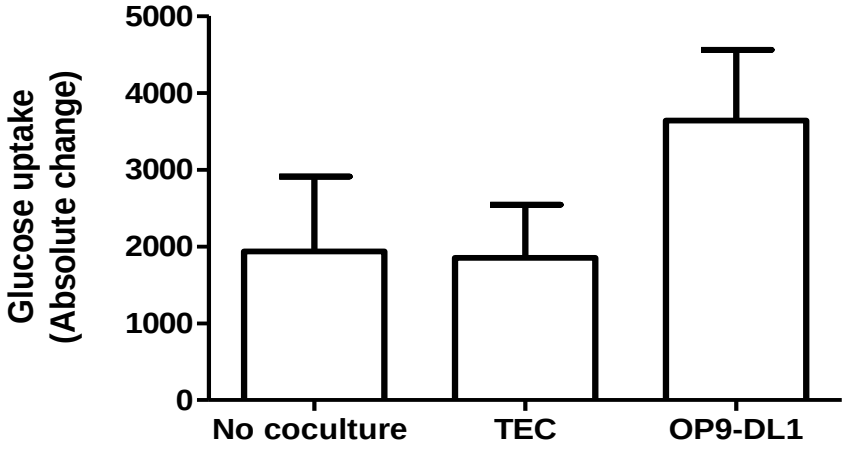

SP8 proliferation
% Dividing cells (> 1 division)
0
20
40
60
80
100
p=0.017
p=0.004
Media
IL-7 1 ng/ml
IL-7 10 ng/ml
IL-7 25 ng/ml

Figure 15. Interleukin-7 induced glucose uptake. A) Glucose uptake was measured in thymocytes co-cultures with TEC ± IL-7 or OP9-DL1 ± IL-7. Controls consisted of thymocytes cultured alone ± IL-7. B) Absolute change in glucose uptake was calculated as: IL-7 (CPM)- control (CPM). Data are expressed as mean ± SD, n=3 for cell cultured alone and with OP9-DL1 and n=2 for TEC. Data were analyzed by student t-test.

A)


10000
8000
6000
4000
2000
0
^{3}H-D-glucose (CPM)
p=0.2
p=0.1
p=0.02
No co-culture
TEC
OP9-DL1
Control
IL-7

B)


5000
4000
3000
2000
1000
0
Glucose uptake
(Absolute change)
No coculture
TEC
OP9-DL1

3.4.5 IL-7 induces STAT-5 phosphorylation in thymocytes.

The signalling pathways activated by IL-7 in mature T-cells have been well defined[62,63,87]. In order to confirm the pathways in human thymocytes, cells were incubated with IL-7 (1-10 ng/ml) and intracellular STAT-5 phosphorylation was measured by flow cytometry after 15 minutes, at which time STAT-5 was rapidly phosphorylated (Figure 16). To determine the responsiveness of individual thymic subsets to IL-7, bulk thymocytes were also stained with CD3, CD4 and CD8. STAT-5 was not phosphorylated in TN, ISP4 and $CD3^-$DP cells in response to IL-7 (Figure 16B). Although $CD3^+$DP cells have low CD127 expression, they responded to IL-7 stimulation as measured by STAT-5 phosphorylation. STAT-5 phosphorylation was observed in both SP4 and SP8 thymocytes following IL-7 stimulation.

3.4.6 IL-7 induced STAT-5 phosphorylation is similar in both co-culture systems

To determine if human thymocytes in co-culture remained functional *ex vivo*, thymocytes were co-cultured with TEC or OP9-DL1 cells and pSTAT-5 in response to IL-7 stimulation was measured. IL-7 stimulation did not induce phosphorylation of STAT-5 in thymocytes when cultured alone (Figure 17). Consistent with previous reports [84,193,197] thymocytes that were co-cultured with OP9-DL1 cells had a significantly higher level of basal pSTAT-5 compared to thymocytes co-cultured with TEC, as measured by the % of $pSTAT\text{-}5^+$ cells (5.3 ± 1.8 % vs. 1.0 ± 0.4 %; p=0.03) (Figure 17). The % of $pSTAT\text{-}5^+$ cells following IL-7 stimulation was similar between co-culture conditions (OP9-DL1: 17.4 ± 5.8 % vs. TEC: 11.3 ± 1.1 %; p=0.26). The

Figure 16. Interleukin-7 stimulates STAT-5 phosphorylation (pSTAT-5) in thymic subsets. A) Bulk thymocytes were stimulated with IL-7 (1 ng/ml) for 15 minutes and STAT-5 phosphorylation was measured by flow cytometry. B) IL-7 induced pStat-5 in thymocyte subsets was measured by stimulating bulk thymocytes with IL-7 (1 ng/ml) and staining bulk thymocytes with CD3, CD4 and CD8 and gating on specific subsets for intracellular pStat-5 by flow cytometry. The more immature subsets TN, ISP4 and CD3-DP had minimal levels of IL-7 induced Stat-5 phosphorylation. Whereas the more mature subsets CD3-DP, SP4 and SP8 subsets had measurable levels of IL-7 induced STAT-5 phsophorylation.

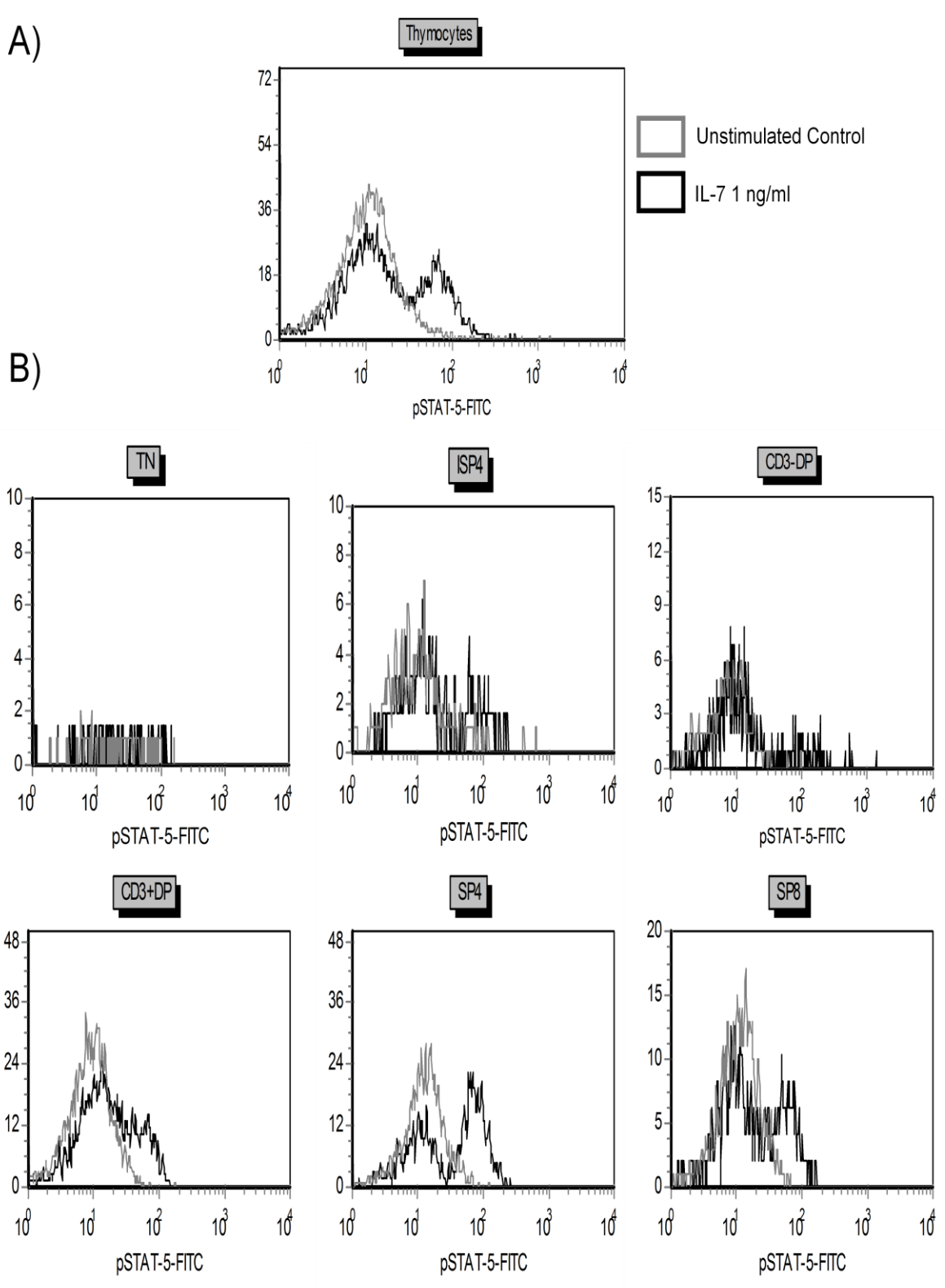
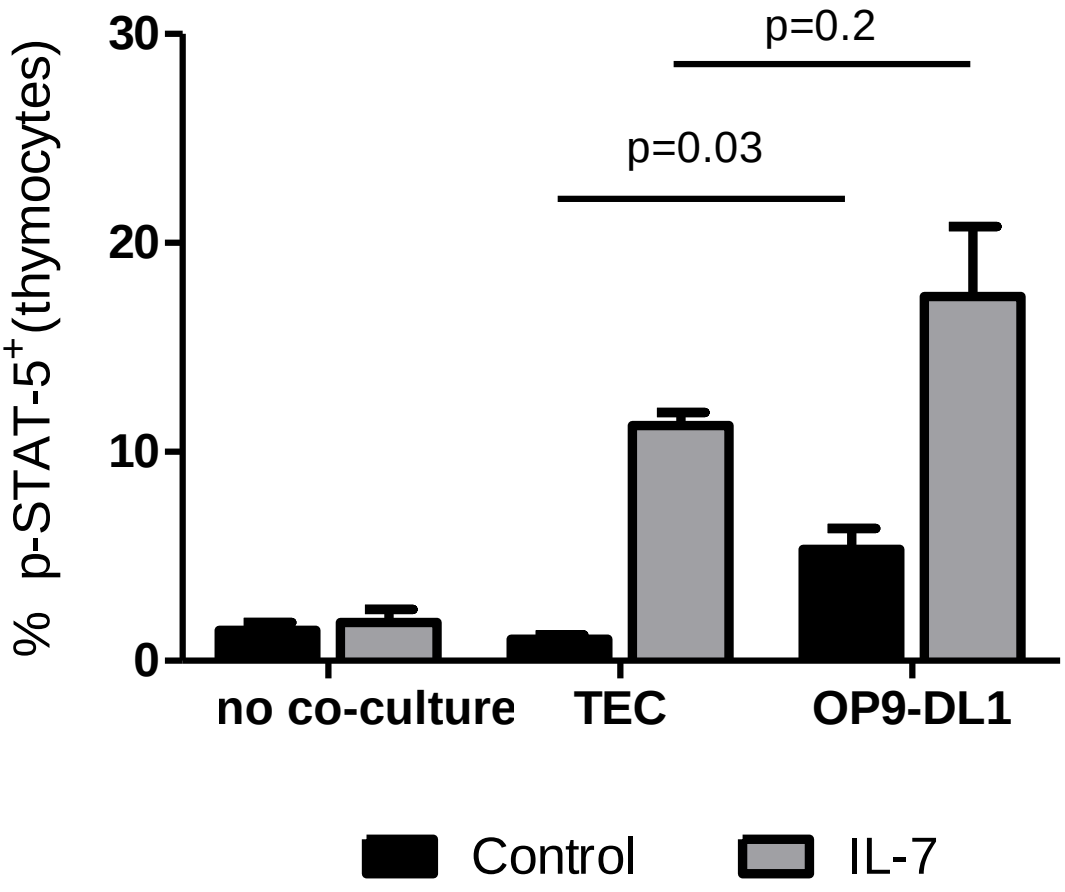

A)
Thymocytes
72
54
36
18
0
Unstimulated Control
IL-7 1 ng/ml
pSTAT-5-FITC
B)
TN
ISP4
CD3-DP
CD3+DP
SP4
SP8
pSTAT-5-FITC

Figure 17 Interleukin-7 stimulates pSTAT-5 in thymic subsets. Thymocytes were co-cultured with OP9-DL1 or TEC cells for 96 hours and then stimulated with IL-7 (1 ng/ml) for 15 minutes. The level of STAT-5 phosphorylation was measured by intracellular staining. Graphical summary of the mean ± SD assessing the percentage of cells expressing pSTAT-5. Thymocytes within OP9-DL1 co-cultures had a higher level of basal STAT-5 phosphorylation than thymocytes with TECs $p=0.03$. There was no difference in the level of pSTAT-5 following IL-7 stimulation between culture systems $p=0.2$. $n=3$ statistical significance calculated by a paired student t-test.


p=0.2
p=0.03
30
20
10
0
% p-STAT-5+(thymocytes)
no co-culture
TEC
OP9-DL1
Control
IL-7

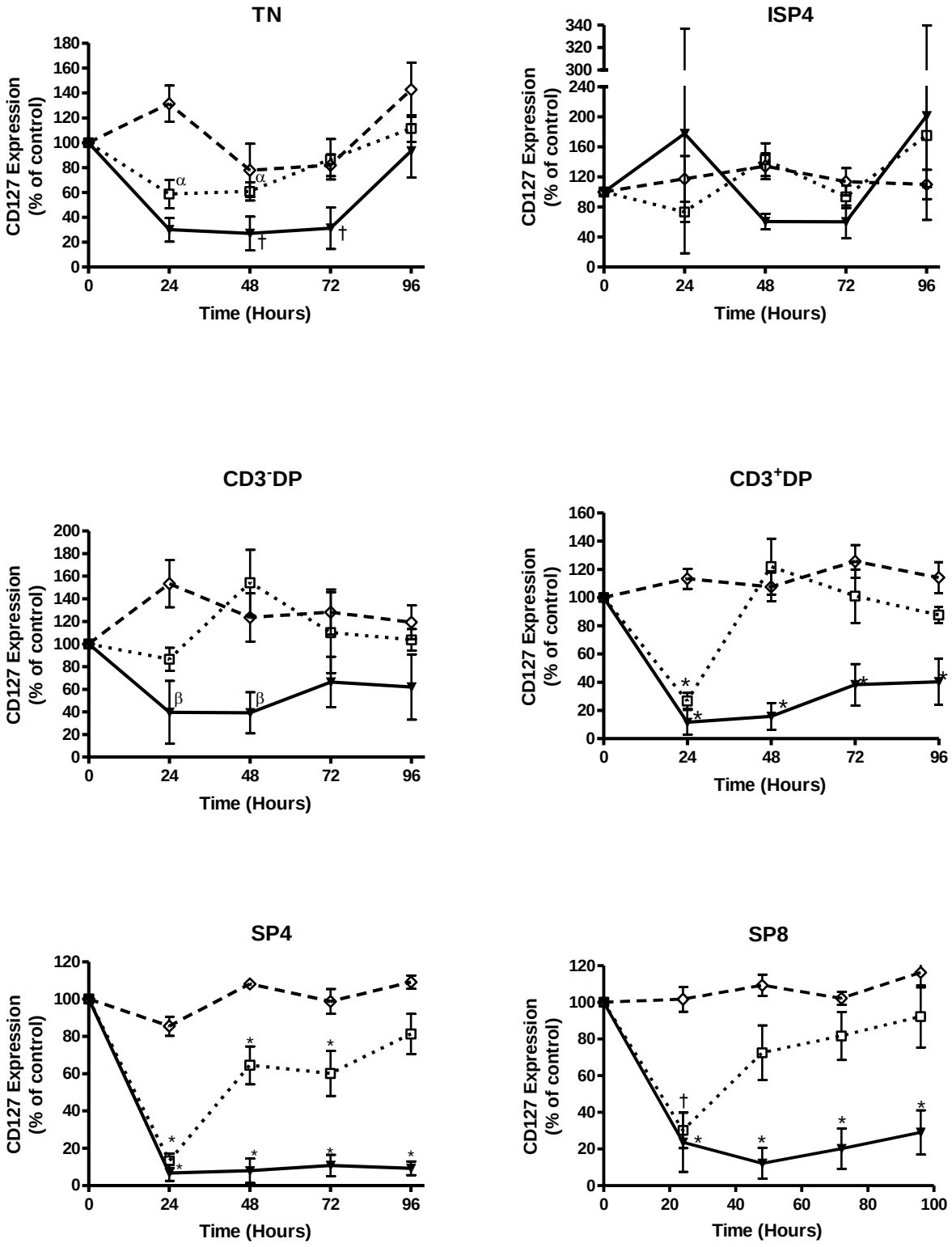
absolute increase in pSTAT-5 induced by IL-7 was also similar in both co-culture conditions (OP9-DL1: 10.2 % ± 1.4 % increase and TEC: 12.1 % ± 6.6 % increase). These results indicate that in co-cultures both TEC and OP9-DL1 cells provide the required signals for thymocytes to remain responsive to IL-7. This is in contrast to what is seen when thymocytes are cultured alone where they lose the capacity to respond to IL-7.

3.5 The regulation of CD127 expression on the surface of thymocytes

3.5.1 Interleukin-7 downregulates CD127 expression on thymocytes

Interleukin-7 has been shown to downregulate CD127 expression on $CD4^+$ and $CD8^+$ T-cells *in vivo* and *in vitro* [68,164,198]. Since IL-7 in the periphery is available in a limited quantity, it is thought that cells that have already received IL-7 signals downregulate CD127 to avoid redundant signalling and maximize the availability of IL-7, thereby activating the decrease in CD127 expression in an altruistic manner[73]. The same mechanism may hold true for thymocytes within the thymus. To evaluate if IL-7 affects CD127 expression on thymocytes, thymocyte/TEC co-cultures were incubated with increasing concentrations IL-7 (100- 5000 pg/ml) for up to 96 hours and cells were analysed by flow cytometry every 24 hours (Figure 18). The kinetics of CD127 decreases varied with the concentration of IL-7. At the lower concentration of 100 pg/ml IL-7 did not significantly affect CD127 expression on the surface of thymic subsets. IL-7 (1000 pg/ml) generally transiently decreased CD127 expression and IL-7 at 5000 pg/ml resulted in a more sustained downregulation of CD127 over 96 hours.

Figure 18 Interleukin-7 downregulates CD127 expression on thymocyte subsets. Thymocytes/TEC were stimulated with increasing concentrations of IL-7 (100-5000 pg/ml) for 96 hours. CD127 expression on individual subsets was determined by staining bulk thymocytes with CD3, CD4 and CD8 and gating on individual subsets. CD127 was normalized to the percent of CD127 expression on thymocytes cultured in media alone. Data represented as mean ± SD n=5 (* $p < 0.0001$, a $p = 0.0005$, † $p = 0.002$, b $p = 0.04$ by ANOVA and $p \leq 0.05$ by Dunnett's test versus time 0).


TN
ISP4
CD3-DP
CD3+DP
SP4
SP8
CD127 Expression (% of control)
Time (Hours)
IL-7 100 pg/ml
IL-7 1000 pg/ml
IL-7 5000 pg/ml

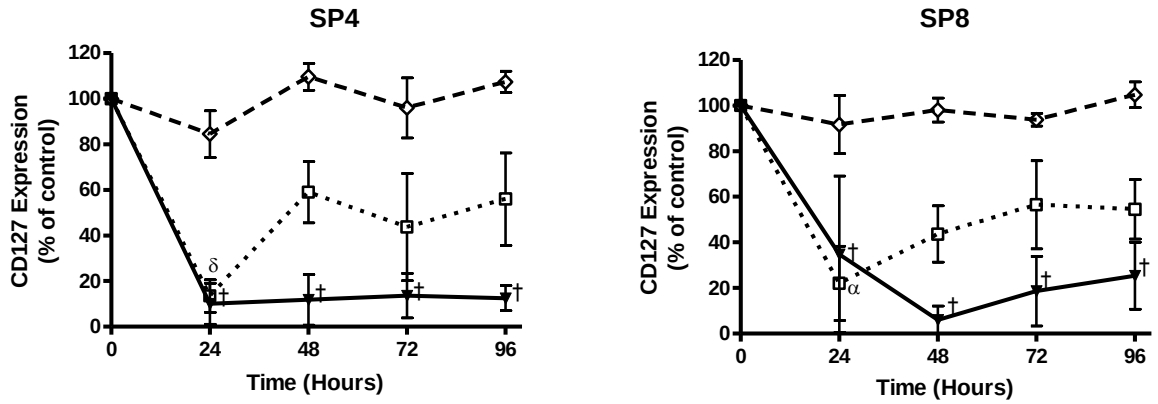
On TN cells, IL-7 (1000 pg/ml) stimulation resulted in a transient decrease in CD127, observed over 48 hours with a recovery above control levels after 96 hours of culture p=0.0005 (Figure 18). At higher concentrations of IL-7 (5000 pg/ml) the decrease in CD127 expression was much more pronounced and was maintained for 72 hours, recovering to control levels by 96 hours. CD127 expression on ISP4 cells was quite erratic; therefore no specific IL-7 effect could be confirmed. The decreased expression of CD127 on $CD3^-$DP only reached statistical significance at the higher concentration of IL-7 (5000 pg/ml) following 24 hours of culture p=0.04The effect of IL-7 on the expression of CD127 on $CD3^+$DP, SP4 and SP8 cells was similar, with IL-7 (1000 pg/ml) transiently decreasing CD127 expression within 24 hours p <0.0001. Subsequently CD127 surface expression recovered by 48 hours and at higher concentration (5000 pg/ml) sustained the decrease up to 96 hours (Figure 18). The downregulation of CD127 expression on thymocytes occurred regardless of the source of TEC (Figure 19 A,B). Thymocytes co-cultured with OP9-DL1 responded more readily to IL-7 by decreasing CD127 expression on thymocytes with 10 fold less IL-7 than what was required to decrease CD127 in thymocytes/TEC co-cultures (Figure 19C). Therefore IL-7 may be responsible for the regulation of CD127 expression on thymocytes similar to what is seen on mature circulating T-cells.

3.5.2 TNF-α downregulates CD127 expression on thymocytes.

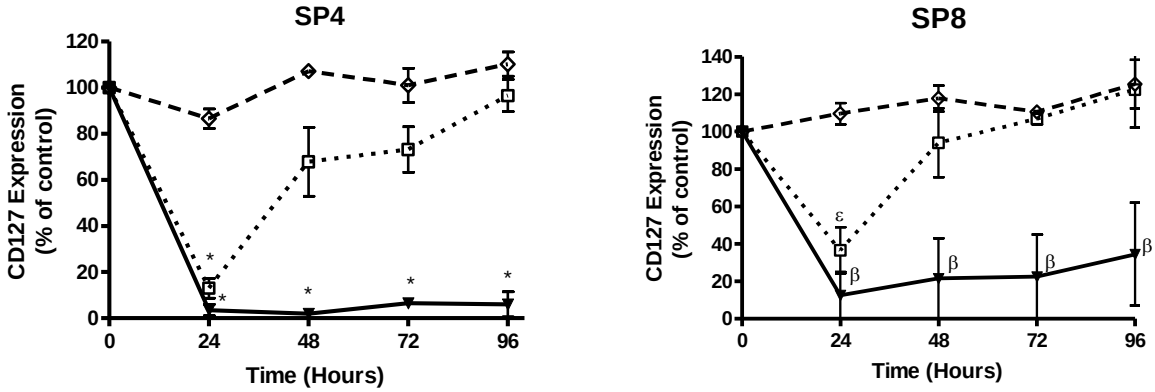
Thymic dysfunction in HIV infection may be caused by direct viral cytopathic effects or could be due to soluble factors released following infection. HIV infection affects

Figure 19. The effect of co-culture systems on IL-7 downregulation of CD127 expression. Thymocytes were co-cultured with A) Allogeneic TEC n=5 , B) Autologous TEC n=4 or C) OP9-DL1 cells n= 3 and stimulated with increasing concentrations of IL-7 (100-5000 pg/ml). The expression of CD127 on mature SP4 and SP8 cells was measured over 96 hours. CD127 was normalized to the percent of CD127 expression on thymocytes cultured in media alone. (* $p < 0.0001$, § $p=0.001$, † $p=0.002$, ε $p=0.004$, b $p=0.01$, a $p=0.02$, ¶ $p=0.03$ and d $p=0.08$ by ANOVA and $p \leq 0.05$ by Dunnett's test versus time 0).

A) Autologous TEC co-culture



B) Autologous TEC co-culture



C) OP9-DL1 co-culture

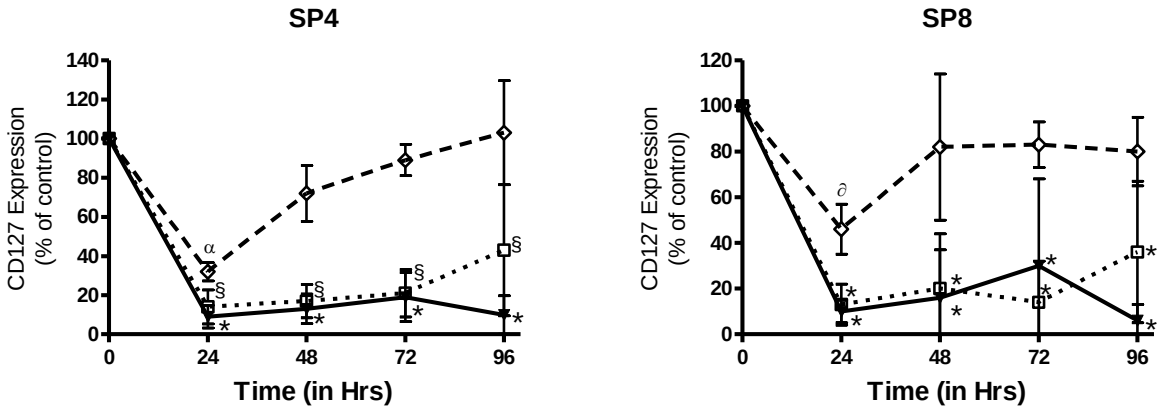
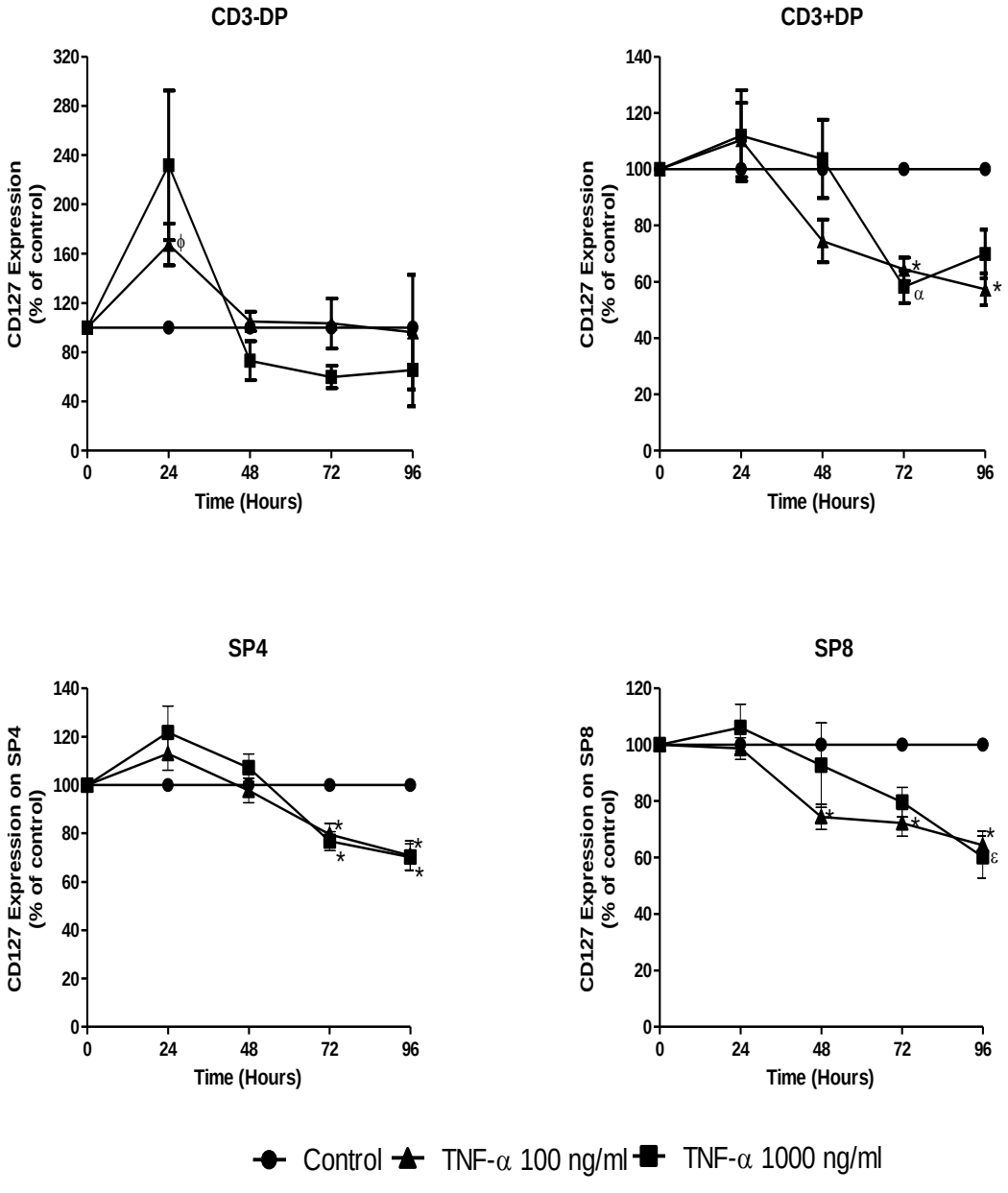


Figure 20. TNF-α decreases CD127 expression on thymocytes. Bulk thymocytes were incubated with increasing concentrations of TNF–α (100,1000 ng/ml) for 96 hours. CD127 expression on individual subsets was determined by staining bulk thymocytes with CD3, CD4 and CD8 and gating on individual subsets. Graphs are showing the mean ± SD. TNF-α decreased CD127 on $CD3^{+}$DP. SP4 and SP8 subsets. CD127 was normalized to the percent of CD127 expression on thymocytes cultured in media alone. (n=3 * $p < 0.0001$, ϕ $p=0.007$, ε $p=0.004$ and a $p=0.002$, by ANOVA and $p \leq 0.05$ by Dunnett's test versus time 0).


CD3-DP
CD127 Expression (% of control)
320
280
240
200
160
120
80
40
0
0
24
48
72
96
Time (Hours)
ϕ
CD3+DP
CD127 Expression (% of control)
140
120
100
80
60
40
20
0
0
24
48
72
96
Time (Hours)
α
*
SP4
CD127 Expression on SP4 (% of control)
140
120
100
80
60
40
20
0
0
24
48
72
96
Time (Hours)
*
SP8
CD127 Expression on SP8 (% of control)
120
100
80
60
40
20
0
0
24
48
72
96
Time (Hours)
ε
*
Control
TNF-α 100 ng/ml
TNF-α 1000 ng/ml

the cytokine network, resulting in an altered production of immune regulators. TNF-α is a proinflammatory cytokine that plays a role in immune regulation and its production is increased in HIV infection[199]. TNF and TNFR knockout mice showed increased proliferation of TN to IL-7 stimulation, suggesting a role for TNF-α in the regulation of IL-7 homeostasis in thymocyte development[200]. To determine if increased TNF-α production has a role in thymocyte dysfunction, we evaluated the effect of TNF-α on CD127 expression of thymocytes was evaluated. Thymocytes co-cultured with TEC were stimulated with TNF-α (1,10 ng/ml) for up to 96 hours (Figure 20). TNF-α stimulation tended to initially increase CD127 expression on thymocytes within the first 24 hours of culture, but this increase was only statistically significant in $CD3^-$DP cells p=0.007. This initial increase was followed by a gradual decrease in CD127 expression which reached statistical significance by 48 hours in SP8 cells ($p < 0.0001$) and by 72 hours in $CD3^+$DP and SP4 cells ($p < 0.0001$). The effects were not dose dependent over the range of concentrations evaluated.

3.5.3 GM-CSF and IFN-α do not significantly affect CD127 expression on thymocytes.

Other potential host soluble factors that may contribute to thymic dysfunction are IFN-α and GM-CSF. IFN-α production is increased in HIV infection and correlates with serum p24 antigen levels[201]. In addition, IFN-α has been shown to inhibit T-cell development in mice potentially by opposing the IL-7 response[163]. GM-CSF can increase HIV replication in monocyte-derived macrophages (MDM)[202] and can inhibit early T-cell development in mice[203]. To determine if these cytokines affect CD127

Figure 21. IFN–α does not affect CD127 expression on thymocytes. Bulk thymocytes were incubated with increasing concentrations of IFN-α (1-100 ng/ml) for 96 hours. CD127 expression on individual subsets was determined by staining bulk thymocytes with CD3, CD4 and CD8 and gating on individual subsets. Graphs are showing the mean ± SD. INF-α had no significant effect on CD127 expression on thymocytes, n=3.

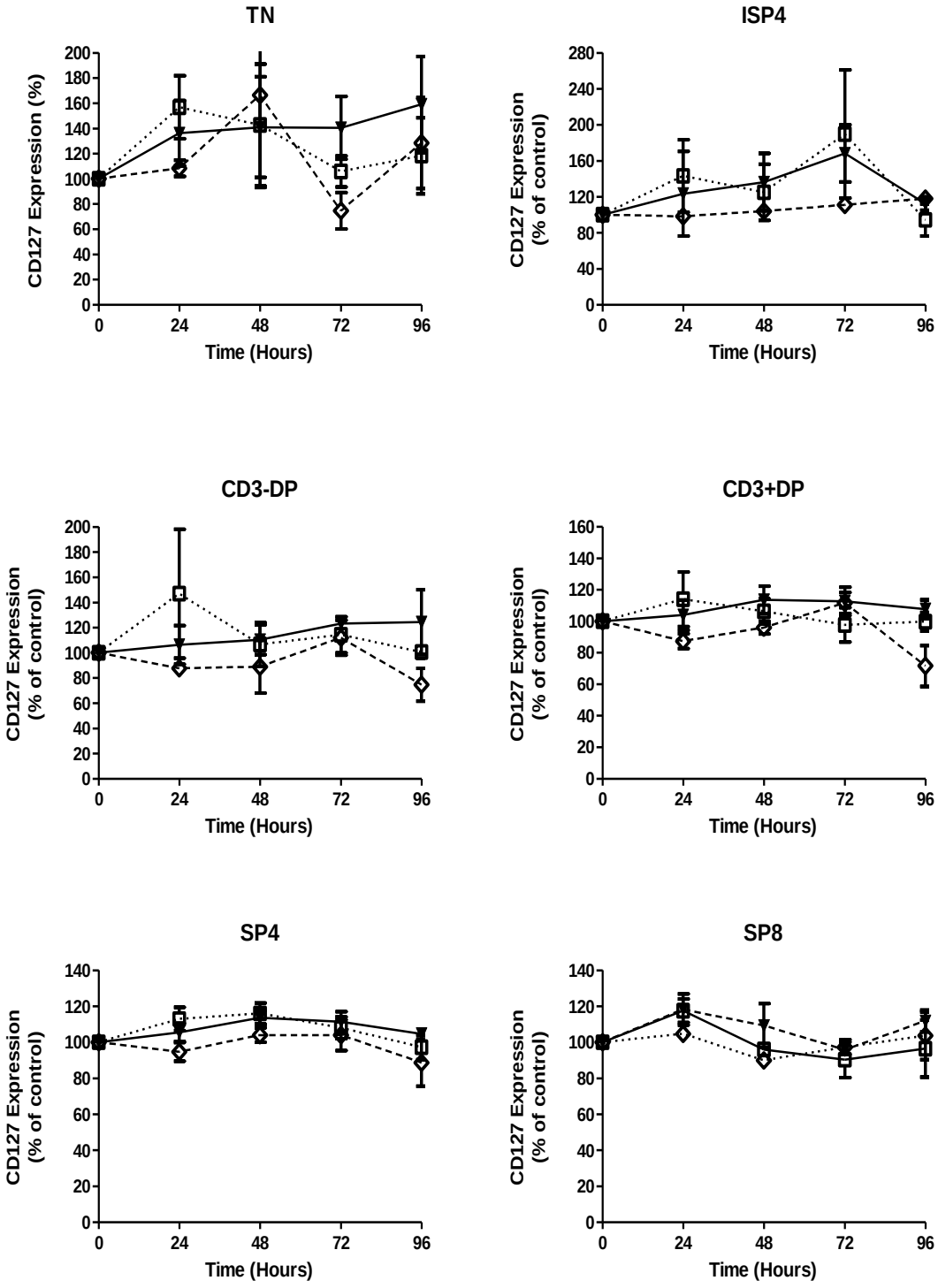
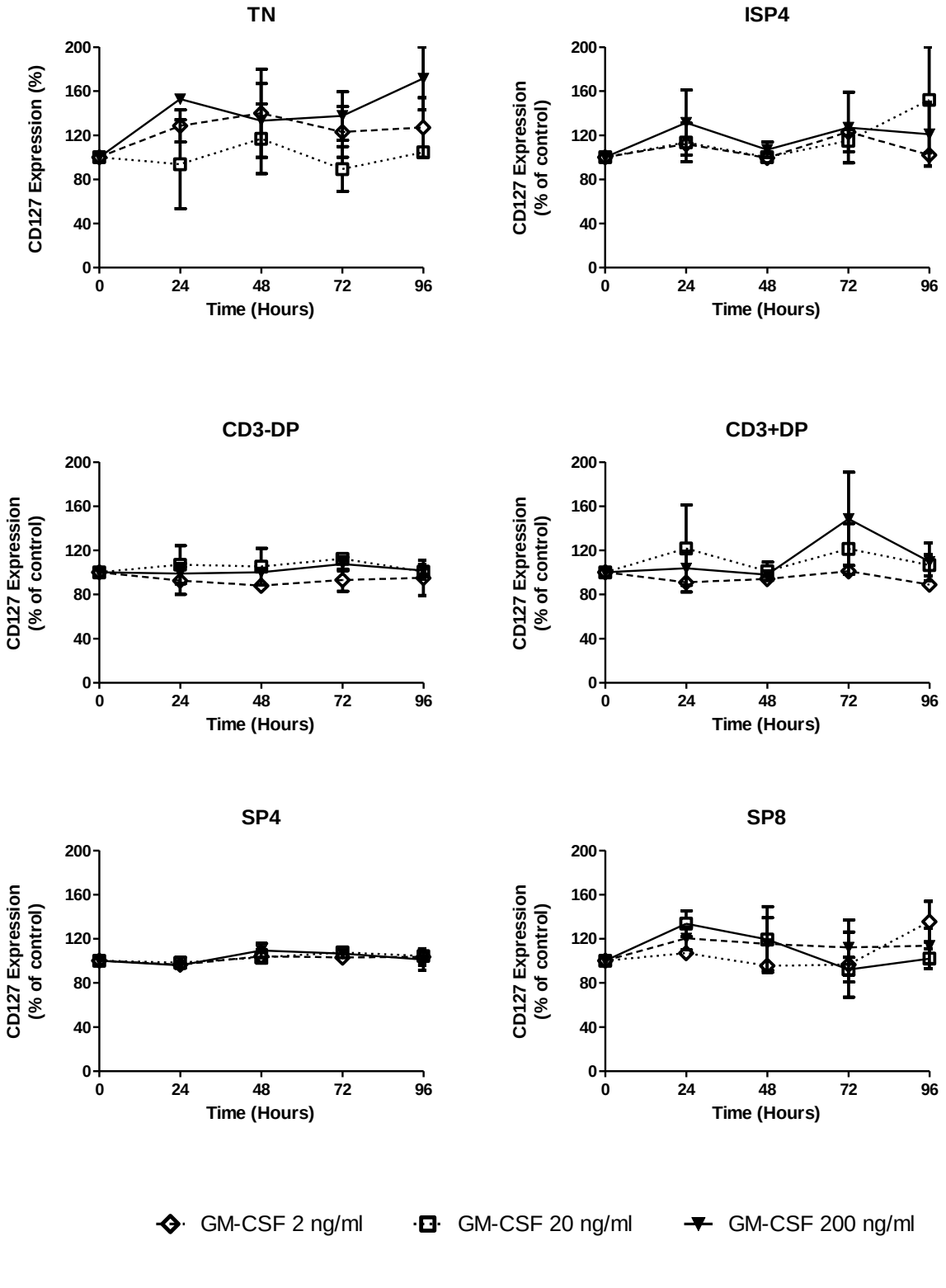

TN
ISP4
CD3-DP
CD3+DP
SP4
SP8
CD127 Expression (%)
CD127 Expression (% of control)
Time (Hours)
0
24
48
72
96
IFN-α 1 ng/ml
IFN-α 10 ng/ml
IFN-α 100 ng/ml

Figure 22. The effect of GM-CSF on CD127 expression on thymocytes. Bulk thymocytes were incubated with increasing concentrations of GM-CSF (2-200 ng/ml) for 96 hours. CD127 expression on individual subsets was determined by staining bulk thymocytes with CD3, CD4 and CD8 and gating on individual subsets. Graphs are showing the mean ± SD. GM-CSF had no significant effect on CD127 expression on thymocytes, n=3.


TN
CD127 Expression (%)
200
160
120
80
40
0
0
24
48
72
96
Time (Hours)
ISP4
CD127 Expression (% of control)
CD3-DP
CD3+DP
SP4
SP8
GM-CSF 2 ng/ml
GM-CSF 20 ng/ml
GM-CSF 200 ng/ml

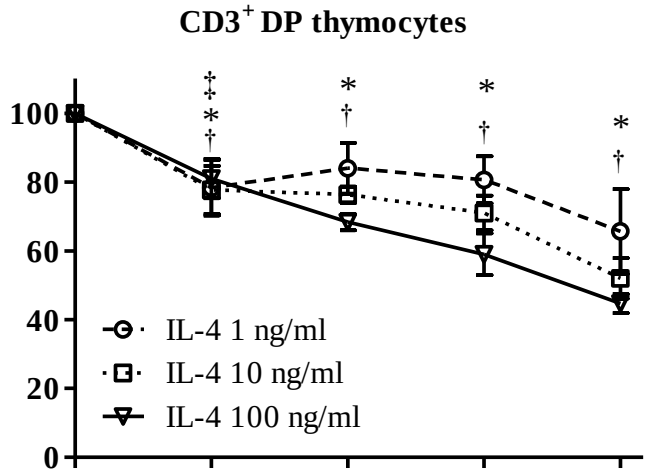
expression of human thymocytes, thymocyte/TEC co-cultures were incubated with increasing concentrations of either IFN-α (0-100 ng/ml) or GM-CSF (0-200 ng/ml) for 96 hours and CD127 expression was measured every 24 hours. IFN-α did not have any significant effect on the level of CD127 expression on thymocyte (Figure 21). CD127 expression on TN and ISP4 tended to increase following IFN-α stimulation, however due to individual variation in CD127 expression, the results did not reach levels of statistical significance. GM-CSF stimulation also tended to increase CD127 expression on TN and ISP4 thymocytes; however this effect did not reach statistical significance (Figure 22). GM-CSF stimulation had no effect on the expression of CD127 on all other thymocyte subsets.

3.5.4 IL-4 downregulates CD127 expression on thymocytes.

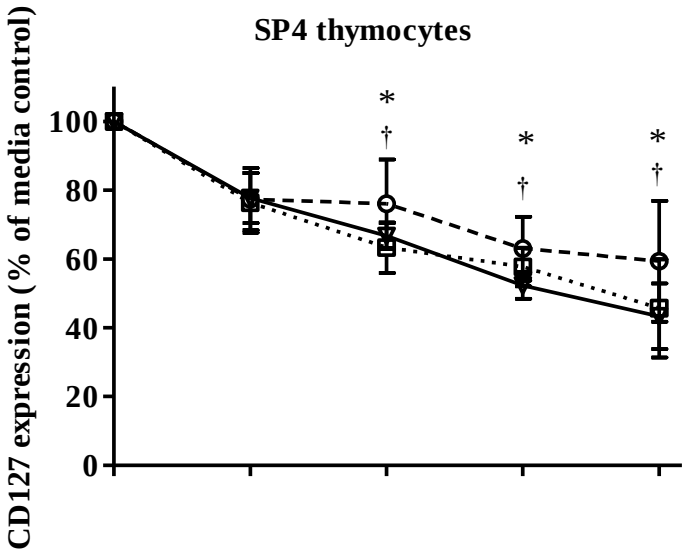
Several cytokines of the γ_C chain family downregulate CD127 transcription in mice including IL-4 [68]. To determine if IL-4 affects CD127 expression on human thymocytes, thymocytes/TEC co-cultures were stimulated with increasing concentrations of IL-4 (1-100ng/ml) for 96 hours. CD127 expression was measured every 24 hours and the change in the % of cells expressing CD127 relative to medium control was evaluated (Figure 23). IL-4 decreased CD127 expression on $CD3^+$DP, SP4 and SP8 cells. The kinetics of downregulation varied among the subsets. With $CD3^+$DP cells the decrease in CD127 occurred after 24 hours, in SP4 cells the decrease was most evident following 48 hours and in SP8 cells the decrease in CD127 was statistically significant only after 72 hours. IL-4 at the highest concentration (100 ng/ml) decreased CD127 expression the most on

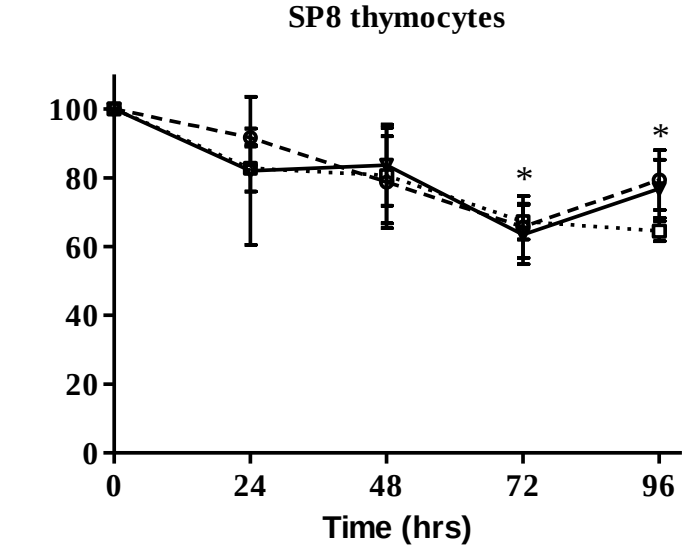
Figure 23. Interleukin-4 induces downregulation of CD127 expression on thymocyte subsets. Freshly isolated thymocytes were cultured with TECs and stimulated with increasing concentrations of IL-4. The expression of CD127 was analyzed by flow cytometry every 24 hrs for 96 hrs. CD127 expression was normalized to the expression of CD127 on unstimulated thymocytes at each time. A) CD127 expression on CD3+DP thymocytes exposed to IL-4 (0 -100 ng/ml) for up to 96 hours (* $p = 0.0006$; † $p < 0.0001$ by ANOVA and $p< 0.05$ by Dunnett's test versus baseline). B) CD127 expression on SP4 thymocytes exposed to IL-4 (0 -100 ng/ml) for up to 96 hours (* $p = 0.012$; † $p = 0.0004$ by ANOVA and p0.05 by Dunnett's test versus baseline). C) CD127 expression on SP8 thymocytes exposed to IL-4 (0 -100 ng/ml) for up to 96 hours (* $p = 0.021$ by ANOVA and $p = 0.05$ by Dunnett's test versus baseline), Data were normalised to CD127 expression of unstimulated thymocytes at each time point. Data shown are mean ± SD (n=3).

A


CD3+ DP thymocytes
100
80
60
40
20
0
IL-4 1 ng/ml
IL-4 10 ng/ml
IL-4 100 ng/ml

B

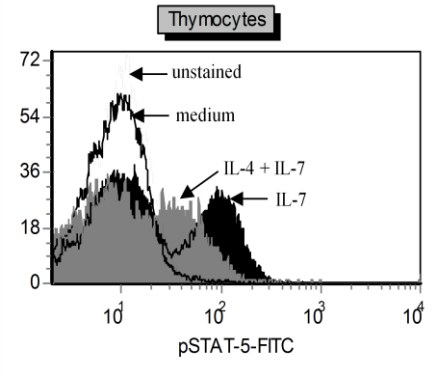

SP4 thymocytes
CD127 expression (% of media control)
100
80
60
40
20
0

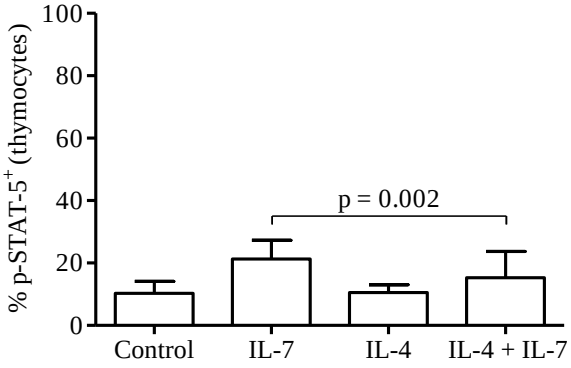

SP8 thymocytes
100
80
60
40
20
0
0
24
48
72
96
Time (hrs)

C

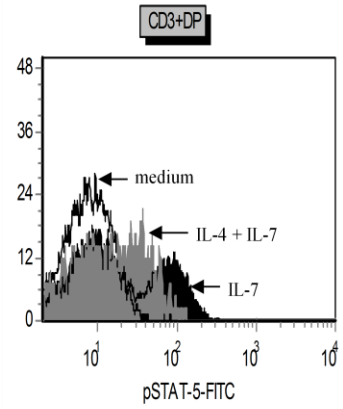
Figure 24 Interleukin-4 inhibits IL-7 induced STAT-5 phosphorylation. Bulk thymocytes were pre-treated with IL-4 (100 ng/ml) for 48 hours, then stimulated with IL-7 (1 ng/ml) for 15 minute prior to intracellular staining for pSTAT-5. Representative histograms are shown demonstrating unstimulated controls (white), IL-7 stimulated (black) or IL-4 + IL-7 stimulation (grey). Summary data show mean ± SD of the percentage of cells that express p-STAT5 A) IL-4 pre-treatment resulted in lower levels of pSTAT-5 in response to IL-7 in bulk thymocytes when compared to control ($p=0.002$ by paired t-test) B) IL-4 pre-treatment resulted in lower levels of pSTAT-5 in response to IL-7 when compared to control in thymic subsets. ($n=4$) Data were analyzed by paired student t-test

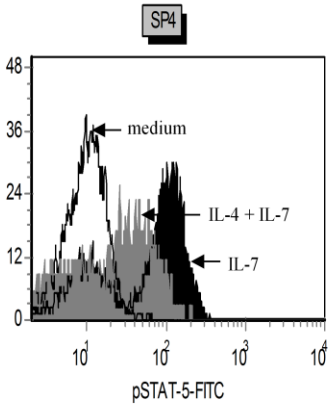
A)

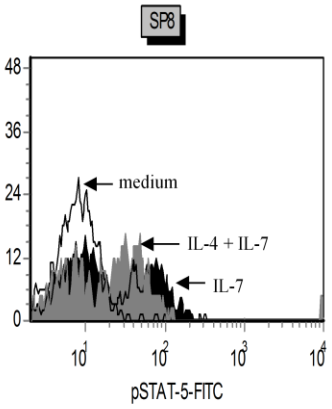

Thymocytes
72
54
36
18
0
unstained
medium
IL-4 + IL-7
IL-7
10^1
10^2
10^3
10^4
pSTAT-5-FITC

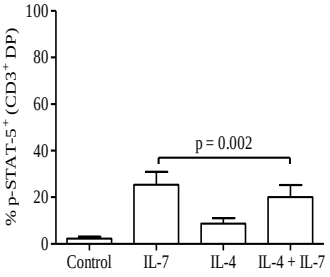

100
80
60
40
20
0
% p-STAT-5+ (thymocytes)
p = 0.002
Control
IL-7
IL-4
IL-4 + IL-7

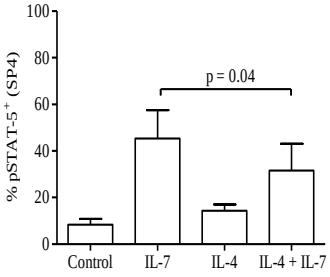
B)

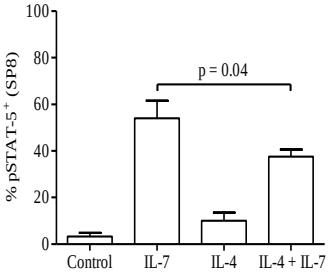

CD3+DP
48
36
24
12
0
medium
IL-4 + IL-7
IL-7
10^1
10^2
10^3
10^4
pSTAT-5-FITC


SP4
48
36
24
12
0
medium
IL-4 + IL-7
IL-7
10^1
10^2
10^3
10^4
pSTAT-5-FITC


SP8
48
36
24
12
0
medium
IL-4 + IL-7
IL-7
10^1
10^2
10^3
10^4
pSTAT-5-FITC


100
80
60
40
20
0
% p-STAT-5+ (CD3+ DP)
p = 0.002
Control
IL-7
IL-4
IL-4 + IL-7


100
80
60
40
20
0
% pSTAT-5+ (SP4)
p = 0.04
Control
IL-7
IL-4
IL-4 + IL-7


100
80
60
40
20
0
% pSTAT-5+ (SP8)
p = 0.04
Control
IL-7
IL-4
IL-4 + IL-7

CD3+DP and SP4 cells 44.7 % ± 4.7 % and 43.3 % ± 16.4 % respectively and to a lesser degree on SP8 cells with 76.8 % ± 16.9 % cells expressing CD127 after 96 hours.

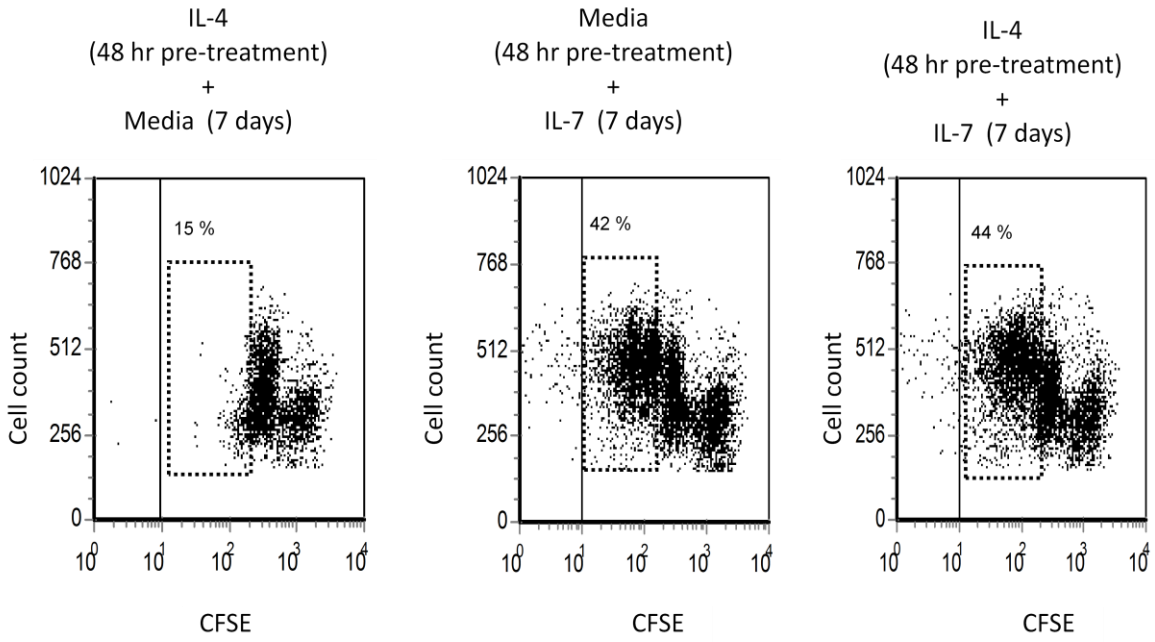
3.5.5 IL-4 inhibits IL-7 induced STAT-5 phosphorylation.

Since IL-4 downregulates CD127 expression on thymocytes, thymocytes were pre-treated with IL-4 to determine its effect on IL-7 activity signalling through IL-7R. Thymocyte/TEC cultures were pre-treated with IL-4 (100 ng/ml) for 48 hours and then stimulated with IL-7 (1000 pg/ml) for 15 minutes. The level of STAT-5 phosphorylation was measured by intracellular flow cytometry (Figure 24 A). IL-4 stimulation does not result in the phosphorylation of STAT-5 (Figure 24 A). Bulk thymocytes pre-treated with IL-4 (100 ng/ml) had lower pSTAT-5 expression compared to cultures treated with IL-7 without IL-4 pre-incubation. To distinguish the effect of IL-4 on thymic subsets, cells were stained with CD3, CD4 and CD8 and pSTAT-5 of individual subsets was evaluated (Figure 24 B). IL-4 pre-treatment resulted in statistically significant decrease in pSTAT-5 following IL-7 stimulation in $CD3^{+}$DP, SP4 and SP8 cells when compared to no IL-4 pre-treatment.

3.5.6 IL-4 does not affect IL-7 induced proliferation in thymocytes

Bulk thymocyte/TEC cultures were pre-treated with IL-4 (100ng/ml) for 48 hours. Thymocytes were then stained with CFSE and stimulated with IL-7 (10 ng/ml) for 7 days (Figure 25). Thymocytes cultured in medium alone did not proliferate. Cells

Figure 25.Interleukin-4 does not affect IL-7 induced proliferation in thymocytes. Bulk thymocytes were pre-treated with IL-4 (100 ng/ml) for 48 hours. IL-7 induced proliferation was measured on unfractionated thymocytes by CFSE dilution following 7 days of culture. Data are presented as a representative histogram of CFSE staining as well as a summary of mean ± SD of the % dividing cells that have undergone more than one division. IL-4 alone did not induce thymocyte proliferation. IL-4 pre-treatment had no affect on the ability of IL-7 to induce thymocyte proliferation . n=5 , (* paired student t-test compared to unstimulated control.)


IL-4
(48 hr pre-treatment)
+
Media (7 days)
Media
(48 hr pre-treatment)
+
IL-7 (7 days)
IL-4
(48 hr pre-treatment)
+
IL-7 (7 days)
15 %
42 %
44 %
Cell count
CFSE
1024
768
512
256
0

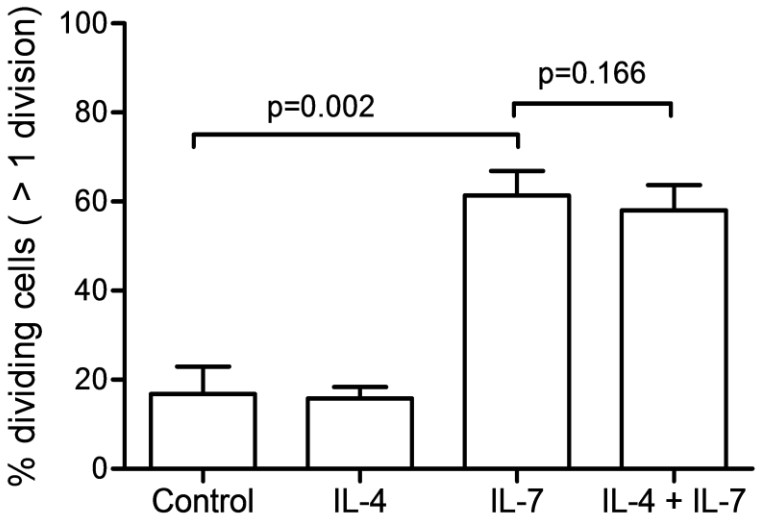
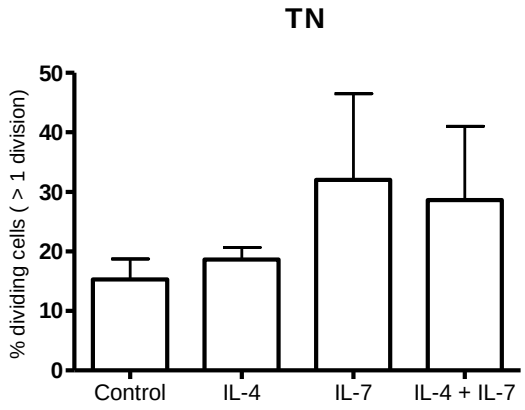
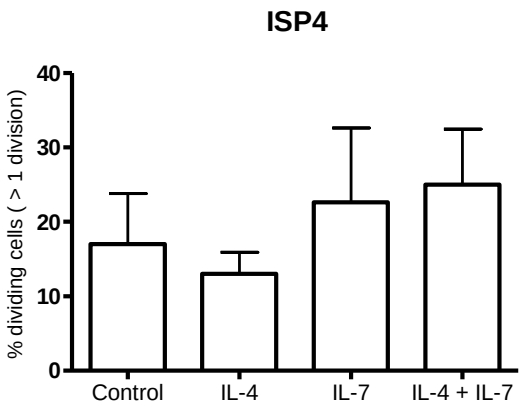
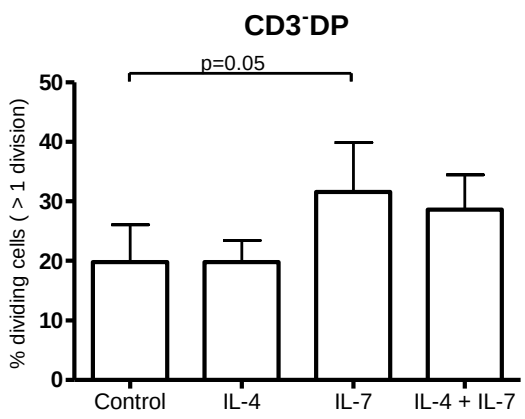
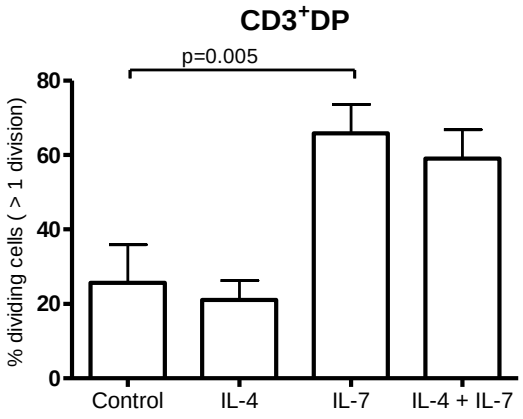

% dividing cells (> 1 division)
p=0.002
p=0.166
Control
IL-4
IL-7
IL-4 + IL-7
100
80
60
40
20
0

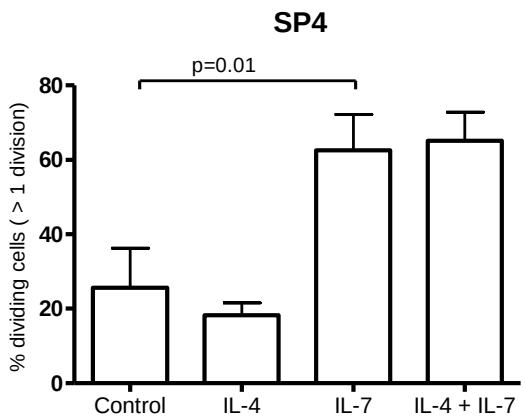
Figure 26.Interleukin-4 does not affect IL-7 induced proliferation in thymocyte subsets. Bulk thymocytes were pre-treated with IL-4 (100 ng/ml) for 48 hours. IL-7 induced proliferation was measured on individual thymic subsets gating on CD3, CD4 and CD8 expression and measuring CFSE dilution following 7 days of culture. Summary data are presented as mean ± SD of the percentage of cells that have undergone more than one division. IL-4 pre-treatment had no effect on the ability of IL-7 to induce thymocyte proliferation in individual thymic subsets. n=5 , (* paired student t-test compared to unstimulated control.).

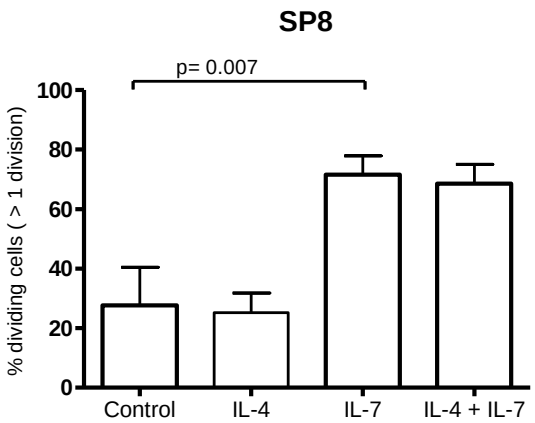

TN
% dividing cells (> 1 division)
50
40
30
20
10
0
Control
IL-4
IL-7
IL-4 + IL-7


ISP4
% dividing cells (> 1 division)
40
30
20
10
0
Control
IL-4
IL-7
IL-4 + IL-7


CD3-DP
p=0.05
% dividing cells (> 1 division)
50
40
30
20
10
0
Control
IL-4
IL-7
IL-4 + IL-7


CD3+DP
p=0.005
% dividing cells (> 1 division)
80
60
40
20
0
Control
IL-4
IL-7
IL-4 + IL-7


SP4
p=0.01
% dividing cells (> 1 division)
80
60
40
20
0
Control
IL-4
IL-7
IL-4 + IL-7


SP8
p= 0.007
% dividing cells (> 1 division)
100
80
60
40
20
0
Control
IL-4
IL-7
IL-4 + IL-7

stimulated with IL-4 alone did not proliferate, consistent with previous reports indicating that IL-4 alone does not induce thymocyte proliferation [204]. IL-7 stimulation of bulk thymocytes induced proliferation with 61.4 % ± 12.2% of cells undergoing more than one division (Figure 26). IL-4 pre-treatment of the cells had no effect on IL-7 driven proliferation with 58.0 % ± 12.7 % of bulk thymocytes undergoing more than one division. To determine if there was a difference within the individual subsets, cells were stained with CD3, CD4 and CD8 prior to flow cytometry analysis. IL-4 had no significant effect on IL-7 driven proliferation in any of the thymic subsets (Figure 26). However, IL-7 signalling through the pSTAT-5 pathway tends to lead to differentiation and increased survival as opposed to increased proliferation[84].

3.6 In vitro HIV-1 infection of thymocytes

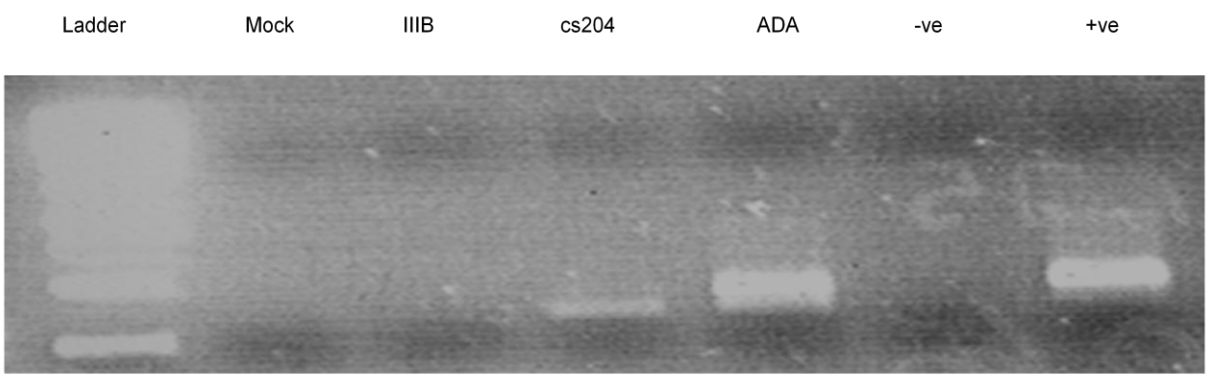
Thymocytes are targets of HIV-1 infection as demonstrated *in vivo*, *in vitro* and in SCID/hu mouse models[144-146,155,171]. To determine if HIV-1-induced thymic dysfunction is attributed in part to disruptions in the IL-7 pathway, thymocytes were infected *in vitro* with HIV-1 and IL-7 responsiveness of the cells was characterized.

3.6.1 Thymocytes are infected in vitro by HIV-1

Thymocytes were infected *in vitro* at an m.o.i of 0.01 and co-cultured with TEC for up to 96 hours. Co-culture conditions provide the required stimulus in order for thymocytes to be infected [171]. There was no consistent induction of p24 antigen in

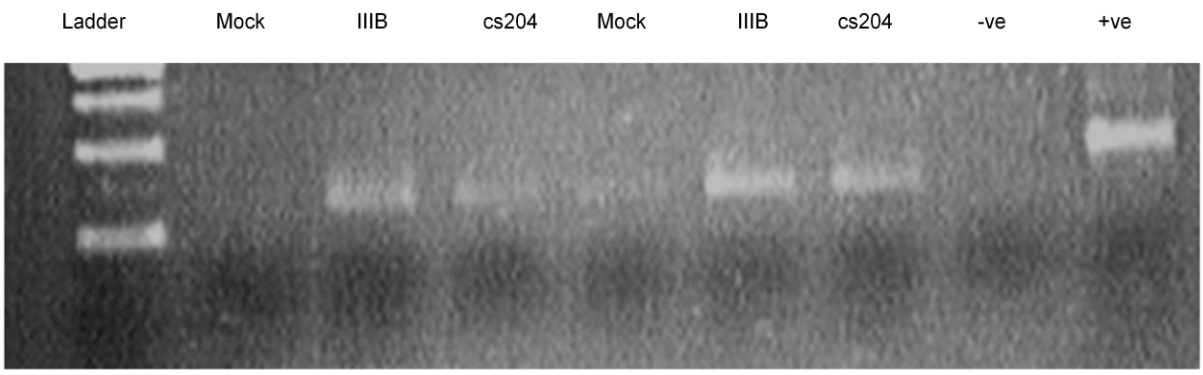
Figure 27. Thymocytes are infected with HIV-1 following 96 hours of co-culture. Thymocytes were incubated with HIV_{IIIB}, HIV_{cs204}, HIV_{ADA} or mock and co-cultured with thymic epithelial cells for up to 96 hours. DNA was isolated from the infected cells and the presence of HIV-1 was measured by nested PCR. Results are representative of 4 separate experiments.

Experiment #1

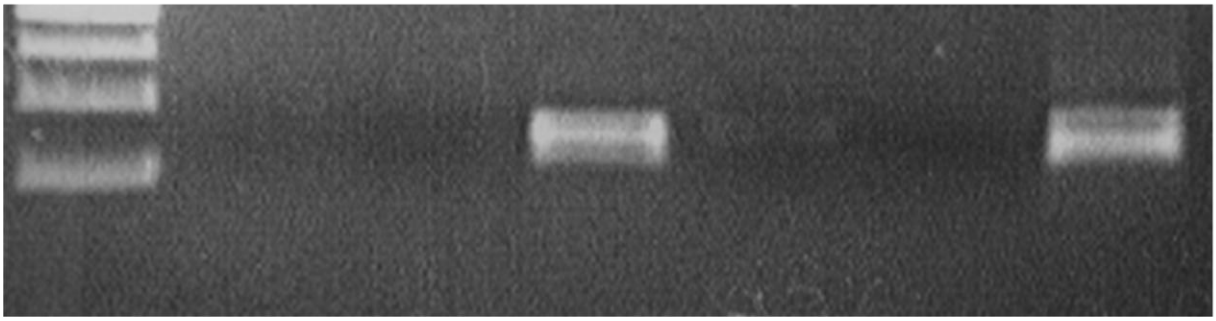


Experiment #2

Experiment #3



Experiment #4



the supernatant of infected cultures as measured by ELISA (data not shown). To demonstrate infection of thymocytes, the presence of HIV-1 DNA in thymocytes was measured by nested PCR. As seen in Figure 27, the presence of HIV-1 DNA could be detected in thymocytes following incubation with the virus but not in mock-infected cultures. Several different strains of HIV-1 were utilised and infection could be detected with all strains, however detection of HIV-1 DNA was not present in all experiments. The p89.6 plasmid, which contains the entire 9.7 kb provirus of HIV-1, was used as a positive control for HIV-1 in the nested PCR reactions.

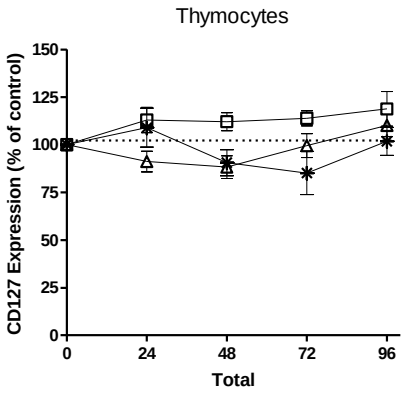
3.6.2CD127 expression on thymocytes following in vitro HIV-1-1 infection

It has been widely reported that CD127 expression is decreased on circulating $CD4^+$ and $CD8^+$ T-cells of HIV-1-infected individuals compared to healthy controls [74-76,159-163,165]. If the same

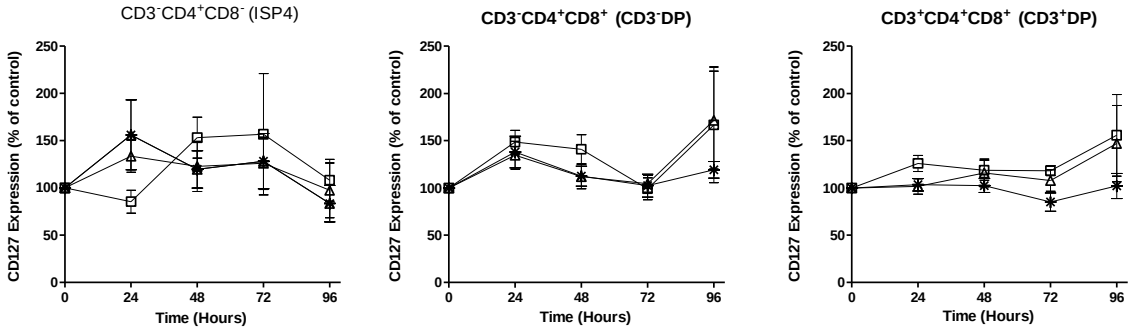
mechanisms occur in thymocytes, this may contribute to HIV-1-induced thymic dysfunction. Therefore, the effect of *in vitro* HIV-1 infection on CD127 expression on thymocytes was investigated. Since HIV viral tropism has been implicated with differential effects on thymocyte infection and susceptibility[149,157,158], the effects of HIV-1 infection of thymocytes with an X4 tropic strain (IIIB), an R5 tropic strain (ADA) and a dual tropic strain (cs204) were compared. There was a statistically significant decrease in CD127 on bulk thymocytes infected with IIIB at an m.o.i of 0.01 following 48-72 hours of culture ($p=0.05$) (Figure 28A). The decrease was approximately 15 % and the change was transient, returning to baseline levels by 72

Figure 28. The effect of HIV-1 on CD127 expression of thymocytes. Thymocytes were incubated with HIV_{IIIB}, HIV_{cs204}, HIV_{ADA} or mock and co-cultured with thymic epithelial cells for up to 96 hours. CD127 expression on thymocyte was measured as percent change with respect to mock infected cultures. (A) A transient decrease in CD127 expression was observed when cultures were infected with IIIB (n=6) p=0.05 by analysis of variance. B-C) Thymic subsets were measured by staining total infected thymocytes with CD3, CD4 and CD8 and gating on specific subsets for CD127 expression. A decrease in CD127 expression was observed on SP8 when infected with IIIB (n=3) p= 0.039 or ADA (n=6) p=0.001 by analysis of variance.

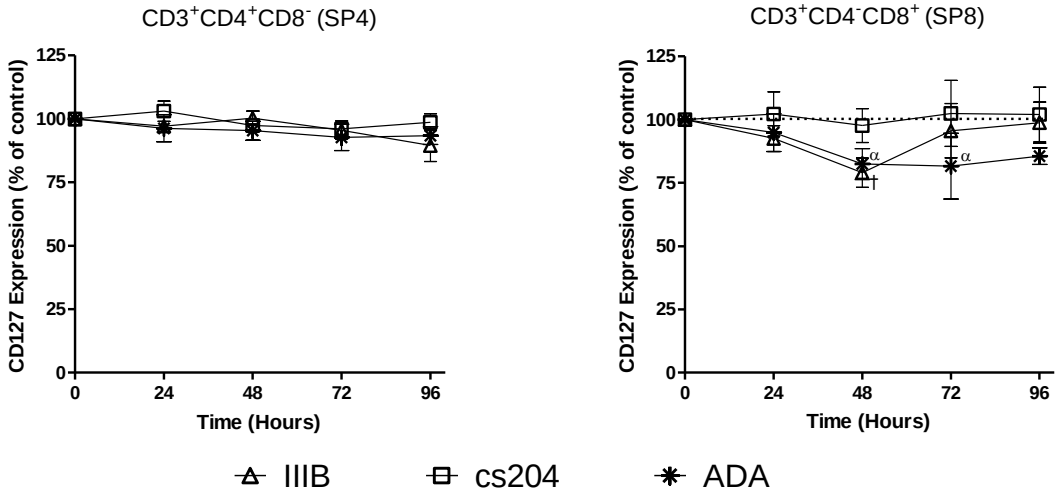
A)


Thymocytes
CD127 Expression (% of control)
150
125
100
75
50
25
0
0
24
48
72
96
Total

B)


CD3-CD4+CD8- (ISP4)
CD3-CD4+CD8+ (CD3-DP)
CD3+CD4+CD8+ (CD3+DP)
CD127 Expression (% of control)
250
200
150
100
50
0
0
24
48
72
96
Time (Hours)

C)


CD3+CD4+CD8- (SP4)
CD3+CD4-CD8+ (SP8)
CD127 Expression (% of control)
125
100
75
50
25
0
0
24
48
72
96
Time (Hours)
α
IIIB
cs204
ADA

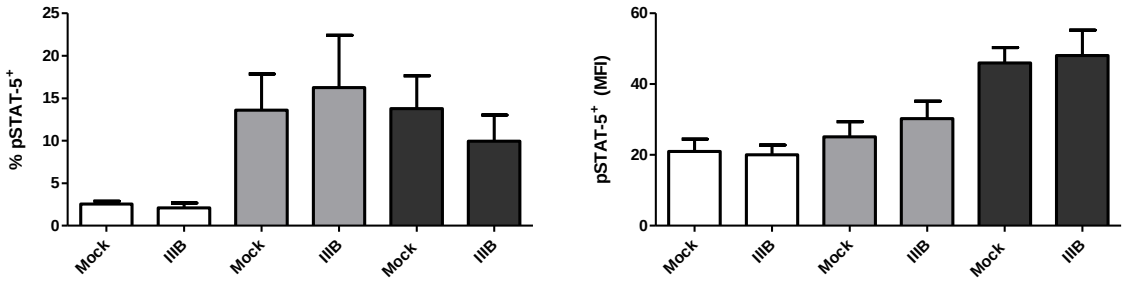
hours. Although there appears to be a slight decrease in CD127 expression when cells were infected with ADA or the dual tropic cs204, this decrease did not reach statistical significance (p=0.2). The variation seen within the bulk thymocyte population could also be influenced by the effects on individual thymic subsets; therefore, bulk thymocytes were infected with HIV and CD127 expression was measured on thymic subsets by flow cytometry. The effect of *in vitro* HIV-1 infection on CD127 expression on the most immature thymocytes varied greatly and no statistically significant change in CD127 expression was detected in ISP4 and $CD3^{+}$DP cells (Figure 28B). The $CD3^{-}$DP subset, which has the lowest level of CD127 expression, had a statistically significant increase in CD127 expression following *in vitro* IIIB infection compared to mock-infected controls (p= 0.036) (Figure 28B). SP4 thymocytes had no change in CD127 expression in the presence of any virus strain (Figure 28C). Thymocyte infection with IIIB and ADA resulted in a decrease in CD127 expression on SP8 cells (p= 0.039 and p=0.001 respectively) while the dual tropic strain cs204 had no effect on CD127 expression on SP8 cells.

3.6.3 The effect of in vitro HIV-1 infection on IL-7 induced STAT-5 phosphorylation following 96 hours of culture.

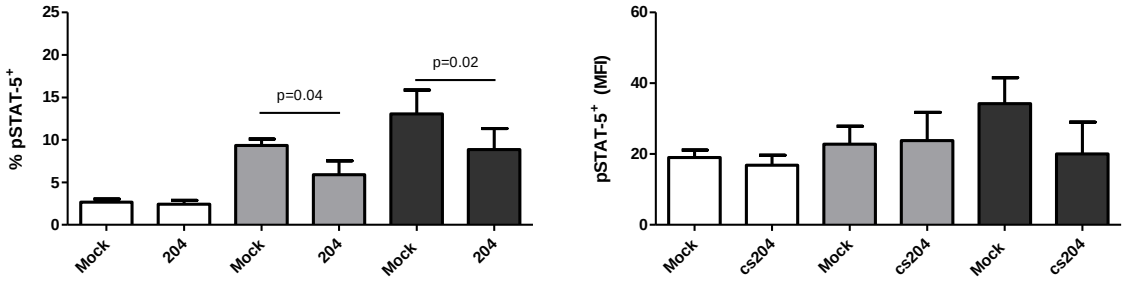
Although *in vitro* HIV-1 infection only resulted in a modest decrease in CD127 expression in thymocytes, there is still rationale to hypothesize that *in vitro* HIV-1 infection can alter IL-7 signalling. In fact, HIV-1 infection has been shown to disrupt IL-7 signalling independently of CD127 expression on $CD8^{+}$ T-cells (Vranjkovic et al , unpublished). Therefore, IL-7 responsiveness of thymocytes, as measured by

Figure 29. The effect of *in vitro* HIV infection on IL-7 induced STAT-5 phosphorylation in thymocytes. IL-7 induced pSTAT-5 phosphorylation was measured on HIV infected thymocytes following 96 hours of culture. A) Data are presented as a graphical summary of mean ± SD, Left panel: % pSTAT-5 expression; Right panel: MFI. Thymocytes were infected with A) HIV_{IIIB} B) HIV_{cs204} C) HIV_{ADA}, n=5. (Data were analyzed by paired student t-test between mock and infected cells)

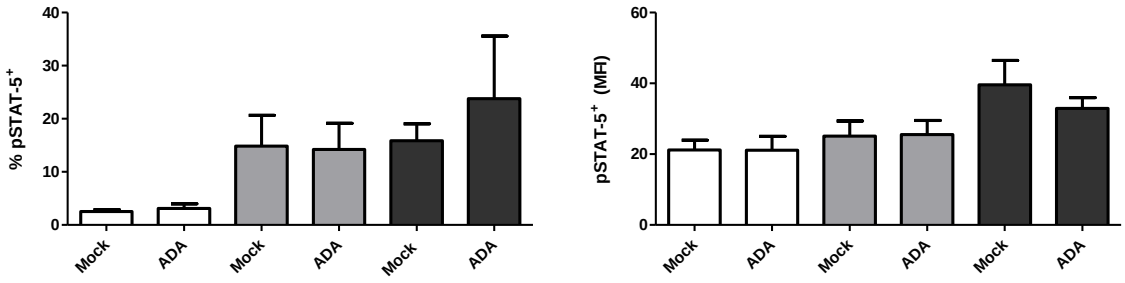
A)HIV_{IIIB}



B) HIV_{cs204}



C) HIV_{ada}



Control IL-7 1ng/ml IL-7 10 ng/ml

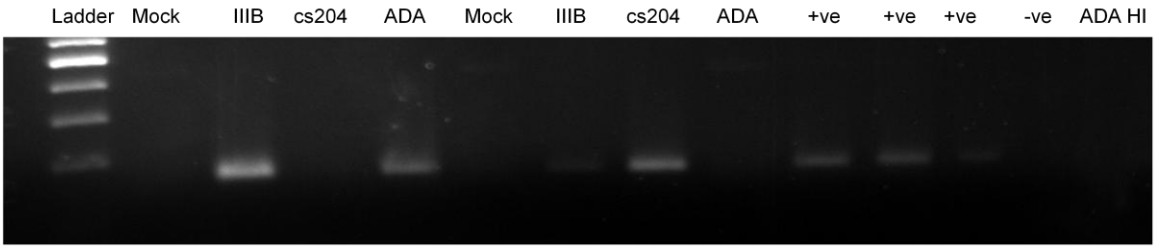
STAT-5 phosphorylation, following *in vitro* HIV-1 infection was evaluated. IL-7 stimulation resulted in the phosphorylation of STAT-5 in bulk thymocyte compared to unstimulated thymocytes as previously described (Figure 29). Incubation of thymocytes with either HIV_{IIIB} or HIV_{ADA} for 96 hours had no effect on the ability of IL-7 to induce pSTAT-5 in bulk thymocytes (Figure 29 A-B). Incubation of thymocytes with HIV_{cs204}, followed by 96 hours of culture, resulted in a lower level of pSTAT-5 expression following IL-7 stimulation compared to mock controls (Figure 29C). The decrease in p-STAT5 was seen when thymocytes were stimulated with either concentration of IL-7 (1 ng/ml or 10 ng/ml). This is indicative of impaired IL-7 responsiveness in thymocytes cultured with $HIV_{cs204.}$

3.6.4 HIV-1 can be detected in thymocytes as early as 24 hours post infection

HIV-1 infection has been measured *in vitro* as early as 3 hours post infection [180]. To determine if *in vitro* HIV-1 infection affects thymocyte function at an earlier time point, the presence of HIV-1 was first measured 24 hours post-infection. HIV-1 DNA could be detected in bulk thymocytes as early as 24 hours post-infection (Figure 30). Infection was detected for all the strains tested, however not in every experiment. As an extra negative control for the specificity of the nested PCR, a heat inactivated (HI) HIV ADA strain was used to infect bulk thymocytes. As expected there were no HIV transcripts detected in these cultures following infection (Figure31), indicating that active viable virus is required in order to detect HIV-1 transcripts in thymocytes.

Figure 30. Thymocytes are infected with HIV-1 following 24 hours of co-culture. Thymocytes were incubated with HIV_{IIIB}, HIV_{cs204}, HIV_{ADA} or mock and co-cultured with thymic epithelial cells for 24 hours. DNA was isolated from the infected cells and the presence of HIV-1 was measured by nested PCR. Results are representative of 3 separate experiments. Water was used as a negative control and DNA isolated from HIV infected PBMC's was used as the positive control.

Experiment 1 Experiment 2



Experiment 3 Experiment 4 Experiment 5

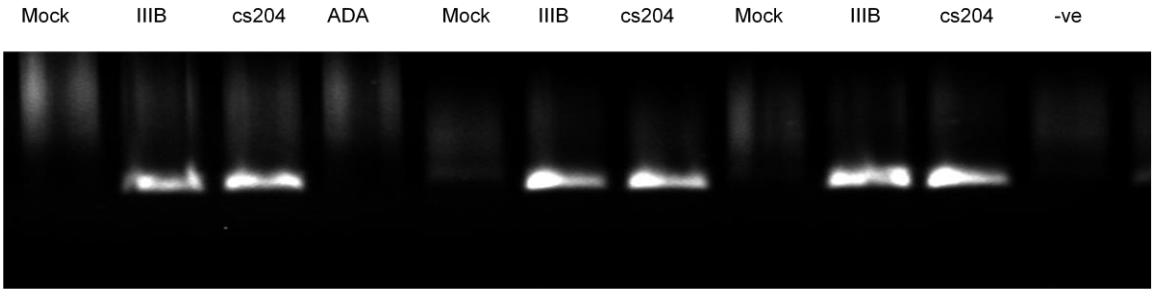
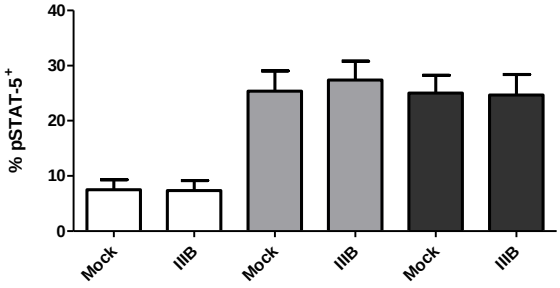
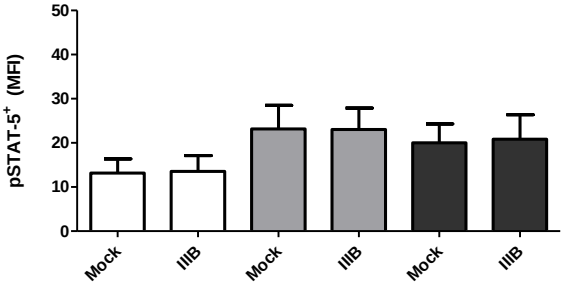


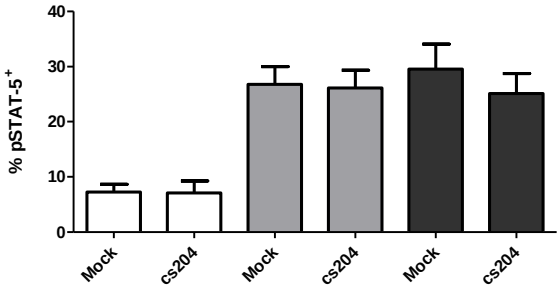
Figure 31. The effect of *in vitro* HIV-1 infection on IL-7 induced pSTAT-5 24 hours post-infection of bulk thymocytes. IL-7 induced pSTAT-5 phosphorylation was measured in HIV infected thymocytes following 24 hours of culture. A) Data are presented as a graphical summary of mean ± SD, Left: panel: % pSTAT-5 expression; Right panel: MFI. Thymocytes were infected with A)HIV_{IIIB} B) HIV_{cs204} C) HIV_{ADA}, n=5. (Data were analyzed by paired student t-test between mock and infected cells)

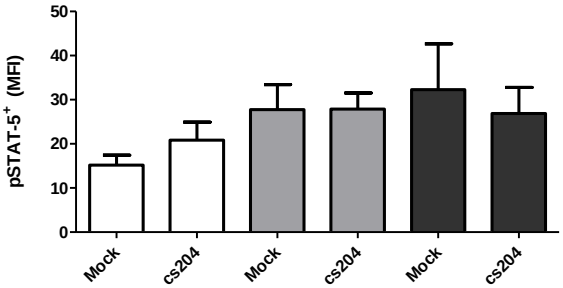
A) HIV_{IIIB} (Bulk thymocytes)



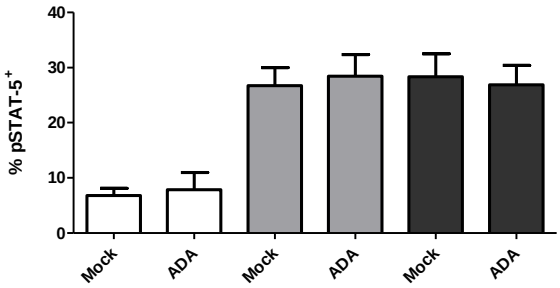


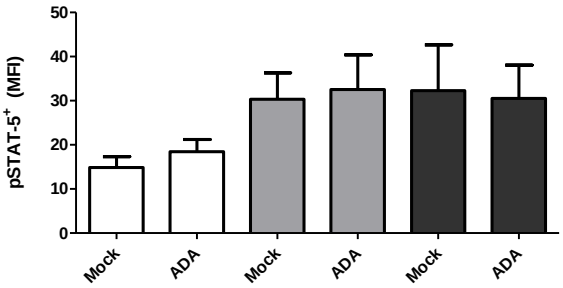
B) HIV_{cs204} (Bulk thymocytes)





C) HIV_{ada} (Bulk thymocytes)

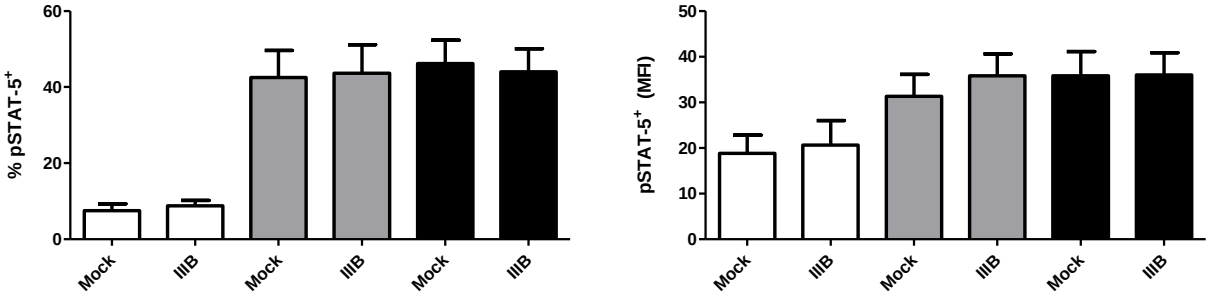




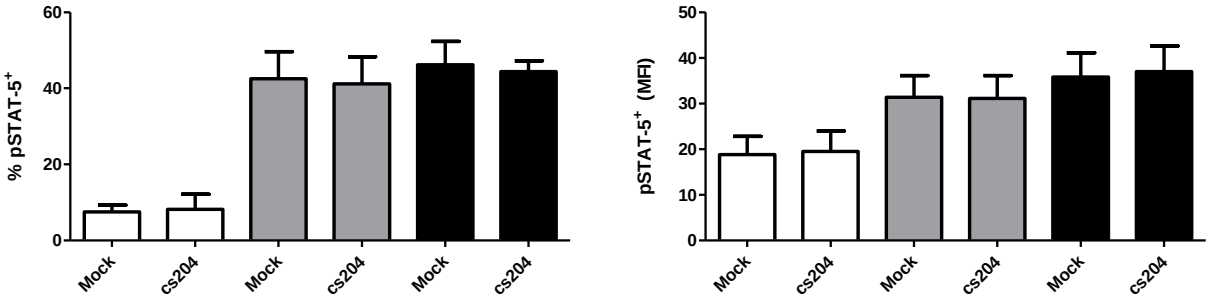
Control IL-7 1ng/ml IL-7 10 ng/ml

Figure 32. The effect of *in vitro* HIV-1 infection on IL-7 induced pSTAT-5 24 hours post-infection in SP4 thymocytes. IL-7 induced pSTAT-5 phosphorylation was measured on HIV infected thymocytes following 24 hours of culture. Unfractionated thymocytes were gated based on CD3, CD4 and CD8 expression to measure the specific effect on SP4 cells. A) Data are presented as a graphical summary of mean ± SD, Left panle: % pSTAT-5 expression; Right panel:MFI. Thymocytes were infected with A)HIV_{IIIB} B) HIV_{cs204} C) HIV_{ADA} n=5. (Data were analyzed by paired student t-test between mock and infected cells)

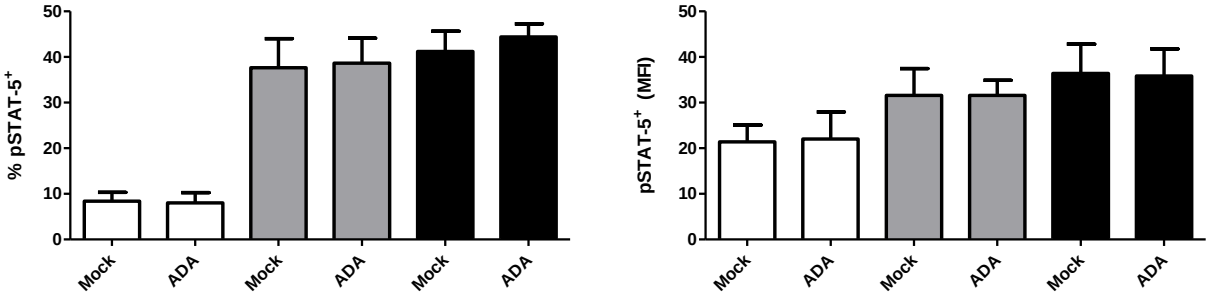
A) HIV_{IIIB} (SP4)



B) HIV_{cs204} (SP4)



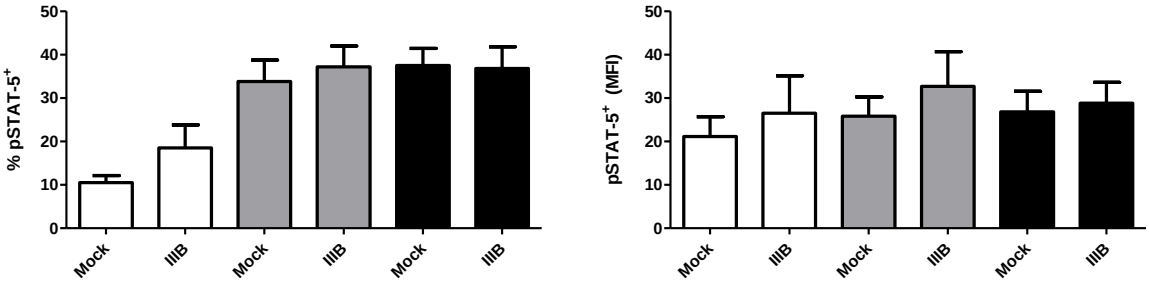
C) HIV_{ada} (SP4)



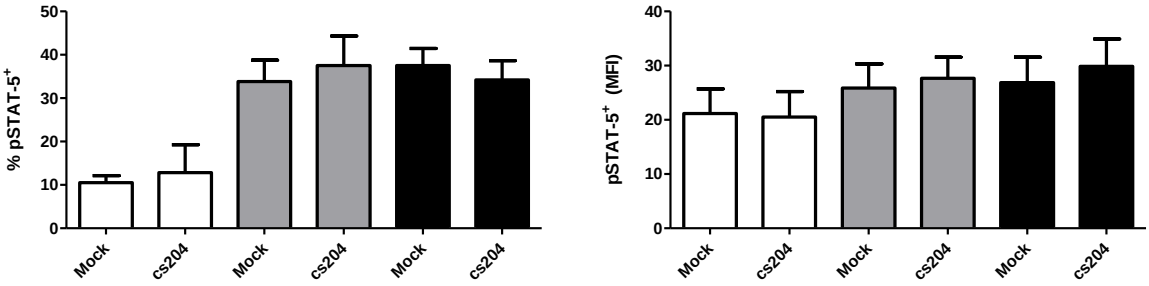
Control IL-7 1ng/ml IL-7 10 ng/ml

Figure 33. The effect of *in vitro* HIV-1 infection on IL-7 induced pSTAT-5 24 hours post-infection in SP8 thymocytes. IL-7 induced pSTAT-5 phosphorylation was measured on HIV infected thymocytes following 24 hours of culture. Unfractionated thymocytes were gated based on CD3, CD4 and CD8 expression to measure the specific effect on SP8 cells. Data are presented as a graphical summary of mean ± SD, Left panle: % pSTAT-5 expression; Right panel: MFI. Thymocytes were infected with A)HIV_{IIIB} B) HIV_{cs204} C) HIV_{ADA}, n=5. (Data were analyzed by paired student t-test between mock and infected cells)

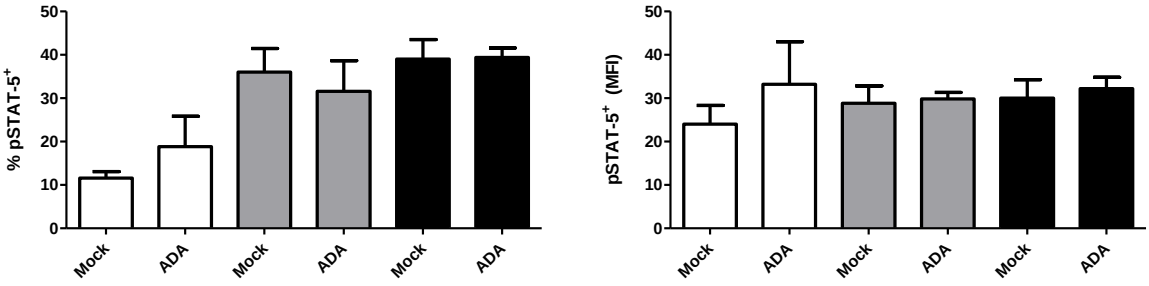
A) HIV_{IIIB} (SP8)



B) HIV_{cs204} (SP8)



C) HIV_{ada} (SP8)



Control IL-7 1ng/ml IL-7 10 ng/ml

3.6.5 IL-7 induced STAT-5 phosphorylation is not affected by HIV infection following 24 hours of infection.

The effect of HIV-1 infection on the IL-7 responsiveness of thymocytes at 24 hours post-infection was evaluated. As seen in Figure 31, HIV-1 infection with any of the strains used had no effect on the level of pSTAT-5 following IL-7 stimulation (1ng/ml or 10 ng/ml). There was also no significant effect of HIV-1 infection on the level of pSTAT-5 following IL-7 stimulation in SP4 (Figure 32) or SP8 cells (Figure 33). This indicates that the mechanisms involved in the affect of HIV infection on thymocyte function require more than just HIV binding, but rather may necessitate the production of a co-factor either host or viral..

3.6.6 The effect of in vitro HIV-1 infection on IL-7 induced Bcl-2 expression following 24 hours of culture.

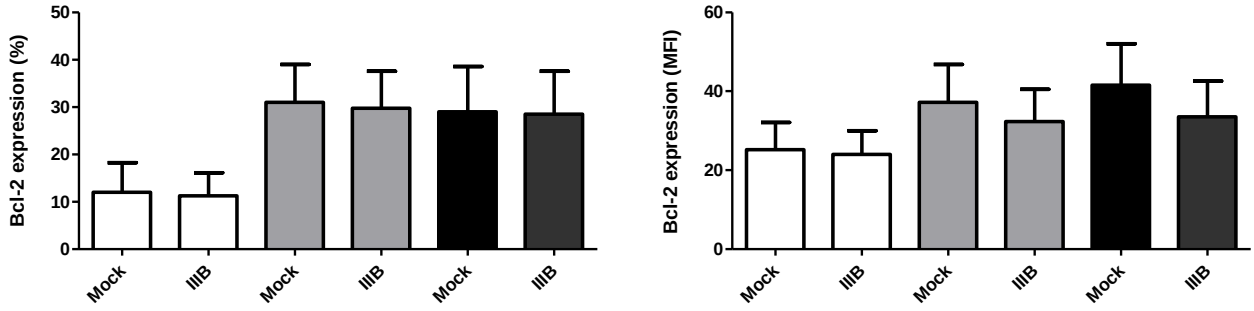
The survival function of IL-7 is mediated through the induction of Bcl-2. As a measure of IL-7 responsiveness of thymocytes, the level of Bcl-2 induction 24 hours post-infection was evaluated. HIV-1 infection of thymocytes with HIV_{IIIB} or HIV_{ADA} (Figure 34 A, C) had no significant effect on the ability of IL-7 to induce Bcl-2 expression in bulk thymocytes. However, HIV_{cs204} infection of thymocytes resulted in lower level of Bcl-2 induction following IL-7 stimulation compared to mock control. The capacity of IL-7 to induce Bcl-2 expression in SP8 cells at a lower concentration (1 ng/ml) was also inhibited following HIV_{cs204} infection p=0.05 (Figure 36). There were trends towards a lower level of Bcl-2 expression following IL-7 stimulation of SP4 p=0.06 (Figure 35) and $CD3^{+}$DP (Figure 37) p=0.07.

3.6.7 The effect of in vitro HIV-1 infection on IL-7 induced PI3K pathway 24 hours post infection.

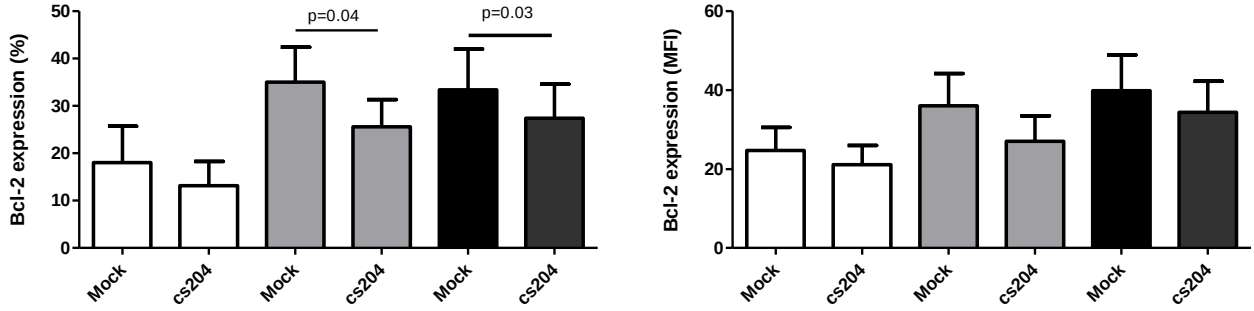
As another measure of IL-7 responsiveness of thymocytes, the phosphorylation of members of the AKT pathway were evaluated. Bulk thymocytes were infected with HIV-1 and 24 hours post-infection thymocytes were stimulated with IL-7 (10ng/ml) and the downstream signalling molecules were analysed by Western blot. HIV infection does not affect the level of AKT, FOXO3a or FOXO1 phosphorylation (Figure 38). However, despite attempts to reduce endogenous signals, the basal expression of these proteins were considerable, making it difficult to assess an IL-7 specific response.

Figure 34. The effect of *in vitro* HIV infection on Bcl-2 expression following IL-7 stimulation 24 hours post infection in bulk thymocytes. BCl-2 expression in thymocytes following IL-7 stimulation was measured following 72 hours of culture. Data are presented as a graphical summary of mean ± SD, Left: panel: % Bcl-2 expression; Right: panel: MFI. Bulk thymocytes were infected with A) HIV_{IIIB} B) HIV_{cs204} or C) HIV_{ADA}, n=5. (Data were analyzed by paired student t-test between mock and infected cells)

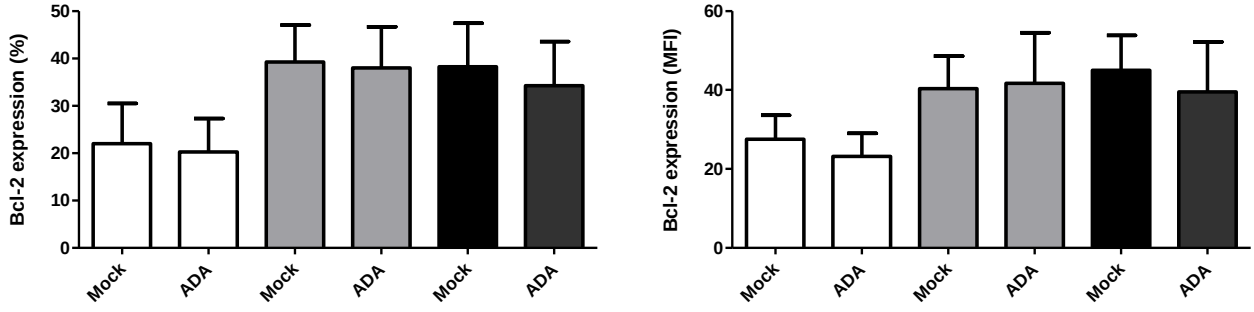
A) HIV_{IIIB} (Bulk thymocytes)



B) HIV_{cs204} (Bulk thymocytes)



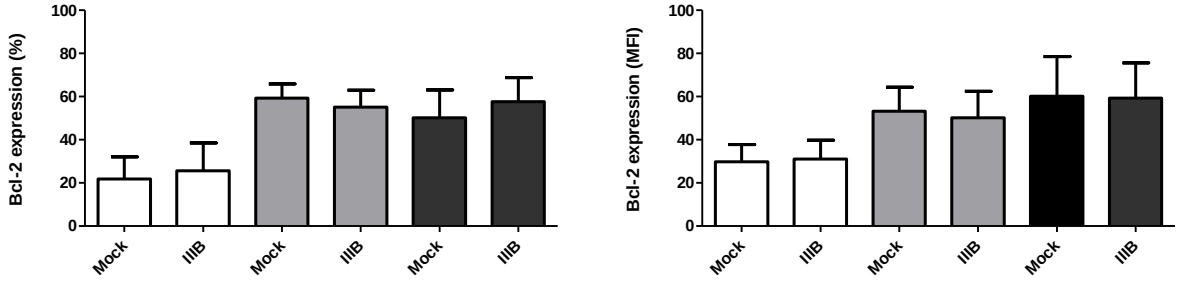
C) HIV_{ada} (Bulk thymocytes)



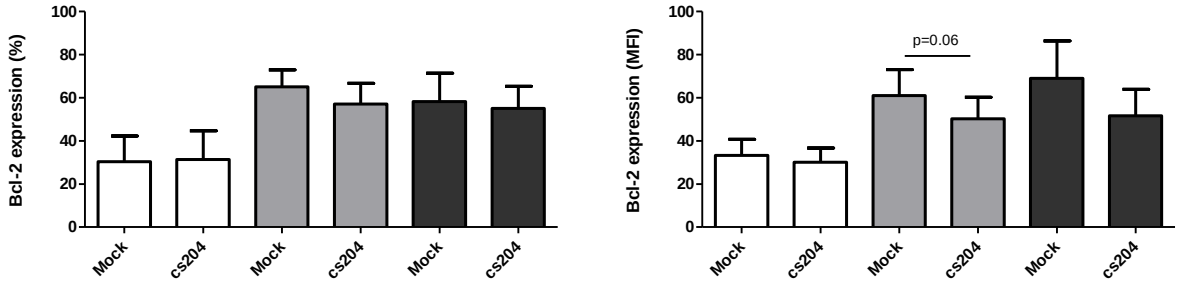
Control IL-7 1 ng/ml IL-7 10 ng/ml

Figure 35. The effect of *in vitro* HIV-1 infection on Bcl-2 expression following IL-7 stimulation in SP4. The level of Bcl-2 expression was measured in HIV infected thymocytes stimulated with IL-7. Unfractionated thymocytes were gated based on CD3, CD4 and CD8 expression to measure the specific effect on SP4 cells. Data are presented as a graphical summary of mean ± SD, Left panel: % Bcl-2 expression; Right panel: MFI Thymocytes were infected with A)HIV_{IIIB} B) HIV_{cs204} or C) HIV_{ADA}, n=5. (Data were analyzed by paired student t-test between mock and infected cells)

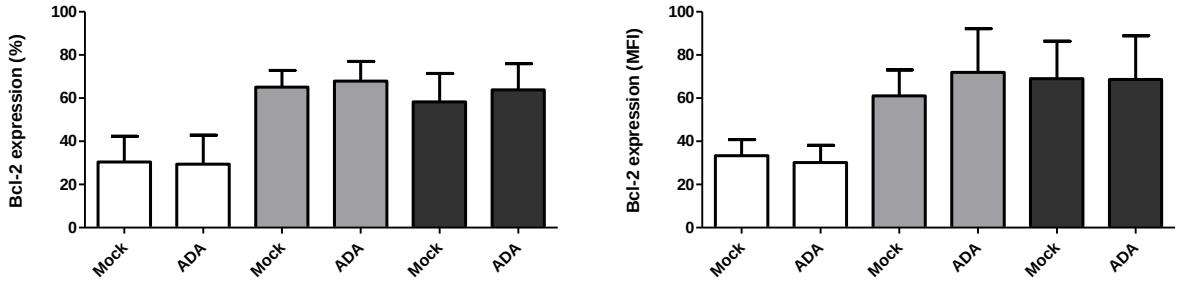
A) HIV_{IIIB} (SP4)



B) HIV_{cs204} (SP4)



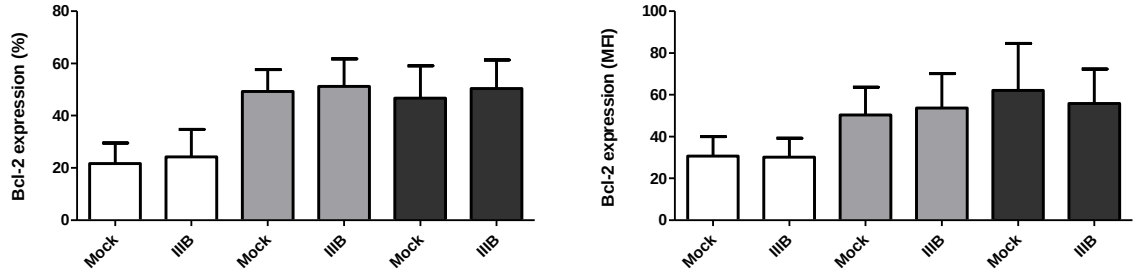
C) HIV_{ada} (SP4)



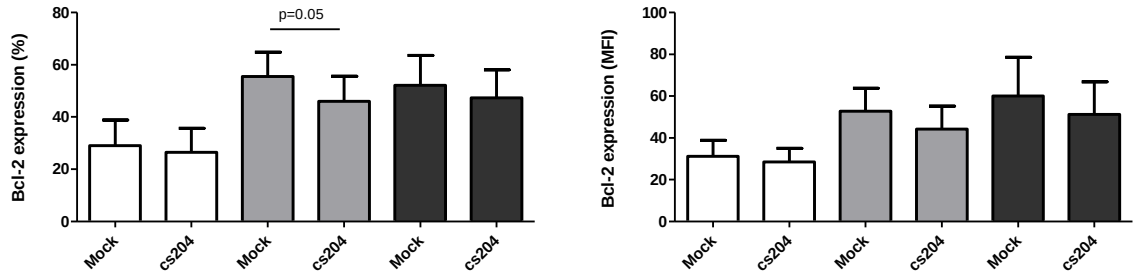
Control IL-7 1 ng/ml IL-7 10 ng/ml

Figure 36. The effect of *in vitro* HIV-1 infection on Bcl-2 expression following IL-7 stimulation in SP8. The level of Bcl-2 expression was measured in HIV infected thymocytes stimulated with IL-7. Unfractionated thymocytes were gated based on CD3, CD4 and CD8 expression to measure the specific effect on SP8 cells. Data are presented as a graphical summary of mean ± SD, Left panel: % Bcl-2 expression; Right panel: MFI. Thymocytes were infected with A) HIV_{IIIB} B) HIV_{cs204} or C) HIV_{ADA}, n=5. (Data were analyzed by paired student t-test between mock and infected cells)

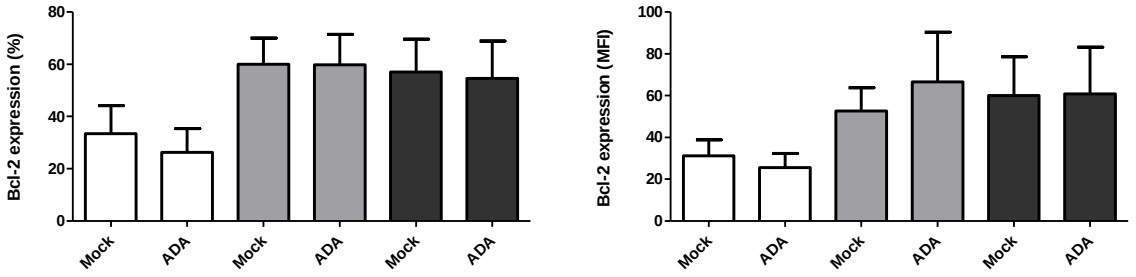
A) HIV_{IIIB} (SP8)



B) HIV_{cs204} (SP8)



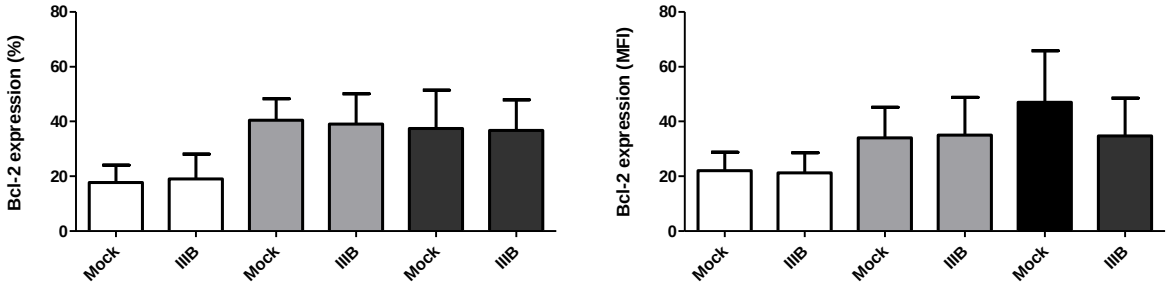
C) HIV_{ada} (SP8)



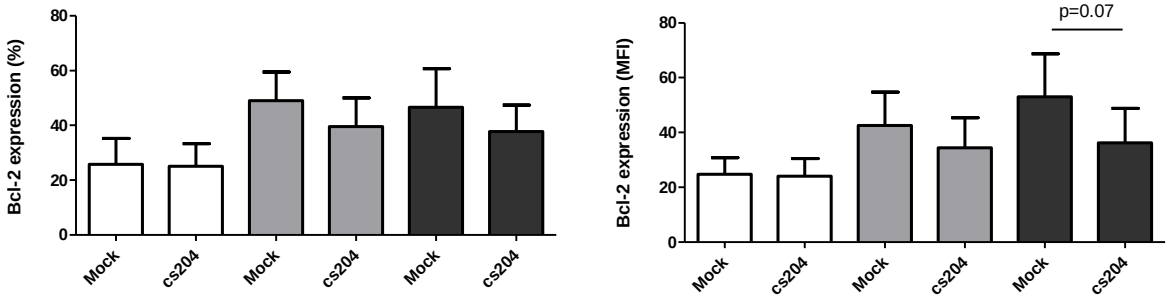
Control IL-7 1 ng/ml IL-7 10 ng/ml

Figure 37. The effect of *in vitro* HIV-1 infection on Bcl-2 expression following IL-7 stimulation in $CD3^+$DP thymocytes. The level of Bcl-2 expression was measured in HIV infected thymocytes stimulated with IL-7. Unfractionated thymocytes were gated based on CD3, CD4 and CD8 expression to measure the specific effect on $CD3^+$DP cells. Data are presented as a graphical summary of mean ± SD, Left panel: % Bcl-2 expression; Right panel: MFI Thymocytes were infected with A) HIV_{IIIB} B) HIV_{cs204} or C) HIV_{ADA}, n=5. (Data were analyzed by paired student t-test between mock and infected cells)

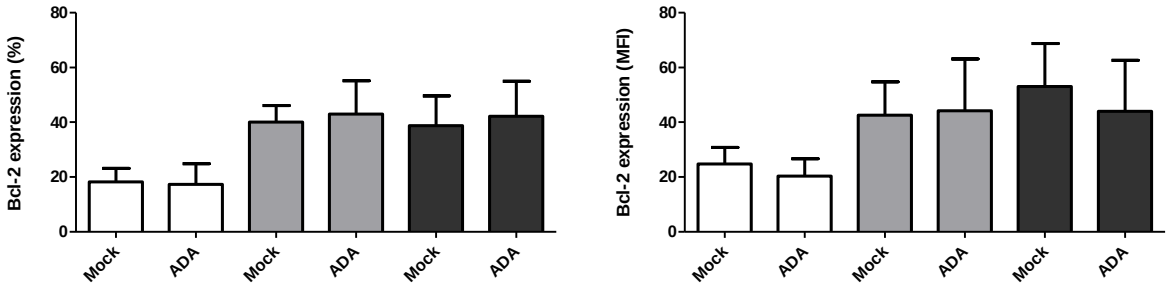
A) HIV_{IIIB} ($CD3^{+}DP$)



B) HIV_{cs204} ($CD3^{+}DP$)

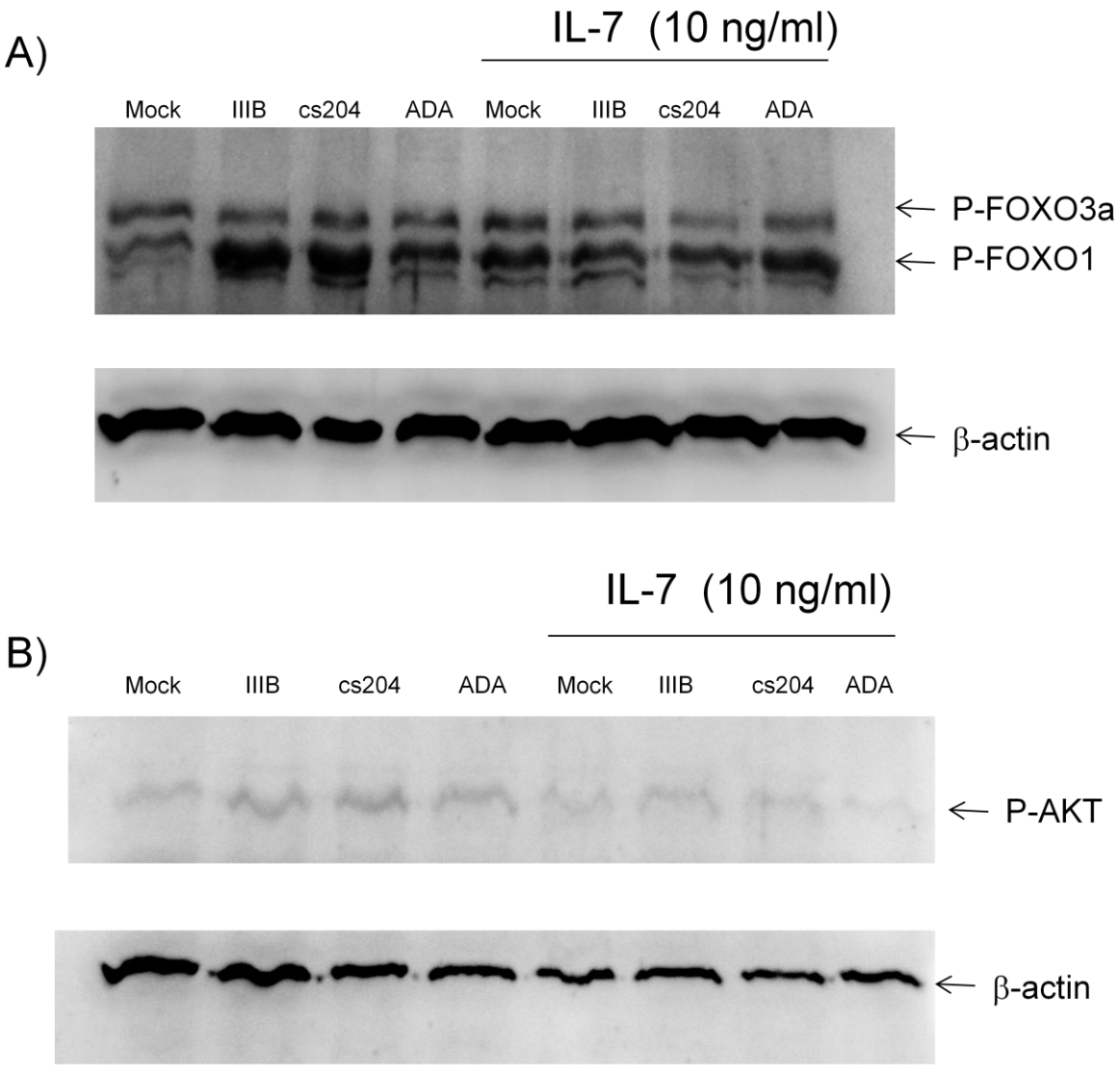


C) HIV_{ada}($CD3^{+}DP$)



Control IL-7 1 ng/ml IL-7 10 ng/ml

Figure 38. The effect of HIV infection on the ability of IL-7 to induce the PI3K pathway in thymocytes. Bulk thymocytes were incubated with HIV_{IIIB}, HIV_{cs204}, HIV_{ADA} or mock and co-cultured with TEC for only 24 hours. After 24 hours of culture, thymocytes are stimulated with IL-7 for 30 minutes. A) FOXO1 and FOXO3a phosphorylation B) AKT phosphorylation. Representative experiment of 3


A)
IL-7 (10 ng/ml)
Mock
IIIB
cs204
ADA
Mock
IIIB
cs204
ADA
P-FOXO3a
P-FOXO1
β-actin
B)
IL-7 (10 ng/ml)
Mock
IIIB
cs204
ADA
Mock
IIIB
cs204
ADA
P-AKT
β-actin

4. Discussion

Although the thymus involutes with age it still remains functional late into adulthood[140]. The potential to increase thymic output to improve immune reconstitution after T-cell depletion is the focus of research in chemotherapy [205], bone marrow transplantation [206-208] and HIV disease [209-211]. This thesis identifies an optimized protocol for thymocyte cellular suspension culture allowing for the study of phenotype and potential functions of individual thymocyte subsets. Establishing such a system will facilitate many aspects of research in the areas of understanding normal human thymopoiesis as well as alterations in thymic function in disease settings such as HIV infection or following radiation or chemotherapy.

4.1 Determining the optimal in vitro system for the study of human thymocytes.

4.1.1 Thymocyte phenotype

The distribution of thymocytes within the human thymus has been previously studied[183] and the results in this thesis are consistent with these reports. The majority of thymocytes isolated from thymic tissue are DP cells, which are at a stage in the maturation process where immature thymocytes undergo massive expansion to optimize the number of cells that are undergoing negative selection[31]. CD127 was expressed on all the thymic subsets to a degree that is consistent with previously published data [183,184]. The expression profile of CD127 on thymocytes corresponds to the functional requirements of each subset for IL-7 signalling. Initially progenitor cells require IL-7 for survival and expansion which is reflected in

the expression of CD127 on TN and ISP4 subsets. The DP subsets, that are undergoing massive apoptosis due to negative selection, down modulate CD127 on their cell surface possibly to attenuate the survival signals provided by IL-7. Human DP thymocytes express low levels of CD127 and in mice, CD127 expression on the DP subsets is completely lost presumably to avoid IL-7 survival signals and the advent of self-reactive immune cells. To avoid self-reactive immune cells, human DP thymocytes upregulate the molecule suppressor of cytokine signal 1 (SOCS-1) which may contribute to the selection process by dampening the survival signals received by DP thymocytes[212]. SP4 and SP8 cells have the highest level of CD127 expression of all the thymic subsets, which is consistent with the functional requirement for IL-7 by mature circulating $CD4^+$ and $CD8^+$ T-cells

4.1.2 Isolation and storage of thymocytes

T-cell development requires a specialized microenvironment that is mainly provided by the thymic stroma [13]. A number of culture systems have been developed to study T-cell development *in vitro*, however they primarily involve murine models. These models are not readily transferable to certain research fields in humans, such as HIV research since mice are not readily infected by HIV. *In vitro* studies of human thymocytes require a co-culture system with TEC in order to maintain activation and functional signals [40,213-215]. The process of isolating TEC necessitates an outgrowth period of several days, creating a need to store isolated thymocytes. The work within this thesis establishes the optimal storage and culture conditions needed for *in vitro* suspension thymocyte cultures.

These results indicate that storage of whole human thymic tissue for future extraction of thymocytes alteres the distribution of the cell populations within the immature T-cell pool. This suggests that in order to study the function of individual thymocyte subsets, thymocytes need to be isolated from fresh whole thymic tissue. Cryopreserving thymocytes at -80°C, in LN_2 or storing them in medium at 37°C prior to TEC co-cultures also results in alterations in the distribution of the thymocyte subsets. Although associated with minor changes, storage of isolated thymocytes at 4°C resulted in greater viability than any other condition and maintained a phenotype more closely resembling that of freshly isolated thymocytes. This indicates that for *in vitro* studies of thymocytes, if storage of thymocytes is required, a temperature of 4°C is the most appropriate and supports published methods for thymocyte/autologous TEC cultures [51,143]. However, storing thymocytes at 4ºC for 7-10 days did result in a slight reduction in CD127 expression, suggesting that freshly isolated thymocytes cultured with allogeneic TEC may be optimal for functional studies of IL-7/IL-7R activity in thymocytes.

4.1.3 Optimal co-culture conditions for thymocyte studies

In the present study, co-culture of freshly isolated thymocytes with allogeneic TEC, or storing thymocytes at 4°C prior to co-culture with autologous *vs* allogeneic TEC, did not differentially affect the viability or subset distribution of cells within the *in vitro* culture system over 96 hours. This suggests that the survival signals released by interaction of thymocytes with TEC are independent of the source of the TEC. It has previously been established that thymocytes are able to bind both allogeneic and

autologous TEC independently of MHC I and MHC II molecules [216]. While "self" may be a requirement for some aspect of thymocyte function or development through the selection processes, it may not be necessary for activation signals released by cell-to-cell contact since the co-culture of thymocytes with either autologous or allogeneic TEC results in a similar induction of NF-κB [143]. This indicates that both allogeneic and autologous TEC supply signals that can activate thymocytes.

In order to avoid any potential detrimental effects of storage, *in vitro* co-culture systems with a TEC cell line would be optimal. There has been limited success in establishing human TEC cell lines. Thymic epithelial cells have been transformed using Simian Vacuolating virus large T antigen (SV40T) [217], however little is published on the use and functionality of these lines. A potential cell line that may be a candidate is the mouse stromal cell line OP9-DL1. The OP9-DL1 culture system has been widely used for the study of commitment of precursor T-cells and subsequent development of cells of T-cell lineage [54]. Although the system is based on a mouse stromal cell line, it has been demonstrated to support the development of human hematopoietic stem cells, and human cord blood in long term cultures [55,56]. In the present study, the capability of OP9-DL1 cells to support the function of human thymocytes in a short term culture was evaluated. These results confirmed the importance of a co-culture system for functional thymocyte studies since thymocytes cultured alone exhibite significantly reduced viability and respond poorly to IL-7. Several factors released upon co-culture of thymocytes with TEC play a role in thymocyte viability, proliferation and activation. Such factors include hormones

such as thymulin [41,218,219] and cytokines, including IL-1, IL-6, and IL-7 (reviewed in [218]). The viability of thymocytes was the greatest with OP9-DL1 co-culture; therefore this cell line appears to be a good candidate for co-culture with a heterogeneous population of thymocytes. Curiously, thymocyte cell numbers over time were lower with OP9-DL1 cells than with TEC co-cultures. The reason for this is not clear as thymocyte apoptosis with OP9-DL1 cultures was lower than that observed in TEC co-culture. Since cell number is a balance of cell proliferation and cell death, this decrease may be due to a decrease in cell proliferation. Optimal T-cell development in long term OP9-DL1 co-cultures requires exogenous cytokines such as IL-7 and stem cell factor (SCF) [220]. The lack of stimulatory signals provided by IL-7 or other cytokines may result in limited thymocyte proliferation and thereby explain the decrease in cell number seen in the short term culture system. Further studies could be conducted in order to evaluate if the proliferative capacity of thymocytes is altered with the presence of OP9-DL1 cells in co-culture.

As mentioned above, co-culture with OP9-DL1 cells protects thymocytes from apoptosis. *In vivo,* the majority of thymocytes are undergoing apoptosis due to the processes of positive and negative selection. Thymocytes studied *ex-vivo* may have already received certain signals and have entered the apoptotic pathway prior to isolation and co-culture. Notch signalling leads to protection from T-cell receptor (TCR)-induced apoptosis as well as glucocorticoid induced apoptosis, hence thymocytes, which express notch, co-cultured with OP9-DL1 may be expected to have reduced apoptosis[221-224].

The distribution of thymic subsets following short term culture of unfractionated thymocytes co-cultured with either TEC or OP9-DL1 was evaluated. Thymocyte subset distribution was slightly different within the OP9-DL1 co-culture when compared to co-culture with TEC. The proportion of $CD3^{+}$DP cells was lower, while the proportion of SP4 cells was higher in thymocytes that were co-cultured with OP9-DL1 cells. Since the maturation and development of T-cells require long term culture [225], the change in phenotype observed with 96 hours of co-culture is probably not due to maturation of cells from $CD3^{+}$DP to SP4 cells. One possible explanation could be a weak proliferation in the $CD3^{+}$DP subset, resulting in an alteration in the proportion of cells within the other subsets of thymocytes co-cultured with OP9-DL1 cells.

The expression of CD127 is highly regulated on thymocytes [67]. The present data demonstrated a lower level of CD127 expression on SP4 cells co-cultured with OP9-DL1 compared to thymocytes co-cultured with TEC. Multiple cytokines have regulatory effects on the expression of CD127. IL-2, IL-4 and IL-7 stimulation results in decreased CD127 expression on the surface of both thymocytes and mature $CD4^{+}$ and $CD8^{+}$ T-cells[49,69-71,192,226]. Therefore, the lower levels of CD127 on SP4 cells may be due to cytokines produced in the supernatants of OP9-DL1/thymocyte cultures. In fact, mean CD127 expression was lower in all subsets within the OP9-DL1 co-culture system, but this difference only reached statistical significance within the SP4 subset. Further studies could be conducted in order to evaluate the activation state of the cells as a potential mechanism of altered CD127 expression.

4.1.4. IL-7 activity in thymocytes in suspension TEC cultures

IL-7 is a pleiotropic cytokine that plays several roles in thymic development such as increasing survival, proliferation and metabolic processes such as glucose uptake[63]. IL-7 stimulation induced Bcl-2 expression of bulk thymocytes, as well as within the majority of the thymic subsets and IL-7 alone was able to induce the proliferation of thymocytes. Unlike $CD8^+$ T-cells, thymocytes do not require a secondary stimulus such as PHA in order for IL-7 to induce proliferation [72]. The proliferative effect of IL-7 on thymocytes is specific, since IL-4 alone did not induce proliferation but required additional stimulation (e.g. PHA) to exert this activity[204].

The majority of the subsets responded to IL-7 stimulation. However $CD3^-$DP cells did not proliferate or phosphorylate STAT-5 in response to IL-7. The lack of IL-7 activity within this subset could be due to the low levels of CD127 receptor expression (Figure 2). However $CD3^+$DP cells express similar levels of CD127 and these cells were able to respond to IL-7 stimulation. The lack of IL-7 responsiveness of the $CD3^-$DP subset may be due to the increase in SOCS-1 expression within this subset which limits the responsiveness of the cells[212]. SOCS-1 suppressing Jak signals can modulate cytokine responsiveness [227], a function that is important in limiting self-reacting T-cells in positive and negative selection. Following TCR engagement, SOCS-1 signalling is decreased allowing thymocytes to respond to IL-7 [228].

4.1.5 IL-7 responsiveness of thymocytes is affected by co-culture conditions

This report demonstrates the importance of using a co-culture system in studies of thymocyte function since thymocytes that are cultured without the benefit of co-culture are unresponsive to IL-7 stimulation as measured by STAT-5 phosphorylation and Bcl-2 induction. Interactions between TEC or OP9-DL1 cells and thymocytes were sufficient for thymocytes to remain responsive to IL-7. Thymocytes co-cultured with OP9-DL1 cells had higher levels of basal STAT-5 phosphorylation than thymocytes co-cultured with TEC. This may be due to soluble factors that are released following thymocyte/OP9-DL1 cell interactions which lead to cell activation and hence STAT-5 phosphorylation. The co-culture system utilised had no significant effect on the level of STAT-5 phosphorylation following IL-7 stimulation, indicating that both culture system are able to maintain thymocyte IL-7 responsiveness.

Notch signalling cooperates with IL-7 to increase proliferation of immature thymocytes and Bcl-2 induction [194,229]. Co-culture of thymocytes with OP9-DL1 cells resulted in higher level of Bcl-2 expression when compared to cells cultured with TEC or cultured alone. Bcl-2 is upregualted in thymocytes in order to protect cells from apoptosis [230,231] and Notch signalling leads to increases in Bcl-2 expression in thymocytes[232]. Hence, the decrease in apoptosis that is observed in co-culture with OP9-DL1 cells may be due to this increased level of Bcl-2. Although IL-7 responsiveness was increased in thymocytes within OP9-DL1 co-cultures, as

measured by higher expression of Bcl-2 following IL-7 stimulation, there was no significant difference in the level of IL-7 induced STAT-5 phosphorylation between the two culture systems. Therefore the greater level of Bcl-2 induction observed in OP9-DL1 co-culture does not appear to be due to increased IL-7 signalling via the JAK/STAT pathway. As another gauge of IL-7 function, the effect of IL-7 stimulation on CD127 expression of thymocytes within an OP9-DL1 co-culture, was measured. IL-7 stimulation resulted in a rapid decrease in CD127 expression on the surface of all thymocyte subsets, similar to what has been observed when thymocytes are co-cultured with TEC. However, thymocytes in co-culture with OP9-DL1 cells were more sensitive to IL-7 with a decrease in CD127 observed at a lower concentration than when cells were co-cultured with TEC. Although Notch signalling can enhance CD127 expression [233], it was not sufficient to overcome the decrease induced by IL-7, suggesting a complex interplay between Notch and IL-7 signalling.

4.2.1 IL-7 downregulates CD127 on thymic subsets

Recently, the decrease in CD127 expression on the surface of CD8 T-cells in HIV infection has been suggested to play a role in impaired CTL activity. Since IL-7 has been proposed as a potential therapy in HIV infection, the understanding of the regulation of its receptor is very important in order to determine the potential efficacy of such therapies. The results in this report indicate that IL-7 stimulation leads to decreased CD127 surface expression on thymocytes which is consistent with the effect of IL-7 on other cell types [68,69,71,76,163,234]. IL-7 regulation of its own receptor has been termed altruistic, since the mechanisms of downregulation appear to occur

in order to optimize the bioavailability of a limited amount of IL-7 [73]. However, IL-7 is not the only cytokine which down regulates its own receptor expression. IL-15, another member of the γ_C cytokine family, also decreases the expression of its own receptor IL-15Rα [235]. Several mechanisms have been proposed for the downregulation of CD127 following IL-7 stimulation. Our group has proposed that IL-7 stimulation induces CD127 receptor shedding, resulting in a down regulation of CD127 at the cell surface [69]. Recently, it has also been proposed that IL-7Rα expression is regulated through a continuous shuttling of receptor from the surface. A steady state of receptor expression is maintained through a balance of receptor degradation in the endosome and shuttling of newly synthesized receptor back to the surface. Following IL-7 stimulation, the rate of receptor degradation in the endosome is increased and newly shuttling is decreased, resulting in decreased surface expression [234]. Both mechanisms may hold true and further studies need to be conducted in order to elucidate the aspects that may favour one pathway from the other. This may be relevant to HIV infection since IL-7 levels are increased in plasma[168], which may contribute to decrease CD127 expression and possibly impair thymic function.

4.2.2 TNF-α stimulation decreases CD127 expression on thymocytes

The disruption in thymic function following HIV infection may be due to host factors that are induced in HIV infection. Cytokine dysregulation observed during HIV infection has been implicated in several aspects of immune deregulation. The results in this report demonstrate that TNF-α decreases CD127 expression on

mature thymocytes. TNF-α is upregulated during HIV-1 infection, therefore these results suggest that it may be one host factor contributing to CD127 downregulation following HIV infection. This is in contrast to what has been previously reported for the effect of TNF-α on CD127 expression on mature T-cells[68,69]. This may be due to the difference in cell type or the difference in activation states of the cells. The *in vitro* co-culture systems provides activation and survival signals through interaction of thymocytes with TEC. One of these signals may have primed the thymocytes to respond to TNF-α which then resulted in decreased CD127 expression. Only the more mature subsets were affected by TNF-α. This may be due to the expression pattern of TNF receptors on thymic subsets. The majority of thymocytes express tumour necrosis factor receptor 1 (TNF-R1), however only $CD3^+$ subsets express TNF-R2 [236]. Therefore the more mature subsets can be expected to be more responsive to TNF-α stimulation. In view of the fact that TNF-α levels are increased in HIV infection and TNF-α decreases CD127 expression on thymocytes, TNF-α may be a host factor contributing to HIV induced thymic dysfunction. This is supported by a study that demonstrated that LPS induced TNF-α production in mice induced apoptosis in DP thymocytes potentially contributing to thymic dysfunction[237]. In addition, immature thymocytes from TNF-R knock-out mice are more responsive to endogenous IL-7[200] supporting the hypothesis that increased TNF-α levels may contribute to thymic dysfunction.

4.2.3 CD127 downregulation on thymocytes is not due to non- specific cytokine stimulation

GM-CSF and IFN-α play a role in HIV immunopathogenesis and have been shown to affect T-cell development [201,202,238]. However, the present results indicate that neither cytokine affected CD127 surface expression on thymocytes. Therefore, the decrease in CD127 expression on thymocytes is not due to non-specific cytokine stimulation. IFN-α has been suggested to inhibit T-cell development in mice by countering the IL-7 response [238]. The results of this thesis demonstrate that *in vitro*, IFN-α does not decrease CD127 expression on thymocytes. Rather, the effect of IFN-α on T-cell and B-cell development is thought to be mediated through the regulation of IL-7 on Bcl-2 expression since over-expression of Bcl-2 rescues the inhibitory affect of IFN-α on B-cell development in mice [239].

4.2.4 IL-4 decreases IL-7 activity in thymocytes

IL-4 is a member of the γ_C cytokine family. In HIV infection, IL-4 production is increased and has been shown to play a role in HIV pathogenesis by increasing the susceptibility of resting T-cells to HIV infection [240]. The results in this report demonstrate that IL-4 stimulation decreased CD127 expression on the surface of thymocytes. Park *et al*. demonstrated that in mice several γ-chain cytokines (IL-2, IL-4, IL-6, IL-7 and IL-15) were able to decrease CD127 expression on T-cells[68]. In humans, IL-7, IL-2 and IL-4 have been shown to decrease CD127 expression on the surface of T-cells[69,70,72,76]. These results indicate that the kinetics of downregulation of CD127 with IL-4 was distinct compared to the effect of IL-7 suggesting a potentially different mechanism of downregulation. CD127 expression was only decreased by 50% and the decrease was not observed until 48 to 72 hours after

stimulation with IL-4. The mechanism of decreased CD127 may be direct, or as suggested by the delay in downregulation compared to the effect of IL-7, may be due to another factor that is induced by IL-4. IL-4 was also able to inhibit IL-7 activity, since IL-4 pre-treatment of thymocytes resulted in a reduced capability for IL-7 to phosphorylate STAT-5. This effect on activity is not thymocyte-specific since IL-4 also reduces IL-7 activity in $CD8^+$ T-cells [72]. The reports regarding the effects of IL-4 on thymopoiesis are contradictory. *In vitro*, IL-4 is stimulatory to thymocytes and increases PHA-induced proliferation[241]. However, *in vivo* IL-4 inhibits the differentiation of murine DP cells[242]. In FTOC, over-expression of IL-4 alters thymocyte development resulting in decreased DP cells and an increase in SP4 cells, suggesting a role for IL-4 in thymocyte dysfunction [243]. Although IL-4 pre-treatment blocked IL-7-induced STAT-5 phosphorylation, it did not affect IL-7-induced proliferation of thymocytes. IL-7 induced proliferation in thymocytes is primarily due to signalling through the PI3K pathway[84]. Therefore IL-4 specifically affects IL-7 activity through the Jak/STAT pathway. IL-4 has also been reported to affect IL-2 signalling selectively through the Jak/STAT pathway, indicating that IL-4 may regulate signalling events of the gamma chain family[222]. These results indicate that IL-4 could possibly block thymocyte development by altering IL-7 activity.

4.3 In vitro HIV infection decreases CD127 on SP8 cells.

Since IL-7 has been proposed as a potential therapy in HIV infection, understanding the regulation of its receptor is very important in order to determine the potential efficacy of such therapies. In this report the effect of *in vitro* HIV infection on the IL-

7/IL-7R system in developing thymocytes was measured. During the course of HIV infection, there is a decrease in CD127 expression on $CD8^+$ T-cells and $CD4^+$ T-cells. It has been proposed that this decrease may be a contributing factor to CTL decline observed in HIV infection. The same mechanisms may hold true in thymocytes, that is HIV infection may decrease CD127 expression of thymocytes thereby altering their development into functional mature $CD4^+$ and $CD8^+$ T-cells.

In this report, *in vitro* HIV infection was shown to decrease CD127 expression on total thymocytes when infected with an X4 tropic strain; however there was no significant change in CD127 expression when cells were infected with the dual tropic or R5 tropic strains. Viral tropism may play a role in viral pathogenecity within the thymus. Varying degrees of thymic dysfunction have been proposed with different strains of HIV (*i.e.* R5, X4 and R5X4 [244]). Models of HIV infection have proposed that X4 strains infect all thymic subsets and results in a much higher level of infection compared to R5 strains [150,158]. The kinetics of infection also vary among viral strains, with X4 tropic viruses leading to a much quicker and more pronounced depletion of thymic cellularity compared to R5 tropic strains which are associated with a delayed depletion [245]. Furthermore, the cytokine microenvironment of the infection differs depending on the viral strains. Infection with X4 strains tends to result in an increase in IL-7 and TNF-α secretion, whereas infection with R5 strains results in IL-10 and TGF-β upregulation [246]. Since many cytokines have been implicated in affecting CD127 regulation on T-cells, the differential effect of the HIV strains on the cytokine milieu may greatly affect the regulation of the CD127

expression. In the periphery, HIV infection is associated with reduced CD127 expression on $CD4^+$ and $CD8^+$ T cells. The present results indicate that *in vitro* HIV infection of thymocytes only resulted in a decrease in CD127 expression on SP8 thymocytes, indicating that reduced CD127 on mature SP8 cells prior to leaving the thymus may be a contributing factor to impaired CTL function in the periphery.

4.4 HIV infection inhibits IL-7 activity independent of CD127 expression

Due to the small magnitude of change in CD127 expression, the effect of HIV infection on downstream signalling elements of IL-7 was measured. Decreased IL-7 activity may occur through a multitude of mechanisms. One hypothesis is that decreased receptor expression could lead to decreased cytokine activity. Another hypothesis is that a block may occur in the IL-7 signalling pathway, resulting in altered IL-7 activity. The results in this report indicate that STAT-5 phosphorylation following IL-7 stimulation was lower in thymocytes that were infected with the cs204 strain when compared to mock-infected controls while having no effect on CD127 expression. This suggests that there may be a block in the IL-7 pathway that is independent of CD127 expression. This data supports the report by Vranjkovic *et al*, which demonstrated reduced IL-7 responsiveness in CD127-expressing $CD8^+$ T-cells from HIV^+ patients. The study demonstrated that isolated $CD8^+CD127^+$ cells from HIV^+ individuals had lower levels of STAT-5 phosphorylation following IL-7 stimulation when compared to those for healthy controls[247]. These data suggest that HIV infection can inhibit the IL-7 pathway. A block in IL-7 signalling also occurs in other disease states such as breast cancer. $CD4^+$ and $CD8^+$ T-cells isolated from

breast cancer patients showed a lack of IL-7 activity as measured by STAT-5 phosphorylation, although the cells still expressed CD127[248]. HIV infection did not affect the ability of IL-7 to induce the PI3K pathway. The PI3K pathway plays a role primarily in IL-7 induced proliferation and glucose uptake[89]. HIV may affect thymocyte function by altering the viability of the cells, consequently lowering the output of functional T-cells from the thymus.

The exact mechanism of HIV inhibition of the IL-7 pathway has yet to be determined, however the results indicate that binding alone is insufficient since there was no effect of HIV on the IL-7 pathway following 24 hours of infection. Rather our data demonstrated that the cells need to be infected for at least 96 hours. This suggests that the mechanism of inhibition requires the production of a factor or factors, supporting the hypothesis that the HIV inhibition may be indirect, *i.e* through the release of soluble factors that can interfere with the IL-7 pathway

4.5 HIV infection inhibits IL-7 induced Bcl-2 expression

To determine if the HIV induced decrease in CD127 affected IL-7-mediated function, the effect on Bcl-2 expression was measured. HIV-specific $CD8^+$ T-cells from HIV^+ individuals have reduced Bcl-2 expression, indicating that the virus may decrease Bcl-2 expression in order to induce cell death in infected cells[249]. There are also reports that HIV infection results in increased Bcl-2 expression in SP4 cells, potentially to enable a viral reservoir. The effect of HIV on Bcl-2 varies amongst the subsets since HIV infection results in a decrease in Bcl-2 expression in $CD3^-$DP

thymocytes, potentially contributing to impairment in T-cell development[185]. To determine if HIV-mediated downregulation of CD127 on SP8 cells affected the ability of IL-7 to increase Bcl-2 expression, infected cells were stimulated with IL-7 and Bcl-2 expression was measured. Although CD127 expression was decreased by HIV_{IIIB} on SP8 cells, the ability of IL-7 to increase Bcl-2 expression was not altered. However, infection with the dual tropic strain cs204 resulted in lower levels of Bcl-2 expression following IL-7 stimulation when compared to mock-infected controls. A similar block in the ability of IL-7 to increase Bcl-2 expression was reported in a study in which $CD4^+$ T-cells from HIV^+ individuals had lower levels of Bcl-2 expression following IL-7 stimulation when compared to healthy controls. The study found no correlation between CD127 expression of $CD4^+$ T cells and IL-7 responsiveness, suggesting that the block in IL-7 activity was not due to the level of CD127 expression [162].

4.6 Conclusion

This report describes the optimization of an *in vitro* system for the study of exogenous stimuli on thymocyte activity such as cytokine responsiveness, survival and proliferation. As such, these results suggest a potential role for disrupted IL-7 signalling in the reduced thymic output in HIV infection. HIV infection of the thymus alters the IL-7/IL-7R signalling pathway by downregulating the CD127 receptor on the surface of thymocytes or by interfering with pSTAT-5 of the JAK/STAT pathway. The exact mechanisms of HIV-1 disruption of the IL-7 pathway have yet to be fully elucidated, however this report suggests a role for the host factors IL-7, IL-4 and

TNF-α ,which are upregulated in HIV, in this dysregulation. The mechanism leading to impaired IL-7 activity in HIV infection is multi factorial and may play a role in dysfunctional thymopoiesis. Cytokine dysregulation following HIV infection is an important factor contributing to an impaired immune system. Understanding the impact of the altered cytokine milieu on the function of immune organs such as the thymus may lead to potential therapies that could improve immune reconstitution.

5.0 Reference

1. Rocha B, Vassalli P, Guy-Grand D. The extrathymic T-cell development pathway. Immunol Today. 1992;13:449-454.
2. Blais ME, Louis I, Perreault C. T-cell development: an extrathymic perspective. Immunol Rev. 2006;209:103-114.
3. Sato K, Ohtsuka K, Hasegawa K, et al. Evidence for extrathymic generation of intermediate T cell receptor cells in the liver revealed in thymectomized, irradiated mice subjected to bone marrow transplantation. J Exp Med. 1995;182:759-767.
4. Guy-Grand D, Azogui O, Celli S, et al. Extrathymic T cell lymphopoiesis: ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. J Exp Med. 2003;197:333-341.
5. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988;241:58-62.
6. Jiang Q, Huang J, Li WQ, Cavinato T, Keller JR, Durum SK. Role of the intracellular domain of IL-7 receptor in T cell development. J Immunol. 2007;178:228-234.
7. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. Science. 2002;297:2256-2259.
8. Payne KJ, Crooks GM. Human hematopoietic lineage commitment. Immunol Rev. 2002;187:48-64.
9. Terstappen LW, Huang S, Picker LJ. Flow cytometric assessment of human T-cell differentiation in thymus and bone marrow. Blood. 1992;79:666-677.
10. Galy A, Verma S, Barcena A, Spits H. Precursors of CD3+CD4+CD8+ cells in the human thymus are defined by expression of CD34. Delineation of early events in human thymic development. J Exp Med. 1993;178:391-401.
11. Dion ML, Poulin JF, Bordi R, et al. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. Immunity. 2004;21:757-768.

12. Havlir DV, Torriani FJ, Schrier RD, et al. Serum interleukin-6 (IL-6), IL-10, tumor necrosis factor (TNF) alpha, soluble type II TNF receptor, and transforming growth factor beta levels in human immunodeficiency virus type 1-infected individuals with Mycobacterium avium complex disease. J Clin Microbiol. 2001;39:298-303.
13. Anderson G, Moore NC, Owen JJ, Jenkinson EJ. Cellular interactions in thymocyte development. Annu Rev Immunol. 1996;14:73-99.
14. Kaufmann SH. gamma/delta and other unconventional T lymphocytes: what do they see and what do they do? Proc Natl Acad Sci U S A. 1996;93:2272-2279.
15. Blom-Potar MC, Bugault F, Lambotte O, Delfraissy JF, Theze J. Soluble IL-7Ralpha (sCD127) and measurement of IL-7 in the plasma of HIV patients. J Acquir Immune Defic Syndr. 2009;51:104-105.
16. Blom B, Verschuren MC, Heemskerk MH, et al. TCR gene rearrangements and expression of the pre-T cell receptor complex during human T-cell differentiation. Blood. 1999;93:3033-3043.
17. Ramiro AR, Trigueros C, Marquez C, San Millan JL, Toribio ML. Regulation of pre-T cell receptor (pT alpha-TCR beta) gene expression during human thymic development. J Exp Med. 1996;184:519-530.
18. Anderson G, Owen JJ, Moore NC, Jenkinson EJ. Thymic epithelial cells provide unique signals for positive selection of CD4+CD8+ thymocytes in vitro. J Exp Med. 1994;179:2027-2031.
19. von Boehmer H. Positive selection of lymphocytes. Cell. 1994;76:219-228.
20. Nossal GJ. Negative selection of lymphocytes. Cell. 1994;76:229-239.
21. Ashton-Rickardt PG, Bandeira A, Delaney JR, et al. Evidence for a differential avidity model of T cell selection in the thymus. Cell. 1994;76:651-663.
22. Singer A, Adoro S, Park JH. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. Nat Rev Immunol. 2008;8:788-801.
23. He X, He X, Dave VP, et al. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature. 2005;433:826-833.
24. Wang L, Wildt KF, Zhu J, et al. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. Nat Immunol. 2008;9:1122-1130.
25. Egawa T, Tillman RE, Naoe Y, Taniuchi I, Littman DR. The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. J Exp Med. 2007;204:1945-1957.
26. Park JH, Adoro S, Guinter T, et al. Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. Nat Immunol;11:257-264.
27. Sato T, Ohno S, Hayashi T, et al. Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. Immunity. 2005;22:317-328.
28. Setoguchi R, Tachibana M, Naoe Y, et al. Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development. Science. 2008;319:822-825.

29. Hare KJ, Jenkinson EJ, Anderson G. An essential role for the IL-7 receptor during intrathymic expansion of the positively selected neonatal T cell repertoire. J Immunol. 2000;165:2410-2414.
30. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. Nat Rev Immunol. 2006;6:127-135.
31. Haynes BF, Markert ML, Sempowski GD, Patel DD, Hale LP. The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. Annu Rev Immunol. 2000;18:529-560.
32. Ritter MA, Palmer DB. The human thymic microenvironment: new approaches to functional analysis. Semin Immunol. 1999;11:13-21.
33. Moore NC, Anderson G, Smith CA, Owen JJ, Jenkinson EJ. Analysis of cytokine gene expression in subpopulations of freshly isolated thymocytes and thymic stromal cells using semiquantitative polymerase chain reaction. Eur J Immunol. 1993;23:922-927.
34. Uittenbogaart CH, Higashitani S, Schmid I, Vollger LW, Boone T, Clement LT. Interleukin-4 induces expression of the CD45RA antigen on human thymocyte subpopulations. Int Immunol. 1990;2:1179-1187.
35. Singer KH, Denning SM, Whichard LP, Haynes BF. Thymocyte LFA-1 and thymic epithelial cell ICAM-1 molecules mediate binding of activated human thymocytes to thymic epithelial cells. J Immunol. 1990;144:2931-2939.
36. Cohen-Kaminsky S, Berrih-Aknin S. Production of interleukin 1 (IL1) by human thymic epithelial cells (TEC). Adv Exp Med Biol. 1988;237:299-305.
37. Le PT, Lazorick S, Whichard LP, et al. Human thymic epithelial cells produce IL-6, granulocyte-monocyte-CSF, and leukemia inhibitory factor. J Immunol. 1990;145:3310-3315.
38. Wiles MV, Ruiz P, Imhof BA. Interleukin-7 expression during mouse thymus development. Eur J Immunol. 1992;22:1037-1042.
39. Oosterwegel MA, Haks MC, Jeffry U, Murray R, Kruisbeek AM. Induction of TCR gene rearrangements in uncommitted stem cells by a subset of IL-7 producing, MHC class-II-expressing thymic stromal cells. Immunity. 1997;6:351-360.
40. Schnittman SM, Denning SM, Greenhouse JJ, et al. Evidence for susceptibility of intrathymic T-cell precursors and their progeny carrying T-cell antigen receptor phenotypes TCR alpha beta + and TCR gamma delta + to human immunodeficiency virus infection: a mechanism for CD4+ (T4) lymphocyte depletion. Proc Natl Acad Sci U S A. 1990;87:7727-7731.
41. Dardenne M, Savino W, Gagnerault MC, Itoh T, Bach JF. Neuroendocrine control of thymic hormonal production. I. Prolactin stimulates in vivo and in vitro the production of thymulin by human and murine thymic epithelial cells. Endocrinology. 1989;125:3-12.
42. van Ewijk W, Hollander G, Terhorst C, Wang B. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. Development. 2000;127:1583-1591.
43. Poliani PL, Facchetti F, Ravanini M, et al. Early defects in human T-cell development severely affect distribution and maturation of thymic stromal cells:

possible implications for the pathophysiology of Omenn syndrome. Blood. 2009;114:105-108.
44. Jenkinson EJ, Anderson G, Owen JJ. Studies on T cell maturation on defined thymic stromal cell populations in vitro. J Exp Med. 1992;176:845-853.
45. Jenkinson EJ, Owen JJ. T-cell differentiation in thymus organ cultures. Semin Immunol. 1990;2:51-58.
46. Kingston R, Jenkinson EJ, Owen JJ. A single stem cell can recolonize an embryonic thymus, producing phenotypically distinct T-cell populations. Nature. 1985;317:811-813.
47. Anderson KL, Moore NC, McLoughlin DE, Jenkinson EJ, Owen JJ. Studies on thymic epithelial cells in vitro. Dev Comp Immunol. 1998;22:367-377.
48. Meilin A, Shoham J, Schreiber L, Sharabi Y. The role of thymocytes in regulating thymic epithelial cell growth and function. Scand J Immunol. 1995;42:185-190.
49. Young CD, Angel JB. Optimization of culture and storage conditions for an in vitro system to evaluate thymocyte phenotype and function. J Immunol Methods. 2006;312:157-166.
50. Salomon DR, Crisa L, Mojcik CF, Ishii JK, Klier G, Shevach EM. Vascular cell adhesion molecule-1 is expressed by cortical thymic epithelial cells and mediates thymocyte adhesion. Implications for the function of alpha4beta1 (VLA4) integrin in T-cell development. Blood. 1997;89:2461-2471.
51. Rothe M, Chene L, Nugeyre MT, Braun J, Barre-Sinoussi F, Israel N. Contact with thymic epithelial cells as a prerequisite for cytokine-enhanced human immunodeficiency virus type 1 replication in thymocytes. J Virol. 1998;72:5852-5861.
52. Mohtashami M, Zuniga-Pflucker JC. Three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development. J Immunol. 2006;176:730-734.
53. Balciunaite G, Ceredig R, Fehling HJ, Zuniga-Pflucker JC, Rolink AG. The role of Notch and IL-7 signaling in early thymocyte proliferation and differentiation. Eur J Immunol. 2005;35:1292-1300.
54. Zuniga-Pflucker JC. T-cell development made simple. Nat Rev Immunol. 2004;4:67-72.
55. Awong G, La Motte-Mohs RN, Zuniga-Pflucker JC. In vitro human T cell development directed by notch-ligand interactions. Methods Mol Biol. 2008;430:135-142.
56. La Motte-Mohs RN, Herer E, Zuniga-Pflucker JC. Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. Blood. 2005;105:1431-1439.
57. Kroemer RT, Kroncke R, Gerdes J, Richards WG. Comparison of the 3D models of four different human IL-7 isoforms with human and murine IL-7. Protein Eng. 1998;11:31-40.
58. Link A, Vogt TK, Favre S, et al. Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. Nat Immunol. 2007;8:1255-1265.

59. Kroncke R, Loppnow H, Flad HD, Gerdes J. Human follicular dendritic cells and vascular cells produce interleukin-7: a potential role for interleukin-7 in the germinal center reaction. Eur J Immunol. 1996;26:2541-2544.
60. Heufler C, Topar G, Grasseger A, et al. Interleukin 7 is produced by murine and human keratinocytes. J Exp Med. 1993;178:1109-1114.
61. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038-1044.
62. Hofmeister R, Khaled AR, Benbernou N, Rajnavolgyi E, Muegge K, Durum SK. Interleukin-7: physiological roles and mechanisms of action. Cytokine Growth Factor Rev. 1999;10:41-60.
63. Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. Blood. 2002;99:3892-3904.
64. Goodwin RG, Friend D, Ziegler SF, et al. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. Cell. 1990;60:941-951.
65. McElroy CA, Dohm JA, Walsh ST. Structural and biophysical studies of the human IL-7/IL-7Ralpha complex. Structure. 2009;17:54-65.
66. Ryan DH, Nuccie BL, Ritterman I, Liesveld JL, Abboud CN, Insel RA. Expression of interleukin-7 receptor by lineage-negative human bone marrow progenitors with enhanced lymphoid proliferative potential and B-lineage differentiation capacity. Blood. 1997;89:929-940.
67. Sudo T, Nishikawa S, Ohno N, Akiyama N, Tamakoshi M, Yoshida H. Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc Natl Acad Sci U S A. 1993;90:9125-9129.
68. Park JH, Yu Q, Erman B, et al. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. Immunity. 2004;21:289-302.
69. Vranjkovic A, Crawley AM, Gee K, Kumar A, Angel JB. IL-7 decreases IL-7 receptor alpha (CD127) expression and induces the shedding of CD127 by human CD8+ T cells. Int Immunol. 2007;19:1329-1339.
70. Xue HH, Kovanen PE, Pise-Masison CA, et al. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. Proc Natl Acad Sci U S A. 2002;99:13759-13764.
71. Swainson L, Verhoeyen E, Cosset FL, Taylor N. IL-7R alpha gene expression is inversely correlated with cell cycle progression in IL-7-stimulated T lymphocytes. J Immunol. 2006;176:6702-6708.
72. Crawley AM, Vranjkovic A, Young C, Angel JB. Interleukin-4 downregulates CD127 expression and activity on human thymocytes and mature CD8(+) T-cells. Eur J Immunol.
73. Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. Nat Rev Immunol. 2007;7:144-154.
74. Boutboul F, Puthier D, Appay V, et al. Modulation of interleukin-7 receptor expression characterizes differentiation of CD8 T cells specific for HIV, EBV and CMV. Aids. 2005;19:1981-1986.

75. MacPherson PA, Fex C, Sanchez-Dardon J, Hawley-Foss N, Angel JB. Interleukin-7 receptor expression on CD8(+) T cells is reduced in HIV infection and partially restored with effective antiretroviral therapy. J Acquir Immune Defic Syndr. 2001;28:454-457.
76. Sasson SC, Zaunders JJ, Zanetti G, et al. Increased Plasma Interleukin-7 Level Correlates with Decreased CD127 and Increased CD132 Extracellular Expression on T Cell Subsets in Patients with HIV-1 Infection. J Infect Dis. 2006;193:505-514.
77. Golden-Mason L, Burton JR, Jr., Castelblanco N, et al. Loss of IL-7 receptor alpha-chain (CD127) expression in acute HCV infection associated with viral persistence. Hepatology. 2006;44:1098-1109.
78. Koesters SA, Alimonti JB, Wachihi C, et al. IL-7Ralpha expression on CD4+ T lymphocytes decreases with HIV disease progression and inversely correlates with immune activation. Eur J Immunol. 2006;36:336-344.
79. Lang KS, Recher M, Navarini AA, et al. Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation. Eur J Immunol. 2005;35:738-745.
80. Koesters SA, Alimonti JB, Wachihi C, et al. IL-7Ralpha expression on CD4(+) T lymphocytes decreases with HIV disease progression and inversely correlates with immune activation. Eur J Immunol. 2006;36:336-344.
81. Franchimont D, Galon J, Vacchio MS, et al. Positive effects of glucocorticoids on T cell function by up-regulation of IL-7 receptor alpha. J Immunol. 2002;168:2212-2218.
82. Zhang M, Drenkow J, Lankford CS, et al. HIV regulation of the IL-7R: a viral mechanism for enhancing HIV-1 replication in human macrophages in vitro. J Leukoc Biol. 2006;79:1328-1338.
83. Faller EM, McVey MJ, Kakal JA, MacPherson PA. Interleukin-7 receptor expression on CD8 T-cells is downregulated by the HIV Tat protein. J Acquir Immune Defic Syndr. 2006;43:257-269.
84. Pallard C, Stegmann AP, van Kleffens T, Smart F, Venkitaraman A, Spits H. Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors. Immunity. 1999;10:525-535.
85. Ye SK, Agata Y, Lee HC, et al. The IL-7 receptor controls the accessibility of the TCRgamma locus by Stat5 and histone acetylation. Immunity. 2001;15:813-823.
86. Giliani S, Mori L, de Saint Basile G, et al. Interleukin-7 receptor alpha (IL-7Ralpha) deficiency: cellular and molecular bases. Analysis of clinical, immunological, and molecular features in 16 novel patients. Immunol Rev. 2005;203:110-126.
87. Palmer MJ, Mahajan VS, Trajman LC, Irvine DJ, Lauffenburger DA, Chen J. Interleukin-7 receptor signaling network: an integrated systems perspective. Cell Mol Immunol. 2008;5:79-89.
88. Venkitaraman AR, Cowling RJ. Interleukin-7 induces the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. Eur J Immunol. 1994;24:2168-2174.

89. Barata JT, Silva A, Brandao JG, Nadler LM, Cardoso AA, Boussiotis VA. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. J Exp Med. 2004;200:659-669.
90. Venkitaraman AR, Cowling RJ. Interleukin 7 receptor functions by recruiting the tyrosine kinase p59fyn through a segment of its cytoplasmic tail. Proc Natl Acad Sci U S A. 1992;89:12083-12087.
91. Benbernou N, Muegge K, Durum SK. Interleukin (IL)-7 induces rapid activation of Pyk2, which is bound to Janus kinase 1 and IL-7Ralpha. J Biol Chem. 2000;275:7060-7065.
92. Crawley JB, Rawlinson L, Lali FV, Page TH, Saklatvala J, Foxwell BM. T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. J Biol Chem. 1997;272:15023-15027.
93. Fleming HE, Paige CJ. Pre-B cell receptor signaling mediates selective response to IL-7 at the pro-B to pre-B cell transition via an ERK/MAP kinase-dependent pathway. Immunity. 2001;15:521-531.
94. Crawley JB, Willcocks J, Foxwell BM. Interleukin-7 induces T cell proliferation in the absence of Erk/MAP kinase activity. Eur J Immunol. 1996;26:2717-2723.
95. Puel A, Ziegler SF, Buckley RH, Leonard WJ. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. Nat Genet. 1998;20:394-397.
96. Moore TA, von Freeden-Jeffry U, Murray R, Zlotnik A. Inhibition of gamma delta T cell development and early thymocyte maturation in IL-7 -/- mice. J Immunol. 1996;157:2366-2373.
97. Peschon JJ, Morrissey PJ, Grabstein KH, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med. 1994;180:1955-1960.
98. Bhatia SK, Tygrett LT, Grabstein KH, Waldschmidt TJ. The effect of in vivo IL-7 deprivation on T cell maturation. J Exp Med. 1995;181:1399-1409.
99. Pandey A, Ozaki K, Baumann H, et al. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. Nat Immunol. 2000;1:59-64.
100. Nagaoka H, Yu W, Nussenzweig MC. Regulation of RAG expression in developing lymphocytes. Curr Opin Immunol. 2000;12:187-190.
101. Huang J, Muegge K. Control of chromatin accessibility for V(D)J recombination by interleukin-7. J Leukoc Biol. 2001;69:907-911.
102. Durum SK, Candeias S, Nakajima H, et al. Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility. J Exp Med. 1998;188:2233-2241.
103. Wang J, Huang DS, Wood S, Giger PT, Watson RR. Kinetics of cytokine production by thymocytes during murine AIDS caused by LP-BM5 retrovirus infection. Immunol Lett. 1995;47:187-192.
104. Suda T, Zlotnik A. IL-7 maintains the T cell precursor potential of CD3-CD4-CD8- thymocytes. J Immunol. 1991;146:3068-3073.
105. Khaled AR, Kim K, Hofmeister R, Muegge K, Durum SK. Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. Proc Natl Acad Sci U S A. 1999;96:14476-14481.

106. Navikas V, Link J, Persson C, et al. Increased mRNA expression of IL-6, IL-10, TNF-alpha, and perforin in blood mononuclear cells in human HIV infection. J Acquir Immune Defic Syndr Hum Retrovirol. 1995;9:484-489.
107. Li WQ, Jiang Q, Khaled AR, Keller JR, Durum SK. Interleukin-7 inactivates the pro-apoptotic protein Bad promoting T cell survival. J Biol Chem. 2004;279:29160-29166.
108. von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med. 1995;181:1519-1526.
109. Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell. 1997;89:1033-1041.
110. Singer A. New perspectives on a developmental dilemma: the kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. Curr Opin Immunol. 2002;14:207-215.
111. Takada K, Takahama Y. Another zinc finger in the pie of CD4-CD8 lineage choice. Nat Immunol;11:370-371.
112. He X, Park K, Wang H, et al. CD4-CD8 lineage commitment is regulated by a silencer element at the ThPOK transcription-factor locus. Immunity. 2008;28:346-358.
113. Sakaguchi S, Hombauer M, Bilic I, et al. The zinc-finger protein MAZR is part of the transcription factor network that controls the CD4 versus CD8 lineage fate of double-positive thymocytes. Nat Immunol;11:442-448.
114. Fry TJ, Connick E, Falloon J, et al. A potential role for interleukin-7 in T-cell homeostasis. Blood. 2001;97:2983-2990.
115. Hickman CJ, Crim JA, Mostowski HS, Siegel JP. Regulation of human cytotoxic T lymphocyte development by IL-7. J Immunol. 1990;145:2415-2420.
116. Buentke E, Mathiot A, Tolaini M, Di Santo J, Zamoyska R, Seddon B. Do CD8 effector cells need IL-7R expression to become resting memory cells? Blood. 2006;108:1949-1956.
117. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol. 2003;4:1191-1198.
118. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med. 2006;203:1701-1711.
119. Urmacher C, Myskowski P, Ochoa M, Jr., Kris M, Safai B. Outbreak of Kaposi's sarcoma with cytomegalovirus infection in young homosexual men. Am J Med. 1982;72:569-575.
120. Barre-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science. 1983;220:868-871.
121. Gallo RC, Salahuddin SZ, Popovic M, et al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science. 1984;224:500-503.

122. Pantaleo G, Graziosi C, Fauci AS. New concepts in the immunopathogenesis of human immunodeficiency virus infection. N Engl J Med. 1993;328:327-335.
123. Coffin JM. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science. 1995;267:483-489.
124. Ferrari G, King K, Rathbun K, et al. IL-7 enhancement of antigen-driven activation/expansion of HIV-1-specific cytotoxic T lymphocyte precursors (CTLp). Clin Exp Immunol. 1995;101:239-248.
125. Carmichael A, Jin X, Sissons P, Borysiewicz L. Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. J Exp Med. 1993;177:249-256.
126. Pantaleo G, Graziosi C, Demarest JF, et al. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature. 1993;362:355-358.
127. Costin JM. Cytopathic mechanisms of HIV-1. Virol J. 2007;4:100.
128. Feinberg MB, Jarrett RF, Aldovini A, Gallo RC, Wong-Staal F. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell. 1986;46:807-817.
129. Fisher AG, Feinberg MB, Josephs SF, et al. The trans-activator gene of HTLV-III is essential for virus replication. Nature. 1986;320:367-371.
130. Muesing MA, Smith DH, Cabradilla CD, Benton CV, Lasky LA, Capon DJ. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature. 1985;313:450-458.
131. Ratner L, Haseltine W, Patarca R, et al. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature. 1985;313:277-284.
132. Strebel K, Klimkait T, Martin MA. A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science. 1988;241:1221-1223.
133. Wong-Staal F, Chanda PK, Ghrayeb J. Human immunodeficiency virus: the eighth gene. AIDS Res Hum Retroviruses. 1987;3:33-39.
134. Jenkins M, Hanley MB, Moreno MB, Wieder E, McCune JM. Human immunodeficiency virus-1 infection interrupts thymopoiesis and multilineage hematopoiesis in vivo. Blood. 1998;91:2672-2678.
135. Cheng-Mayer C, Rutka JT, Rosenblum ML, McHugh T, Stites DP, Levy JA. Human immunodeficiency virus can productively infect cultured human glial cells. Proc Natl Acad Sci U S A. 1987;84:3526-3530.
136. Collman R, Hassan NF, Walker R, et al. Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. J Exp Med. 1989;170:1149-1163.
137. Rosenzweig M, Clark DP, Gaulton GN. Selective thymocyte depletion in neonatal HIV-1 thymic infection. Aids. 1993;7:1601-1605.
138. Kourtis AP, Ibegbu C, Nahmias AJ, et al. Early progression of disease in HIV-infected infants with thymus dysfunction. N Engl J Med. 1996;335:1431-1436.
139. Gaulton GN. Viral pathogenesis and immunity within the thymus. Immunol Res. 1998;17:75-82.

140. Douek DC, McFarland RD, Keiser PH, et al. Changes in thymic function with age and during the treatment of HIV infection. Nature. 1998;396:690-695.
141. Vigano A, Vella S, Saresella M, et al. Early immune reconstitution after potent antiretroviral therapy in HIV-infected children correlates with the increase in thymus volume. Aids. 2000;14:251-261.
142. McCune JM. Thymic function in HIV-1 disease. Semin Immunol. 1997;9:397-404.
143. Chene L, Nugeyre MT, Guillemard E, Moulian N, Barre-Sinoussi F, Israel N. Thymocyte-thymic epithelial cell interaction leads to high-level replication of human immunodeficiency virus exclusively in mature CD4(+) CD8(-) CD3(+) thymocytes: a critical role for tumor necrosis factor and interleukin-7. J Virol. 1999;73:7533-7542.
144. Valentin H, Nugeyre MT, Vuillier F, et al. Two subpopulations of human triple-negative thymic cells are susceptible to infection by human immunodeficiency virus type 1 in vitro. J Virol. 1994;68:3041-3050.
145. Knutsen AP, Roodman ST, Freeman JJ, Mueller KR, Bouhasin JD. Inhibition of thymopoiesis of CD34+ cell maturation by HIV-1 in an in vitro CD34+ cell and thymic epithelial organ culture model. Stem Cells. 1999;17:327-338.
146. Bonyhadi ML, Su L, Auten J, McCune JM, Kaneshima H. Development of a human thymic organ culture model for the study of HIV pathogenesis. AIDS Res Hum Retroviruses. 1995;11:1073-1080.
147. Sodora DL, Milush JM, Ware F, et al. Decreased levels of recent thymic emigrants in peripheral blood of simian immunodeficiency virus-infected macaques correlate with alterations within the thymus. J Virol. 2002;76:9981-9990.
148. Diehl LJ, Mathiason-Dubard CK, O'Neil LL, Obert LA, Hoover EA. Induction of accelerated feline immunodeficiency virus disease by acute-phase virus passage. J Virol. 1995;69:6149-6157.
149. Pedroza-Martins L, Gurney KB, Torbett BE, Uittenbogaart CH. Differential tropism and replication kinetics of human immunodeficiency virus type 1 isolates in thymocytes: coreceptor expression allows viral entry, but productive infection of distinct subsets is determined at the postentry level. J Virol. 1998;72:9441-9452.
150. Schmitt N, Nugeyre MT, Scott-Algara D, et al. Differential susceptibility of human thymic dendritic cell subsets to X4 and R5 HIV-1 infection. Aids. 2006;20:533-542.
151. Meissner EG, Zhang L, Jiang S, Su L. Fusion-induced apoptosis contributes to thymocyte depletion by a pathogenic human immunodeficiency virus type 1 envelope in the human thymus. J Virol. 2006;80:11019-11030.
152. Mullberg J, Rauch CT, Wolfson MF, et al. Further evidence for a common mechanism for shedding of cell surface proteins. FEBS Lett. 1997;401:235-238.
153. Kitazawa H, Muegge K, Badolato R, et al. IL-7 activates alpha4beta1 integrin in murine thymocytes. J Immunol. 1997;159:2259-2264.
154. Ho Tsong Fang R, Colantonio AD, Uittenbogaart CH. The role of the thymus in HIV infection: a 10 year perspective. Aids. 2008;22:171-184.
155. Stanley SK, McCune JM, Kaneshima H, et al. Human immunodeficiency virus infection of the human thymus and disruption of the thymic microenvironment in the SCID-hu mouse. J Exp Med. 1993;178:1151-1163.

156. Maroder M, Scarpa S, Screpanti I, et al. Human immunodeficiency virus type 1 tat protein modulates fibronectin expression in thymic epithelial cells and impairs in vitro thymocyte development. Cell Immunol. 1996;168:49-58.
157. Kourtis AP, Ibegbu CC, Scinicarrielo F, Lee FK. Effects of different HIV strains on thymic epithelial cultures. Pathobiology. 2001;69:329-332.
158. Schmitt N, Chene L, Boutolleau D, et al. Positive regulation of CXCR4 expression and signaling by interleukin-7 in CD4+ mature thymocytes correlates with their capacity to favor human immunodeficiency X4 virus replication. J Virol. 2003;77:5784-5793.
159. Carini C, McLane MF, Mayer KH, Essex M. Dysregulation of interleukin-7 receptor may generate loss of cytotoxic T cell response in human immunodeficiency virus type 1 infection. Eur J Immunol. 1994;24:2927-2934.
160. Albuquerque AS, Cortesao CS, Foxall RB, Soares RS, Victorino RM, Sousa AE. Rate of increase in circulating IL-7 and loss of IL-7Ralpha expression differ in HIV-1 and HIV-2 infections: two lymphopenic diseases with similar hyperimmune activation but distinct outcomes. J Immunol. 2007;178:3252-3259.
161. Benoit A, Abdkader K, Sirskyj D, et al. Inverse association of repressor growth factor independent-1 with CD8 T cell interleukin (IL)-7 receptor [alpha] expression and limited signal transducers and activators of transcription signaling in response to IL-7 among [gamma]-chain cytokines in HIV patients. Aids. 2009;23:1341-1347.
162. Colle JH, Moreau JL, Fontanet A, Lambotte O, Delfraissy JF, Theze J. The correlation between levels of IL-7Ralpha expression and responsiveness to IL-7 is lost in CD4 lymphocytes from HIV-infected patients. Aids. 2007;21:101-103.
163. Vingerhoets J, Bisalinkumi E, Penne G, et al. Altered receptor expression and decreased sensitivity of T-cells to the stimulatory cytokines IL-2, IL-7 and IL-12 in HIV infection. Immunol Lett. 1998;61:53-61.
164. Paiardini M, Cervasi B, Albrecht H, et al. Loss of CD127 expression defines an expansion of effector CD8+ T cells in HIV-infected individuals. J Immunol. 2005;174:2900-2909.
165. Rethi B, Fluur C, Atlas A, et al. Loss of IL-7Ralpha is associated with CD4 T-cell depletion, high interleukin-7 levels and CD28 down-regulation in HIV infected patients. Aids. 2005;19:2077-2086.
166. Dunham RM, Cervasi B, Brenchley JM, et al. CD127 and CD25 expression defines CD4+ T cell subsets that are differentially depleted during HIV infection. J Immunol. 2008;180:5582-5592.
167. Sharma TS, Hughes J, Murillo A, et al. CD8+ T-cell interleukin-7 receptor alpha expression as a potential indicator of disease status in HIV-infected children. PLoS One. 2008;3:e3986.
168. Napolitano LA, Grant RM, Deeks SG, et al. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat Med. 2001;7:73-79.
169. Bolotin E, Annett G, Parkman R, Weinberg K. Serum levels of IL-7 in bone marrow transplant recipients: relationship to clinical characteristics and lymphocyte count. Bone Marrow Transplant. 1999;23:783-788.

170. Fluur C, De Milito A, Fry TJ, et al. Potential Role for IL-7 in Fas-Mediated T Cell Apoptosis During HIV Infection. J Immunol. 2007;178:5340-5350.
171. Chene L, Nugeyre MT, Barre-Sinoussi F, Israel N. High-level replication of human immunodeficiency virus in thymocytes requires NF-kappaB activation through interaction with thymic epithelial cells. J Virol. 1999;73:2064-2073.
172. Muthukumar A, Wozniakowski A, Gauduin MC, et al. Elevated interleukin-7 levels not sufficient to maintain T-cell homeostasis during simian immunodeficiency virus-induced disease progression. Blood. 2004;103:973-979.
173. Juffroy O, Bugault F, Lambotte O, et al. Dual mechanism of impairment of interleukin-7 (IL-7) responses in human immunodeficiency virus infection: decreased IL-7 binding and abnormal activation of the JAK/STAT5 pathway. J Virol;84:96-108.
174. Levy Y, Lacabaratz C, Weiss L, et al. Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. J Clin Invest. 2009;119:997-1007.
175. Sereti I, Dunham RM, Spritzler J, et al. IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. Blood. 2009;113:6304-6314.
176. Camargo JF, Kulkarni H, Agan BK, et al. Responsiveness of T Cells to Interleukin-7 Is Associated with Higher CD4(+) T Cell Counts in HIV-1-Positive Individuals with Highly Active Antiretroviral Therapy-Induced Viral Load Suppression. J Infect Dis. 2009;199:1872-1882.
177. Hepler PK, Palevitz BA. Metabolic inhibitors block anaphase A in vivo. J Cell Biol. 1986;102:1995-2005.
178. Gendelman HE, Orenstein JM, Martin MA, et al. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. J Exp Med. 1988;167:1428-1441.
179. HIV protocols. New Jersey: Humana Press; 1999.
180. Brussel A, Sonigo P. Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. J Virol. 2003;77:10119-10124.
181. Plum J, De Smedt M, Verhasselt B, et al. Human T lymphopoiesis. In vitro and in vivo study models. Ann N Y Acad Sci. 2000;917:724-731.
182. Res P, Spits H. Developmental stages in the human thymus. Semin Immunol. 1999;11:39-46.
183. Okamoto Y, Douek DC, McFarland RD, Koup RA. Effects of exogenous interleukin-7 on human thymus function. Blood. 2002;99:2851-2858.
184. Napolitano LA, Stoddart CA, Hanley MB, Wieder E, McCune JM. Effects of IL-7 on early human thymocyte progenitor cells in vitro and in SCID-hu Thy/Liv mice. J Immunol. 2003;171:645-654.
185. Guillemard E, Nugeyre MT, Chene L, et al. Interleukin-7 and infection itself by human immunodeficiency virus 1 favor virus persistence in mature CD4(+)CD8(-)CD3(+) thymocytes through sustained induction of Bcl-2. Blood. 2001;98:2166-2174.
186. Itoh T, Nakamura M, Yagi H, et al. Establishment of a mouse thymic epithelial cell line, IT-76MHC and a brief review on cultured thymic epithelial cells. Cell Mol Biol (Noisy-le-grand). 2001;47:1-18.

187. Braun J, Valentin H, Nugeyre MT, Ohayon H, Gounon P, Barre-Sinoussi F. Productive and persistent infection of human thymic epithelial cells in vitro with HIV-1. Virology. 1996;225:413-418.
188. Ropke C. Thymic epithelial cell culture. Microsc Res Tech. 1997;38:276-286.
189. Marino JH, Tan C, Taylor AA, et al. Differential IL-7 responses in developing human thymocytes. Hum Immunol. 2010;71:329-333.
190. Kim K, Lee CK, Sayers TJ, Muegge K, Durum SK. The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. J Immunol. 1998;160:5735-5741.
191. von Freeden-Jeffry U, Solvason N, Howard M, Murray R. The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. Immunity. 1997;7:147-154.
192. Crawley AM, Katz T, Parato K, Angel JB. IL-2 receptor gamma chain cytokines differentially regulate human CD8+CD127+ and CD8+CD127- T cell division and susceptibility to apoptosis. Int Immunol. 2009;21:29-42.
193. Gu XX, Yue FY, Kovacs CM, Ostrowski MA. The Role of Cytokines which Signal through the Common gamma Chain Cytokine Receptor in the Reversal of HIV Specific CD4 and CD8 T Cell Anergy. PLoS ONE. 2007;2:e300.
194. Biasin M, Boasso A, Piacentini L, et al. IL-4 and CXCR4 upregulation in HIV-infected and uninfected individuals from Maharashtra-Mumbai. Aids. 2003;17:1563-1565.
195. Wofford JA, Wieman HL, Jacobs SR, Zhao Y, Rathmell JC. IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T-cell survival. Blood. 2008;111:2101-2111.
196. Swainson L, Kinet S, Mongellaz C, Sourisseau M, Henriques T, Taylor N. IL-7-induced proliferation of recent thymic emigrants requires activation of the PI3K pathway. Blood. 2007;109:1034-1042.
197. Van De Wiele CJ, Marino JH, Murray BW, Vo SS, Whetsell ME, Teague TK. Thymocytes between the beta-selection and positive selection checkpoints are nonresponsive to IL-7 as assessed by STAT-5 phosphorylation. J Immunol. 2004;172:4235-4244.
198. Fry TJ, Moniuszko M, Creekmore S, et al. IL-7 therapy dramatically alters peripheral T-cell homeostasis in normal and SIV-infected nonhuman primates. Blood. 2003;101:2294-2299.
199. Reddy MM, Sorrell SJ, Lange M, Grieco MH. Tumor necrosis factor and HIV P24 antigen levels in serum of HIV-infected populations. J Acquir Immune Defic Syndr. 1988;1:436-440.
200. Baseta JG, Stutman O. TNF regulates thymocyte production by apoptosis and proliferation of the triple negative (CD3-CD4-CD8-) subset. J Immunol. 2000;165:5621-5630.
201. von Sydow M, Sonnerborg A, Gaines H, Strannegard O. Interferon-alpha and tumor necrosis factor-alpha in serum of patients in various stages of HIV-1 infection. AIDS Res Hum Retroviruses. 1991;7:375-380.

202. Perno CF, Yarchoan R, Cooney DA, et al. Replication of human immunodeficiency virus in monocytes. Granulocyte/macrophage colony-stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by 3'-azido-2'3'-dideoxythymidine (AZT) and other dideoxynucleoside congeners of thymidine. J Exp Med. 1989;169:933-951.
203. Yasuda Y, Kaneko A, Nishijima I, Miyatake S, Arai K. Interleukin-7 inhibits pre-T-cell differentiation induced by the pre-T-cell receptor signal and the effect is mimicked by hGM-CSF in hGM-CSF receptor transgenic mice. Immunology. 2002;106:212-221.
204. Varma C, Chantry D, Brennan F, Turner M, Katz F, Feldmann M. Interleukin 7 and interleukin 4 stimulate human thymocyte growth through distinct mechanisms. Cytokine. 1990;2:55-59.
205. Mackall CL, Fleisher TA, Brown MR, et al. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. N Engl J Med. 1995;332:143-149.
206. Min D, Taylor PA, Panoskaltsis-Mortari A, et al. Protection from thymic epithelial cell injury by keratinocyte growth factor: a new approach to improve thymic and peripheral T-cell reconstitution after bone marrow transplantation. Blood. 2002;99:4592-4600.
207. Tan JT, Dudl E, LeRoy E, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc Natl Acad Sci U S A. 2001;98:8732-8737.
208. Farge D, Henegar C, Carmagnat M, et al. Analysis of immune reconstitution after autologous bone marrow transplantation in systemic sclerosis. Arthritis Rheum. 2005;52:1555-1563.
209. Haynes BF, Hale LP, Weinhold KJ, et al. Analysis of the adult thymus in reconstitution of T lymphocytes in HIV-1 infection. J Clin Invest. 1999;103:453-460.
210. Hazra R, Mackall C. Thymic Function in HIV Infection. Curr HIV/AIDS Rep. 2005;2:24-28.
211. Ruiz-Mateos E, de la Rosa R, Franco JM, et al. Endogenous IL-7 is associated with increased thymic volume in adult HIV-infected patients under highly active antiretroviral therapy. Aids. 2003;17:947-954.
212. Yu Q, Park JH, Doan LL, Erman B, Feigenbaum L, Singer A. Cytokine signal transduction is suppressed in preselection double-positive thymocytes and restored by positive selection. J Exp Med. 2006;203:165-175.
213. Pongracz J, Hare K, Harman B, Anderson G, Jenkinson EJ. Thymic epithelial cells provide WNT signals to developing thymocytes. Eur J Immunol. 2003;33:1949-1956.
214. Muller KP, Kyewski BA. T cell receptor targeting to thymic cortical epithelial cells in vivo induces survival, activation and differentiation of immature thymocytes. Eur J Immunol. 1993;23:1661-1670.
215. Munoz-Blay T, Nieburgs AC, Cohen S. Thymic epithelium in vitro. V. Binding of thymocytes to cultured thymic epithelial cells. Cell Immunol. 1987;109:371-383.
216. Singer KH, Wolf LS, Lobach DF, et al. Human thymocytes bind to autologous and allogeneic thymic epithelial cells in vitro. Proc Natl Acad Sci U S A. 1986;83:6588-6592.

217. Wakkach A, Chastre E, Bruand C, et al. Phenotypic and functional characterization of human thymic stromal cell lines. Cell Mol Biol (Noisy-le-grand). 2001;47:167-178.
218. Yarilin AA, Belyakov IM. Cytokines in the thymus: production and biological effects. Curr Med Chem. 2004;11:447-464.
219. Savino W, Bartoccioni E, Homo-Delarche F, Gagnerault MC, Itoh T, Dardenne M. Thymic hormone containing cells--IX. Steroids in vitro modulate thymulin secretion by human and murine thymic epithelial cells. J Steroid Biochem. 1988;30:479-484.
220. Wang H, Pierce LJ, Spangrude GJ. Distinct roles of IL-7 and stem cell factor in the OP9-DL1 T-cell differentiation culture system. Exp Hematol. 2006;34:1730-1740.
221. Jehn BM, Bielke W, Pear WS, Osborne BA. Cutting edge: protective effects of notch-1 on TCR-induced apoptosis. J Immunol. 1999;162:635-638.
222. Castro A, Sengupta TK, Ruiz DC, Yang E, Ivashkiv LB. IL-4 selectively inhibits IL-2-triggered Stat5 activation, but not proliferation, in human T cells. J Immunol. 1999;162:1261-1269.
223. Jang J, Choi YI, Choi J, et al. Notch1 confers thymocytes a resistance to GC-induced apoptosis through Deltex1 by blocking the recruitment of p300 to the SRG3 promoter. Cell Death Differ. 2006;13:1495-1505.
224. Choi YI, Jeon SH, Jang J, et al. Notch1 confers a resistance to glucocorticoid-induced apoptosis on developing thymocytes by down-regulating SRG3 expression. Proc Natl Acad Sci U S A. 2001;98:10267-10272.
225. Schmitt TM, Zuniga-Pflucker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity. 2002;17:749-756.
226. Crawley AM. Interleukin-4 downregulates CD127 expression and activity on human thymocytes and mature CD8+ T-cells. Eur J Immunol. in press.
227. Honda M, Yamamoto S, Cheng M, et al. Human soluble IL-6 receptor: its detection and enhanced release by HIV infection. J Immunol. 1992;148:2175-2180.
228. Yu Q, Xu M, Sen JM. Beta-catenin expression enhances IL-7 receptor signaling in thymocytes during positive selection. J Immunol. 2007;179:126-131.
229. Garcia-Peydro M, de Yebenes VG, Toribio ML. Notch1 and IL-7 receptor interplay maintains proliferation of human thymic progenitors while suppressing non-T cell fates. J Immunol. 2006;177:3711-3720.
230. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell. 1993;75:229-240.
231. Reed JC. Double identity for proteins of the Bcl-2 family. Nature. 1997;387:773-776.
232. Deftos ML, He YW, Ojala EW, Bevan MJ. Correlating notch signaling with thymocyte maturation. Immunity. 1998;9:777-786.
233. Magri M, Yatim A, Benne C, et al. Notch ligands potentiate IL-7-driven proliferation and survival of human thymocyte precursors. Eur J Immunol. 2009;39:1231-1240.

234. Henriques CM, Rino J, Nibbs RJ, Graham GJ, Barata JT. IL-7 induces rapid clathrin-mediated internalization and JAK3-dependent degradation of IL-7Ralpha in T cells. Blood. 2010;115:3269-3277.
235. Kumaki S, Armitage R, Ahdieh M, Park L, Cosman D. Interleukin-15 up-regulates interleukin-2 receptor alpha chain but down-regulates its own high-affinity binding sites on human T and B cells. Eur J Immunol. 1996;26:1235-1239.
236. Murphy M, Pike-Nobile L, Soo VW, Epstein LB. Characterization of TNF receptors on human thymocytes. Thymus. 1994;23:177-194.
237. Chatzidakis I, Mamalaki C. T cells as sources and targets of TNF: implications for immunity and autoimmunity. Curr Dir Autoimmun;11:105-118.
238. Lin Q, Dong C, Cooper MD. Impairment of T and B cell development by treatment with a type I interferon. J Exp Med. 1998;187:79-87.
239. Wang J, Lin Q, Langston H, Cooper MD. Resident bone marrow macrophages produce type 1 interferons that can selectively inhibit interleukin-7-driven growth of B lineage cells. Immunity. 1995;3:475-484.
240. Unutmaz D, KewalRamani VN, Marmon S, Littman DR. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J Exp Med. 1999;189:1735-1746.
241. Ueno Y, Boone T, Uittenbogaart CH. Selective stimulation of human thymocyte subpopulations by recombinant IL-4 and IL-3. Cell Immunol. 1989;118:382-393.
242. Plum J, De Smedt M, Leclercq G, Tison B. Inhibitory effect of murine recombinant IL-4 on thymocyte development in fetal thymus organ cultures. J Immunol. 1990;145:1066-1073.
243. Tepper RI, Levinson DA, Stanger BZ, Campos-Torres J, Abbas AK, Leder P. IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. Cell. 1990;62:457-467.
244. Ye P, Kourtis AP, Kirschner DE. The effects of different HIV type 1 strains on human thymic function. AIDS Res Hum Retroviruses. 2002;18:1239-1251.
245. Uittenbogaart CH, Anisman DJ, Jamieson BD, et al. Differential tropism of HIV-1 isolates for distinct thymocyte subsets in vitro. Aids. 1996;10:F9-16.
246. Choudhary SK, Choudhary NR, Kimbrell KC, et al. R5 human immunodeficiency virus type 1 infection of fetal thymic organ culture induces cytokine and CCR5 expression. J Virol. 2005;79:458-471.
247. Komsic-Vranjkovic A MPaAJ. Differential regulation of IL-7 receptor (CD127) on CD8+ T-cells: implications for HIV immunopathogenesis (Abstract). J Can J Infect Dis Med Microbiol 2005;16:SA.
248. Vudattu NK, Magalhaes I, Schmidt M, Seyfert-Margolis V, Maeurer MJ. Reduced numbers of IL-7 receptor (CD127) expressing immune cells and IL-7-signaling defects in peripheral blood from patients with breast cancer. Int J Cancer. 2007;121:1512-1519.
249. Petrovas C, Mueller YM, Dimitriou ID, et al. HIV-specific CD8+ T cells exhibit markedly reduced levels of Bcl-2 and Bcl-xL. J Immunol. 2004;172:4444-4453.