# PROTEOMIC ANALYSIS OF IL-7 INDUCED SIGNALING EFFECTORS INVOLVED IN LYMPHOMA DEVELOPMENT

by

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## ABSTRACT

IL-7 is a cytokine that plays a central role in the development, survival and proliferation of T and B cell lymphocytes. Overexpression of IL-7 in mice (Tg IL-7) leads to both increased proliferation of early T and B cell progenitors and T lymphomas. Genetic evidence indicates that known IL-7 receptor (IL-7R)-dependent proteins, including pro-survival protein Bcl-2, may not be solely responsible for the effects of IL-7. Earlier studies found that known IL-7-induced signaling proteins dock to a specific tyrosine (Y449) residue on the IL-7R. We have previously shown in a IL-7R$\alpha^{449F}$ knock-in model that IL-7-induced lymphomas require Y449 phosphorylation and loss of this phosphorylation confers protection from disease. However, the mechanism by which this lymphoma protection occurs remains unclear. Using this genetic model, we aimed to identify novel signaling effectors important for IL-7-mediated lymphocyte development and lymphomagenesis. An iTRAQ proteomic analysis was performed comparing $CD4^-$ $CD8^-$ double negative T cell progenitors from mice overexpressing IL-7 (Tg IL-7) (lymphoma prone) to Tg IL-7 mice with a mutated IL-7 receptor (Tg IL-7//IL-7R$\alpha^{449F}$) (lymphoma protected). Several proteins involved in survival/proliferation and apoptosis as well as cytoskeleton regulation were found to be differentially expressed between the two samples. Three proteins of particular interest, Gimap4, Bit1 and FKBP 51 were validated by immunoblot analysis and are being further characterized regarding their involvement in IL-7 signaling pathways and lymphomagenesis. These proteins have not been previously implicated with IL-7 and as such may represent novel targets for preventing or treating lymphoma development.

## TABLE OF CONTENTS

**ABSTRACT** ..... ii
**TABLE OF CONTENTS** ..... iii
**LIST OF TABLES** ..... iv
**LIST OF FIGURES** ..... v
**ABREVIATIONS** ..... vi
**ACKNOWLEDGEMENTS** ..... vii
**CO-AUTHORSHIP STATEMENT** ..... viii
**CHAPTER 1: Introduction** ..... 1
1.1 The cytokine IL-7 and the immune system ..... 1
1.2 IL-7 and lymphoma ..... 2
1.3 IL-7 signaling pathways ..... 3
1.4 Hypothesis and experimental approach ..... 6
REFERENCES ..... 8
**CHAPTER 2: Proteomics analysis of IL-7 induced signaling effectors** ..... 11
2.1 Introduction ..... 11
2.2 Experimental procedures ..... 15
2.3 Results ..... 19
2.4 Discussion ..... 23
REFERENCES ..... 36
**CHAPTER 3: General discussion** ..... 40
3.1 Analysis of experimental approach ..... 40
3.2 Gene ontology analysis of biological process and molecular function ..... 43
3.3 Identification and validation of survival/apoptosis proteins ..... 45
3.4 Future directions ..... 47
3.5 Candidate screening for other IL-7-induced survival effectors ..... 48
3.6 Conclusion ..... 49
REFERENCES ..... 54
**APPENDIX** ..... 56
UBC animal care acertificate ..... 57
UBC biohazard approval certificate ..... 58

## LIST OF TABLES

**CHAPTER 2 - Proteomics analysis of IL-7 induced signaling effectors**

Table 2.1 Proteins differentially expressed in Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes . . . **30**

**CHAPTER 3 - General discussion**

Table 3.1 Cytoskeleton proteins differentially expressed in Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes . . . . . **51**

## LIST OF FIGURES

**CHAPTER 2 - Proteomics analysis of IL-7 induced signaling effectors**

Figure 2.1 Purification of αβ DN T cell progenitors . . . . . **31**

Figure 2.2 Overview of experimental design . . . . . **32**

Figure 2.3 Protein categorization by gene ontology . . . . . **33**

Figure 2.4 Validation of Gimap4, Bit1 and FKBP51 . . . . . **35**

**CHAPTER 3 - General discussion**

Figure 3.1 Validation of Arp2/3 . . . . . **52**

Figure 3.2 Mcl-1 regulation by IL-7Rα Y449 following acute IL-7 stimulation . . **52**

Figure 3.3 Mcl-1 and Noxa regulation by IL-7Rα Y449 in a steady state setting . . **53**

## ABBREVIATIONS

DAG- directed acyclic graph
DN- $CD4^-$ $CD8^-$ double negative
IL- interleukin
IL-7R- interluekin-7 receptor
iTRAQ- isobaric tags for relative and absolute quantitation
Lin- lineage
MACS- magnetic cell sorting
Tg- transgenic

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## CO-AUTHORSHIP STATEMENT

This thesis is submitted in manuscript format and included contributions from co-authors who I would like to acknowledge here. For Chapter's 1, 2 and 3 I personally conducted all experimental procedures regarding my research project and am responsible for all experimental results, data analysis and manuscript composition unless otherwise indicated below. The following individuals contributed to Chapter 2 as indicated:

-**L.C. Osborne** assisted with DN lysate collections and manuscript feedback.
-**L.J. Foster** assisted with gene ontology analysis and manuscript revisions.
-**N. Abraham** edited this manuscript.

# CHAPTER 1: Introduction

## 1.1 The cytokine IL-7 and the immune system

A properly functioning immune system relies on a delicate system of balance and regulation at the levels of lymphocyte development and lymphocyte survival. Cytokines play a central role in the development, survival and proliferation of lymphocyte lineages. One of these cytokines, interleukin (IL)-7, is of particular interest as defects in IL-7 production or its overexpression can lead to either immunodeficiency or lymphomas, respectively.

IL-7 is a critical growth factor essential for lymphocyte development and survival (1, 2). Loss of IL-7 results in T and B cell immunodeficiency in mice and T cell immunodeficiency in humans (2, 3). The development of both T and B cells are also severely impaired in IL-7 receptor (IL-7R) knock-out mice, which show an early block in T cell development at the $CD4^-CD8^-$ double negative (DN) T cell progenitor stage (4). Conversely, addition of IL-7 leads to increased proliferation of early T and B cell progenitors in mice (5, 6). Indeed, excessive IL-7 signaling has been implicated in a number of human cancers such as T-cell acute lymphoblastic leukemia (T-ALL), Hodgkin's disease and Burkitt's lymphoma (7-13).

Leukemia and lymphomas are the two major cancers of the immune system. Leukemia is cancer of white blood cells originating in the blood, causing abnormally large numbers of lymphocytes to form in the bloodstream or bone marrow (14). Lymphomas, on the other hand, are malignant neoplasms which originate in lymph nodes or lymphoid tissue and are divided into two groups: Hodgkins disease and non-Hodgkin's lymphoma (14). Hodgkin's disease and non-Hodgkin's lymphomas are differentiated by

distinct characteristics in the microscopic appearance of the cancerous cells (15). In 2007, there were 7,000 new cases of non-Hodgkin's lymphoma, 4,500 new cases of leukemia and 890 new cases of Hodgkin's disease in Canada alone (16). In addition, leukemia and lymphoma are the leading cause of death from disease in children. Although there is increasing evidence of IL-7's involvement in immune system cancers, the exact mechanism by which IL-7 is involved in these various forms of cancer remains unclear.

## 1.2 IL-7 and lymphoma

In order to determine the role of IL-7 signaling in tumorigenesis, we study a transgenic mouse model that overexpresses IL-7 (Tg IL-7). The IL-7 transgene is under control of the heavy chain Ig enhancer and promoter with a deletion of a critical Ig intron, resulting in IL-7 being produced in an autocrine manner by both B and T cells (17). IL-7 overexpression results in a few different phenotypes, including progressive alopecia from infiltrating lymphcytes in the skin and generalized lymphoproliferation including both splenalomegaly and lymphadenopathy (17). Thymocyte development is also slightly altered in these mice, with a decrease in double positive (DP) thymocytes and a skewing towards CD8 single positive (SP) thymocytes. The increased T cell proliferation results in aggressive tumor formation and almost 100% mortality by seven months of age as a result of T cell lymphomas (Osborne *et al.*, manuscript in revision). These tumors are mainly thymomas arising from either CD8 SP or CD4/CD8 DP thymocytes (Osborne *et al.*, manuscript in revision). The above data indicates that excessive IL-7 signaling *in vivo* is sufficient to induce the generation of immune cell tumors. It remains to be determined, however, which components of the IL-7 signaling pathway are responsible for this deregulated cellular growth and development in the presence of IL-7 overexpression.

## 1.3 IL-7 signaling pathways

IL-7 is produced by stromal cells found mainly in lymphoid tissues and to a lesser degree in the intestinal epithelium and skin (11, 18, 19). Although the cells responsible for producing IL-7 have yet to be fully characterized, initial studies looking at the nature of IL-7 production found that IL-7 mRNA transcripts are generated at a constant, constitutive level (18, 20). Signaling pathways are induced when IL-7 binds to the IL-7R, a heterodimer composed of the IL-7Rα chain (shared with the cytokine TSLP) and the common γ chain ($\gamma_c$) (shared with IL-2,-4,-9,-15,-21) (19). The two chains are expressed independently on the cell surface and the $\gamma_c$ chain is recruited to the IL-7Rα chain upon IL-7 binding (19). As the IL-7Rα chain does not contain any intrinsic kinase activity, it relies on the activity of non-receptor tyrosine kinases, namely Jak1 (associated with the IL-7Rα chain) and Jak3 (associated with the $\gamma_c$ chain) (19). The IL-7Rα chain has four tyrosine residues located in the cytoplasmic domain: Y390, Y401, Y449 and Y456, but a number of studies clearly demonstrate that Y449 is the main phosphorylation target of the associated tyrosine kinases upon IL-7 ligand binding (21, 22). Once phosphorylated, this Y449XXM motif acts a docking site for two critical signaling pathways, Signal Transducer and Activator of Transcription 5 (STAT5) and potentially phosphatidylinositol -3 kinase (PI3 kinase) (21, 23, 24).

STAT5 is a member of the STAT family of trancription factors and is found in two isoforms, STAT5a and STAT5b. Upon docking to IL-7Rα Y449 and being phosphorylated, STAT5a and STAT5b dimerize and translocate into the nucleus to induce transcription of its target genes, many of which remain unknown. It is thought that one of the primary targets up-regulated by STAT5 is the anti-apoptotic protein Bcl-2 (25,

26), however recent evidence indicates that Bcl-2 induction can occur through STAT5 independent pathways (24). While it has been shown that overexpressing Bcl-2 in IL-7R$^{-/-}$ mice is able to rescue T cell development, a functional T cell compartment is only partially restored, indicating Bcl-2 is incompletely sufficient for normal T cell development to occur (27, 28). Other research has shown overexpressing Bcl-2 in STAT5$^{-/-}$ mice does not complement the striking defects in hematopoietic stem cells and progenitor cells observed in STAT5 deficient mice (29). These results combined provide strong evidence that Bcl-2 is not the sole survival regulator induced by IL-7, implying IL-7 is acting through other unidentified mechanisms.

PI3 kinase has been shown to activate the downstream signaling proteins Akt and S6 kinase (19). Akt triggers a number of pro-survival and growth functions, including inactivation of pro-apoptotic proteins Bad and Bim and the cell cycle inhibitor p27$^{kip1}$ (19). While some studies suggest IL-7 is capable of activating the PI3 kinase/Akt signaling pathway, the majority of this work has been done in transformed cell lines and human thymocytes (10, 30). One group found that mice with a deficiency in the p110$\gamma$ catalytic subunit of PI3 kinase showed a slight deficiency in thymic cellularity, however the T cell population that is especially dependent on IL-7 (the DN development stage) was unaffected in cellularity (31). In addition, we have not thus far been able to detect immediate early PI3 kinase activation by stimulating murine thymocytes *ex vivo* with IL-7 and looking for activated Akt and S6 kinase (Osborne, L., unpublished data). This suggests that PI3 kinase is not a major survival signal in early T cell development and thus other unknown effectors may be responsible for IL-7's survival and proliferation effects.

The IL-7Rα chain can also combine with the thymic stromal lymphopoietin receptor chain (TSLPR) for signaling by the cytokine TSLP. While TSLP has been found to play a significant role in airway epithelium and allergic inflammation in humans (32, 33), T cell development and cellularity is unimpaired in TSLPR KO mice suggesting that the majority of IL-7Rα chain KO effects in the T cell lineage are a result of loss of IL-7 signaling (34, 35). CD4+ T cells from these mice, however, are unable to expand as efficiently in an irradiated host as those from WT mice, indicating this population may require TSLP signaling for normal development (34). In addition $\gamma_c$ chain/TSLPR double KO mice present with a more severe lymphoid defect then $\gamma_c$ chain KO mice further suggesting TSLP is responsible for some degree of lymphoid development (34). It was recently shown that increasing TSLP levels in IL-7 deficient mice rescued B cell development and partially restored thymocyte and splenocyte numbers (36). This suggests that T and B cell progenitors are TSLP responsive and that this cytokine may be playing a bigger role in effects attributed to IL-7 then was previously thought. The contribution of TSLP in our mouse models is currently being further investigated.

Initial studies of IL-7 downstream signaling proteins found that Bcl-2 is incompletely sufficient for thymocyte development and PI3 kinase may not play a major role in early T cell development. Taken together, this evidence suggests that previously undescribed signaling effectors downstream of IL-7 may be involved.

### 1.4 Hypothesis and experimental approach

To assess the role of IL-7-induced signaling effectors, we have previously generated a knock-in mouse model where the Y449 residue on IL-7Rα is substituted with a phenylalanine (IL-7Rα$^{449F}$). Loss of phosphorylated IL-7Rα Y449 completely

abrogates all downstream signaling pathways dependent on the phosphorylated Y449 residue for activation (24). Interestingly, in contrast to IL-7R$^{-/-}$ mice, IL-7R$\alpha^{449F}$ lymphocytes bypass a developmental defect at an early stage in thymopoiesis such that lymphocyte development in these mice is largely unimpaired, albeit with reduced cellularity (24). To determine the involvement of Y449-dependent signaling pathways in lymphoma development, we crossed IL-7R$\alpha^{449F}$ mice with Tg IL7 mice (Tg IL-7/IL-7R$\alpha^{449F}$). Strikingly, IL-7R$\alpha^{449F}$ mice overexpressing IL-7 are completely disease free over their whole lifespan, in marked contrast to Tg IL-7 mice with wild-type IL-7Rα (Osborne et al., manuscript in preparation). This indicated that IL-7-induced lymphoma development is entirely dependent on phosphorylation of Y449 in the IL-7Rα chain and the effectors recruited to this site. To test this hypothesis, a proteomic analysis was performed using iTRAQ to identify quantitative differences in proteins from Tg IL-7 and Tg IL-7/IL-7R$^{449F}$ CD4$^-$CD8$^-$ double negative T cell progenitors. Several proteins involved in survival/proliferation and apoptosis were found to be quantitatively different between the two sample sets and three proteins of particular interest, Gimap4, Bit1 and FKBP51 were validated by western blot analysis. This proteomic data was also analyzed using bioinformatics software to discern larger trends in identified protein differences between these two mouse strains. Identifying unknown survival effectors downstream of IL-7 could lead to both a better understanding of the biology of lymphomagenesis as well as the identification of novel therapeutic targets to treat or prevent lymphoma development.

**Overall Hypothesis:**

IL-7Rα Y449 is required for signaling through previously unidentified pro-survival factors that are important for IL-7 to support lymphocyte development and lymphomagenesis.

**Objective: Identify the signals that are altered in IL-7Rα$^{449F}$ DN T cell progenitors that confer protection from IL-7-mediated lymphomas.**

**Specific Aims:**

**Aim 1.1**: Purify sufficient amounts of DN protein (150 μg) from both Tg IL-7 and Tg IL-7/IL-7R$^{449F}$ mice for iTRAQ analysis.

**Aim 1.2**: Perform gene ontology analysis on proteins identified from iTRAQ analysis comparing total proteins to Tg IL-7/IL-7Rα$^{449F}$ up- and downregulated proteins.

**Aim 1.3**: Identify proteins with putative roles in survival, proliferation or apoptosis that quantitatively differ between Tg IL-7 and Tg IL-7/IL-7Rα$^{449F}$ DN's.

**Aim 1.4**: Validate proteins of interest by immunoblot analysis.

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## CHAPTER 2: Proteomics analysis of IL-7 induced signaling effectors[1]

### 2.1 Introduction

A properly functioning immune system relies on a delicate system of balance and regulation at both the level of lymphocyte development and lymphocyte survival. Cytokines play a central role in the development, survival and proliferation of lymphocyte lineages. One of these cytokines, interleukin (IL)-7, is of particular interest as defects in IL-7 production or its overexpression can lead to either immunodeficiency or lymphomas, respectively.

IL-7 is a growth factor essential for lymphocyte development and survival (1, 2). IL-7 deficiency results in T and B cell immunodeficiency in mice and T cell immunodeficiency in humans (2, 3). Mice lacking IL-7 are strikingly lymphopenic, with thymic cellularity reduced twenty-fold (2). The development of both T and B cells are also severely impaired in IL-7 receptor (IL-7R) knock-out mice, which show an early block in T cell development at the $CD4^{-}CD8^{-}$ (double negative, DN) T cell progenitor stage (4). Conversely, *in vitro* addition of IL-7 leads to increased proliferation of early T and B cell progenitors in mice (5, 6). Indeed, excessive IL-7 signaling has been implicated in a number of human cancers such as T-cell acute lymphoblastic leukemia (T-ALL), Hodgkin's disease and Burkitt's lymphoma (7-13), as well as autoimmune

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pathology (14) although the exact mechanism by which IL-7 is involved remains unclear.

In order to determine the role of IL-7 signaling in tumorigenesis, we study a transgenic mouse model that overexpresses IL-7 (Tg IL-7). These mice present with increased T and B cell proliferation, resulting in aggressive tumor formation and almost 100% mortality by seven months of age as a result of T and B cell lymphomas (15). This indicates that excessive IL-7 signaling *in vivo* is sufficient to induce the generation of immune cell tumors. It remains unclear, however, which components of the IL-7 signaling pathway are responsible for this deregulated cellular growth and development in the presence of IL-7 overexpression. Gaining a better understanding of the molecular processes driving lymphoma development in Tg IL-7 mice could provide critical insight into the biology of lymphomas and thus allow for the development of much needed novel therapeutic strategies.

IL-7 signaling pathways are induced when IL-7 binds to the IL-7R, a heterodimer composed of the IL-7Rα chain and the common γ chain ($\gamma_c$) chain (reviewed in (16)). A number of studies clearly demonstrate that tyrosine 449 (Y449) on the IL-7Rα chain is the main phosphorylation target of the associated tyrosine kinases upon ligand binding (17, 18). Once phosphorylated, this Y449XXM motif may act as a docking site for both Signal Transducer and Activator of Transcription 5 (STAT5) and phosphatidylinositol -3 kinase (PI3 kinase) (17, 19).

One of the primary targets up-regulated by STAT5 is the well known anti-apoptotic protein Bcl-2 (20). PI3 kinase is activated upon binding to phosphorylated Y449 and consequently activates the downstream signaling protein Akt (16). Akt itself triggers a number of pro-survival and growth functions, including inactivation of pro-

apoptotic proteins Bad and Bim and the cell cycle inhibitor $p27^{kip1}$ (16). Initial studies to determine which of the known downstream signaling proteins mediate IL-7 function revealed that deficiencies in either STAT5, PI3 kinase, Bcl-2 or Akt does not phenocopy the severe defects in early T and B cell development observed in IL-7 or IL-7Rα knockout mice (21) (22-25). In addition, overexpression of Bcl-2 in IL-7Rα knockout mice only partially complements thymocyte cellularity (26, 27). These data suggest that these known effectors are not necessary for IL-7 function, that Bcl-2 is incompletely sufficient and that novel signaling effectors downstream of IL-7 may be involved. Given these unknowns, alternative global approaches are required to gain a better understanding of IL-7 signaling pathways.

To assess the role of IL-7-induced signaling effectors, we generated a mouse model where the Y449 residue on IL-7Rα is substituted with a phenylalanine (IL-$7R\alpha^{449F}$). Loss of phosphorylated IL-7Rα Y449 completely abrogates all downstream signaling pathways dependent on the phosphorylated Y449 residue for activation (28). Interestingly, IL-$7R\alpha^{449F}$ lymphocytes bypass a developmental defect at an early stage in thymopoiesis seen in IL-7R knock-out mice, such that lymphocyte development is largely unimpaired (28). To determine the involvement of Y449-dependent signaling pathways in lymphoma development, we crossed IL-$7R\alpha^{449F}$ mice with Tg IL-7 mice (Tg IL-7/IL-$7R\alpha^{449F}$). Strikingly, IL-$7R\alpha^{449F}$ mice overexpressing IL-7 are completely disease free over their whole lifespan, in marked contrast to Tg IL-7 mice with wild-type IL-7Rα that succumb to lymphomas at an early age[1]. This indicated that IL-7-induced lymphoma development is entirely dependent on phosphorylation of Y449 in the IL-7Rα chain and the effectors recruited to this site. Accordingly, we formulated the hypothesis that IL-7Rα

Y449 is required for signaling through previously unidentified pro-survival factors that are important for IL-7 to support lymphocyte development and lymphomagenesis.

To test this hypothesis, a proteomic analysis was performed using isobaric tags for relative and absolute quantitation (iTRAQ) to identify qualitative and quantitative differences in proteins from Tg IL-7 and Tg IL-7/IL-7R$\alpha^{449F}$ $CD4^-CD8^-$ DN T cell progenitors from young, disease-free mice. iTRAQ technology is a major advancement in the study of quantitative gene expression at the proteome level. It is a stable isotope peptide tagging system where the primary amines of peptides are chemically tagged with up to four different tags, and then analyzed by tandem mass spectrometry. This allows simultaneous identification and quantification of proteins from different samples in a single mass spectrometry run. Furthermore, as all tryptic peptides are labeled rather than just cysteine-containing peptides as with older ICAT technology, iTRAQ reagents allow for expanded proteome coverage and increased confidence in protein identification.

DN thymocytes were examined as they are the precursor of mature T cells and as such are most likely to develop T cell lymphoma-forming mutations to generate cancer stem cells (29-32). Due to limited sample and a desire to remain completely unbiased these analyses were performed on whole-cell lysate.

Several proteins involved in survival/proliferation and apoptosis were found to be quantitatively different between the two sample sets and three proteins of particular interest, Gimap4, Bit1 and FKBP51 were validated by western blot analysis. Identifying unknown survival effectors downstream of IL-7 could lead to both a better understanding of the biology of lymphomagenesis as well as the identification of novel therapeutic targets to treat and/or prevent lymphoma development.

## 2.2 Experimental procedures

**Mice:** Animals were housed at the University of British Columbia, Microbiology and Immunology department animal facility in accordance with University of British Columbia Animal Care and Biosafety Committee certificates. Tg IL-7 mice (Tg IL-7 mice) on a FVBN background were obtained from Dr. Philip Leder (15) and backcrossed for 20 generations to C57BL/6 mice (Jackson laboratories, Bar Harbor, ME). IL-7R$\alpha^{449F}$ mice were generated, backcrossed 7 generations to C57BL/6 and then crossed with Tg IL-7 mice to create Tg IL-7/IL-7R$\alpha^{449F}$ mice. Pre-lymphoma mice of 7.5 weeks of age were used to avoid frank lymphomas.

**DN Thymocyte Enrichment**: Thymi were harvested and prepared as single cell suspensions. Individual sample aliquots were stained and assessed by flow cytometry for the absence of frank lymphomas as determined by clonal expansion of thymocyte populations. The remaining cells were labeled with αCD8 and αCD4 autoMACS magnetic beads (Miltenyi Biotec, Auburn, CA). The labeled thymocytes were run through the "DepleteS" program on an autoMACS automated magnetic cell sorter and the negative (unlabeled) fraction collected. The CD4/CD8 depleted thymocytes were then stained for surface markers CD4 and CD8 and lineage (lin) cocktail (B220, Gr-1, Ter119 and γδ T cells) and sorted by flow cytometry. The $Lin^-CD4^-CD8^-$ (DN) thymocyte fraction was collected and lysed in cold NP-40 lysis buffer (1X PBS, 1% NP-40 and 1X proteinase inhibitor cocktail (Calbiochem)). Total cell lysates were pre-cleared by centrifugation (13,000xg) and the supernatant stored at -80°C and later pooled.

**Protein quantification**: Protein concentration was determined using the Pierce BCA protein assay and spectrometry readings were taken at $A_{562}$ on a Hitachi U-2000 spectrophotomer.

**Antibodies:** FITC- and PE- directly conjugated antibodies were obtained from BD Biosciences (Mississauga, ON) (anti-CD4 (RM4-5), CD8α (53-6.7), TER119, B220 (RA3-6B2), γδ TCR, Gr-1 (RB6-8C5). Bit1 and FKBP51 antibodies were obtained from Imgenenx and Abcam, respectively, and Gimap4 antibody was generously supplied by Dr. Heinz Jacobs .

**Flow Cytometry**: Cell samples were sorted on a FACSAria cell sorter, analyzed on an LSRII flow cytometer (BD Biosciences, Mississauga, ON) and data analyzed with FlowJo software (Tree Star).

**Tandem Mass Spectrometry:** iTRAQ labeling and mass spectrometry was performed by the University of Victoria Genome BC Proteomics Centre. Samples were reduced, alkylated, trypsin digested and labeled using the iTRAQ Reagents Multiplex kit according to manufacturers instructions (Applied Biosystems, Foster City, CA). Each sample was labeled with a different isobaric tag (Tg IL-7 proteins with the 117 iTRAQ tag and Tg IL-7/IL-7Rα$^{449F}$ proteins with the 115 iTRAQ tag) and separated first by strong cation exchange high pressure liquid chromatography (HPLC) and then further fractionated using reverse phase microcapillary HPLC (33). The reverse phase microcapillary HPLC was coupled online to an electrospray ionization (ESI)-MS/MS mass spectrometer (API QStar Pulsar, Applied Biosystems) (33). All mass spectrometry runs were performed with the same parameters; detected protein threshold >1.30 (95.0%), methylmethanethiosulphate-modified cysteine as fixed modifications, and biological

modifications 'ID focus' settings. Parameters such as tryptic cleavage specificity, precursor ion mass accuracy and fragment ion mass accuracy are built-in functions of ProteinPilot software.

**Data Analysis:** QStar spectra were processed by ProteinPilot™ Software 1.0[2] (Applied Biosystems, Software Revision 6684), which searched measured versus theoretical fragment spectra from the International Protein Index (IPI) mouse protein database version 3.15 from the European Bioinformatics Institute (68248 entries) using the following criteria: trypsin cleavage specificity, methylmethanethiosulphate-modified cysteine as fixed modifications, and biological modifications 'ID focus' settings. The following criteria were required to consider a protein significant: 2 or more high confidence (>95%) unique peptides had to be identified, the protein identification had to have a $P<0.01$, and the fold difference had to be greater than 1.2. This software corrects all protein ratios and individual peptide ratios for bias as part of the software processing. It corrects for pipetting error when mixing different labeled samples as well as identifies the median average protein ratio and corrects it to unity. It also uses all data from a mass spectrometry run to calculate the bias correction factor for each ratio.

Error factor (EF) is a 95% confidence limit of the measurement error term of a given 117/115 ratio. Rather than report a plus/minus range for an average, which is inaccurate for ratios reported in the linear scale, the EF term indicates that the actual average value lies between (reported ratio)*(ER) and (reported ratio)/(ER) 95% of the time. Error factor is calculated by: Error factor = $10^{95\% \text{ Confidence Error}}$, where 95% Confidence Error = $S_{MW}$ x (Student t Factor for N-1 degrees of freedom). $S_{MW}$ is the

weighted standard deviation of the weighted average of log ratios. The error factor is blank when there is just one value, as standard deviation is undefined in this case.

The Unused protein score is ProteinPilot's measurement of protein identification confidence taking into account all peptide evidence for a protein, excluding any evidence that is better explained by a higher ranking protein. The Unused protein score depends on a number of factors, including where the protein is ranked in the list of detected proteins, what spectra the protein represents, and which of those spectra are already represented by higher ranked proteins.

Gene ontology analysis of identified proteins was performed using Blast2GO V.1.3.1 (34). Protein sequences were acquired using IPI accession numbers and gene ontology numbers were derived by performing a BLASTp search against the nr database with an expectation value maximum $1 \times 10^{-3}$ and an HSP length cutoff of 33. Protein sequences were then annotated according to the following parameters: a pre-eValue-Hit-Filter of $1 \times 10^{-6}$, a pro-Similarity-Hit-Filter of 15, an Annotation Cutoff of 55 and a GO Weight of 5. Directed acyclic graphs (DAG's) were then generated using a sequence filter of 5, a score alpha of 0.6 and a node score filter of 0.

**Western blotting**: DN thymocyte proteins were extracted in NP-40 lysis buffer (1X PBS, 1% NP-40 and 1X proteinase inhibitor cocktail), incubated on ice for 10 minutes and centrifuged to remove cellular debris. Samples were quantified and normalized for total protein level. Proteins were resolved by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Polyclonal antibodies were used for detection and visualized with Alexa Fluor 680 goat anti-rabbit/mouse IgG antibodies (Molecular Probes) on a LI-

COR Odyssey infra-red imager. Protein levels were quantified and normalized to total protein loaded by coomassie blue staining and LI-COR quantitation.

## 2.3 Results

### Purification of αβ DN Thymocytes:

Hematopoietic stem cells give rise to T cell progenitors in the thymus by initially differentiating into $CD4^-CD8^-$ (DN) thymocytes. To evaluate changes in signaling effector levels in Tg IL-7/IL-7R$\alpha^{449F}$ thymocyte progenitors that account for differences in functional and transformation outcomes, DN thymocytes were isolated from both Tg IL-7 and Tg IL-7/IL-7R$\alpha^{449F}$ thymi using sequential purification steps. First, more mature single positive and double positive thymocytes were removed by magnetic cell sorting (MACS) depletion of $CD4^+$ $CD8^+$ T cells (Fig 2.1). This crude DN population was then further purified by flow cytometry cell sorting to remove residual $CD4^+$ or $CD8^+$ cells along with B cells, granulocytes, γδ T cells and red blood cells (Fig 2.1).

Characterization of DN thymocyte populations from the two mouse strains showed that DN cellularity greatly differed between the Tg IL-7 and Tg IL-7/IL-7R$\alpha^{449F}$ mice. Tg IL-7 mice have decreased numbers of DN's as compared to wild type counterparts and unusually high proportions of lineage positive cells (B cells and granulocytes). Tg IL-7/IL-7R$\alpha^{449F}$ mice, on the other hand, have a very similar distribution of thymocyte populations to wild type mice, but have a two-fold increase in overall thymocyte numbers. Figure 2.1 shows representative flow cytometry analysis plots of samples at various steps of MACS and flow cytometry purification. The MACS depletion enriched the DN thymocyte target population: percentages of DN's in Tg IL-

7/IL-7Rα$^{449F}$ were increased from 3.9% to 61% and percentages of Tg IL-7 DN's increased from 2.7% to 9.7%. A number of DN enrichments were performed in order to obtain sufficient amounts of protein from these rare T cell progenitors.

350 μg of Tg IL-7/IL-7Rα$^{449F}$ protein was obtained through 4 separate enrichments, 17 mice in total. Conversely, 150 μg of Tg IL-7 protein (the minimum required to perform iTRAQ analysis) was obtained through 8 separate enrichments, 45 mice in total.

**Protein Identification and Analysis:**

iTRAQ mass spectrometry data was analyzed using Applied Biosystems ProteinPilot$^{TM}$ Software 1.0 which searched against the International Protein Index (IPI) mouse protein database for peptide sequence identification. Proteins of interest were those with 2 or more high confidence unique peptides identified with a $P<0.01$ and a fold difference greater than 1.2.

Proteins were then analyzed by gene ontology using Blast2GO software. This software assigns putative roles to identified proteins according to two ontologies, biological process and molecular function. Results were visualized by directed acyclic graphs (DAG's), which map proteins according to gene ontology terms by grouping proteins under very broad terms and moving down by levels, grouping proteins under increasingly specific terms. DAG's enable the visualization of the hierarchical structure of terms and provides a more global perspective of general trends within a given protein set. Directed acyclic graphs were generated from the total protein set (537), the Tg IL-7/IL-7Rα$^{449F}$ upregulated protein set (51) and the Tg IL-7/IL-7Rα$^{449F}$ downregulated protein set (65). The number of proteins found under each term from level 4 of the

DAG's were then plotted for total proteins and Tg IL-7/IL-7Rα$^{449F}$ upregulated and downregulated proteins (Fig 2.3 a and b).

A number of differences in biological process trends were obvious between total proteins and those up or downregulated in Tg IL-7IL-7Rα$^{449F}$ DN thymocyte progenitors. There was approximately a 25% relative increase in these cells in the number of proteins upregulated in cellular metabolism, macromolecule metabolism and primary metabolism compared to downregulated proteins and roughly a 15% relative increase compared to total proteins. The relative increase in the number of upregulated proteins in Tg IL-7/ IL-7Rα$^{449F}$ DN thymocyte progenitors involved in biosynthesis was more than 50% higher than downregulated proteins. While 5% of the upregulated proteins in these progenitors were involved in cell cycle, there were no downregulated proteins involved in this process. Conversely, there were 8% of Tg IL-7/IL-7Rα$^{449F}$ downregulated proteins involved in cell organization and biogenesis, with no upregulated proteins found. All three protein subsets were similar in terms of the percentage of proteins involved in establishment of localization, regulation of cellular physiological process, regulation of metabolism and transport.

Many differences among the three analyzed protein sets were also apparent upon graphing molecular function DAG data. The percentage of Tg IL-7/IL-7Rα$^{449F}$ downregulated proteins involved in cation binding, metal ion binding and RNA binding were approximately 30% higher relative to both upregulated proteins and total proteins. Conversely, the percentage of proteins involved in purine nucleotide binding was 43% higher in upregulated proteins relative to downregulated proteins. 11% of upregulated proteins were involved in hydrolase activity, acting on acid anhydrides and 14% involved

in unfolded protein binding, with no downregulated proteins in Tg IL-7/IL-7Rα$^{449F}$ progenitors found in these categories. 15% of downregulated proteins were found to be involved in cytoskeletal protein binding.

Several proteins with putative roles in cell survival, death or proliferation were among the up-and downregulated protein sets. Gimap4 was the first protein identified that fit this criteria. It was determined to be 11.3 fold lower in Tg IL-7/IL-7Rα$^{449F}$ DN's than Tg IL-7 DN's, based on the 117/115 iTRAQ tag ratio (Table 1). Gimap4 is a recently discovered small GTPase which may play a role in positive selection and apoptosis, though it's role in the T cell lineage has yet to be fully characterized. (35). The second protein found to fit the above criteria was Annexin A6, whose expression was 2.4 fold lower in Tg IL-7/IL-7Rα$^{449F}$ DN's. Annexin A6 is a family member of $Ca^{2+}$-dependent membrane binding proteins and may play a role in mediating Ras signaling pathways. There is also evidence that annexin A6 can act as a recruiting/scaffolding protein in a number of signaling pathways controlling differentiation, proliferation and apoptosis. (36). Signal transducer and activator of transcription 1 (STAT1) expression was also found to be decreased by 2.3 fold in Tg IL-7/IL-7Rα$^{449F}$ DN's. STAT1 is a transcription factor that is believed to play a role in cytokine response (37). Another protein determined to have decreased expression in the Tg IL-7/IL-7Rα$^{449F}$ DN's was Cdc42, whose expression was 1.3 fold lower. Cdc42 is involved in mediating cell proliferation and cycling (38, 39).

Two proteins of interest were selected from those with increased expression in the Tg IL-7/IL-7Rα$^{449F}$ DN's: FKBP51 and Bit1, with a fold increase of 2.1 and 1.3 respectively. FKBP51 is an immunophilin that is inhibited by either FK506 or rapamycin,

both potent immunosuppressive drugs that inhibit T cell proliferation (40). Inhibition of FKBP51 results in NFκB activation and thus increased apoptosis and decreased cell proliferation. Bit1 is a mitochondrial protein that mediates apoptosis through its interaction with Grouch family proteins AES and TLE1. Upon loss of integrin-mediated cell attachment, Bit1 is released from the mitochondria to form a complex with AES; this Bit1/AES complex inhibits TLE1 and results in increased apoptosis.

**Protein Validation:**

Proteins previously implicated in mediating cell survival or apoptosis that were quantitatively different between Tg IL-7 and Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes according to iTRAQ ratios were validated by 3 separate immunoblots using fresh DN lysates. Quantification of the immunoblots was performed using a LI-COR Odyssey infra-red imager to generate 'Integrated Intensity' values (pixels/mm$^2$). Gimap4 had, on average, 56.1 fold (ranging from 31.1 to 123) lower expression in Tg IL-7/IL-7Rα$^{449F}$ DN's by immunoblot analysis quantification (Fig 2.4). Two proteins of interest were selected from those with increased expression in the Tg IL-7/IL-7Rα$^{449F}$ DN's: Bit1 and FKBP51, with an average fold increase of 2.6 (ranging from 1.8 to 14.3) and 2.0 (ranging from 1.1 to 3.7) respectively (Fig 2.4). Interestingly, in all validations, the differences in protein expression level as determined by immunoblot analysis were found to be greater than originally reported from iTRAQ analysis.

## 2.4 Discussion

IL-7 is an essential cytokine for proper development and functioning of the immune system. We are using a Tg IL-7 mouse model as a model of IL-7 related human

immune system cancers such as T-cell acute lymphoblastic leukemia (T-ALL), Hodgkin's disease and Burkitt's lymphoma (7-13). These mice have increased proliferation in both the T and B cell compartments which leads to lymphoma development and subsequent death by 7 months of age as a result. To investigate the signaling pathways involved in IL-7-induced lymphomas, we generated a mouse model carrying an amino acid substitution in a critical motif in the IL-7Rα chain (IL-7Rα$^{449F}$) important for the initiation of a number of IL-7 signaling pathways. Having observed that Tg IL-7/IL-7Rα$^{449F}$ mice are completely protected from lymphoma development, we aimed to identify novel signaling effectors downstream of this receptor mutation that may contribute to the observed lymphoma protection.

Within the hematopoietic compartment, all mature, terminally differentiated cells arise from a very small population of rare hematopoietic stem cells with the unique ability to self renew. Tumor forming cells also seem to follow a similar hierarchy, with a very small population of cancer stem cells initiating and supporting tumor growth (30, 32). Thymocyte precursors were studied as they are the population likely responsible for initiating and sustaining T cell lymphoma growth. While current therapeutic techniques targeting rapidly dividing cells are often quite successful in eliminating tumors, relapse is a frequent problem, indicating cancer stem cells are not being simultaneously eradicated. It is clear that gaining a better understanding of the molecular and cellular properties of these rare cells would provide tremendous insight into the biology of cancer and allow the development of more effective therapies.

We performed a global proteomic analysis comparing DN thymocyte progenitor whole cell lysate from our genetic models of lymphoma-prone and lymphoma-protected

mice. DN thymocyte progenitors were isolated from 7.5 week old mice to ensure that samples were not clonally transformed cells and hence, misrepresentative. Using iTRAQ and tandem mass spectrometry, we were able to identify both qualitative and quantitative differences between the two whole cell lysate protein samples. Through this approach we aimed to characterize and analyze protein differences in order to better understand the molecular effects of this receptor mutation as well as possibly discover novel IL-7-induced signaling effectors. As whole cell lysates are typically very complex and have a high dynamic range, our discovery of so many pieces of the IL-7 signaling puzzle is very encouraging but also suggests that many more pieces of the puzzle remain to be discovered.

We performed gene ontology analysis comparing all proteins identified to those found to be either up- or downregulated in Tg IL-7/IL-7R$\alpha^{449F}$ DN thymocytes. From biological process analysis, five categories were most clearly affected by the IL-7R$\alpha^{449F}$ knock-in mutation. In the categories of cellular metabolism, macromolecule metabolism, biosynthesis, and primary metabolism the number of upregulated proteins were significantly higher relative to downregulated proteins. In addition, only upregulated proteins were found to be involved in cell cycle. This would suggest that during IL-7 stimulation of normal lymphocytes, phosphorylation of the IL-7R$\alpha$ Y449 residue activates signaling pathways that in some way negatively regulate these processes. The under representation of metabolism and cell cycle proteins in Tg IL-7 DN progenitors may hence account for the reduced cellularity evident in these mice. Clearly the abrogation of these signaling pathways results in an increase in the number of proteins involved in these biological processes. Paradoxically, the upregulation of proteins

involved in these processes is indicative of increased cell growth and proliferation. This apparent conundrum may be compensated for by the putative loss of the ability to induce survival signals and induction of apoptosis and highlights the complex homeostatic mechanisms regulating the DN progenitor pool. Compensation by these processes in Tg IL-7/IL-7R$\alpha^{449F}$ DN thymocytes will be further explored.

A number of downregulated proteins were involved in cell organization and biogenesis while no upregulated proteins were found. This suggests that the phosphorylation of Y449 is required to mediate normal functioning of cell organization and biogenesis and loss of these signals results in a decrease in the level of proteins involved in these processes.

Some general trends were also apparent in the analysis of molecular function between total proteins and those either up or downregulated in Tg IL-7/IL-7R$\alpha^{449F}$ DN thymocytes. First, upregulated proteins involved in purine nucleotide binding were higher relative to downregulated proteins. Only upregulated and total proteins were found to be involved in hydrolase activity, acting on acid anhydrides, and unfolded protein binding. There were significantly more downregulated proteins involved in cation binding, metal ion binding and RNA binding compared to upregulated proteins. There were a number of downregulated proteins involved in cytoskeletal protein binding, with no upregulated proteins found.

The trends revealed by analysis of this latter class of proteins yielded some unexpected and novel insights. Closer analysis of the data set (Supplementary Table 1) revealed that a large number of cytoskeletal proteins such as gelsolin, plastin, actin-related protein 2/3 complex subunits 3,4 and 5 and gamma-actin were downregulated in

Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes, with iTRAQ ratios ranging from 4.8 fold to 1.6 fold lower. As a tumor cell's capacity for migration and invasion is closely associated with changes in the cytoskeleton, a deregulation of cytoskeletal components could be playing a major role in allowing cancer invasion and lymphoma development in Tg IL-7 mice. Downregulation of proteins normally involved in interacting selectively with components of the cytoskeleton such as actin or tubulin in Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes could deregulate processes such as cell adhesion, immune synapse formation, motility and migration potentially affecting transformation development. Such loss of cellular polarity has clear precedent in transformation of adherent cells but a role in lymphoid tumorigenesis has not been established.

We were able to identify proteins from the iTRAQ data set that have been previously implicated in regulating survival, proliferation or death and as such could be potential targets for specifically treating lymphomas. Gimap4 was one of the first proteins of interest to fit the above criteria and was determined to be 56 fold lower in Tg IL-7/IL-7Rα$^{449F}$ thymocyte progenitors compared to Tg IL-7 (Fig 2.4). Previous research on this protein has revealed that it is a small GTPase whose expression increases upon positive selection of T cells (41). Within the four DN populations, Gimap4 was only found to be expressed during the DN IV stage (41). Gimap4 deficient mice exhibit a delay between the transition from apoptotic to dead cells, thus they have greater amounts of apoptotic cells and decreased amounts of dead cells (42). The processes of T cell development, selection and activation appear unaffected in Gimap4 deficient mice (42). To our knowledge, this is the first time a connection has been shown between Gimap4 and IL-7. The results of our quantitative proteomic analysis indicate that Gimap4

expression levels are regulated through phosphorylation of the IL-7Rα chain Y449 residue. As such, the abrogation of Gimap4 induction resulting from loss of Y449-dependent signals may play a major role in lymphoma protection from IL-7 overexpression. While known IL-7 signaling effectors activated by Y449 such as STAT5 or PI3 kinase are involved in numerous different signaling pathways, targeting Gimap4 may confer a high therapeutic index for treating or preventing lymphomas given that mice deficient in Gimap4 have normal, unaffected lymphocytes (42). This will be examined further in ongoing studies.

Two other promising candidates that were identified and validated by immunoblot analysis were Bit1 and FKBP51. These proteins were found to be elevated in Tg IL-7/IL-7Rα$^{449F}$ thymocyte progenitors compared to controls (Fig 2.4). Bit1 is a pro-apoptotic mitochondrial protein that is involved in regulating apoptosis through its interaction with Groucho family transcriptional regulators AES and TLE1. Upon the loss of integrin-mediated cell adhesion, Bit1 is released from the mitochondria and is then able to form a complex with AES, which is speculated to function in a pro-apoptotic manner by inhibiting the anti-apoptotic TLE1 (43). Both proper cell adhesion and the anti-apoptotic proteins Bcl-2 and Bcl-xL appear to prevent the translocation of Bit1 and thus Bit1/AES complex formation (44). Until now Bit1 has not been shown to be regulated by IL-7. Our results suggest that Bit1 is normally negatively regulated in thymocytes by IL-7R Y449-dependent signals and thus upon overexpression of IL-7, inhibition of this pro-apoptotic protein may permit increased cell survival.

FKBP51 is a T cell specific immunophilin which can bind to the immunosuppressive drugs FK506 or rapamycin (40). The immunophilin-drug complex

mediates its effects by inhibiting calcineurin, a key signaling molecule in T cell activation (40). There is evidence that FKBP51 is required for IκBα degradation and consequently NFκB activation (45). Furthermore, FKBP51 overexpression has been shown to lead to NFκB activation (46). Such activation of NFκB may induce pro-apoptotic pathways, depending on the cellular context, and render Tg IL7/IL-7Rα$^{449F}$ thymocyte progenitors unable to support lymphoma development (47, 48). Whether IL-7 stimulation of normal, non-Tg thymocytes can induce Gimap4, NFκB activation via FKBP51 and suppress Bit1 is currently under investigation.

A global proteomic analysis was used to identify novel IL-7-induced signaling effectors. While a more focused receptor pull down approach would address very immediate, early events proximal to the IL-7R, we favored a global approach as we were more interested in evaluating later events, distal to the IL-7R to determine the range of effector families that were affected. We were able to identify a number of proteins that are specifically dependent on the IL-7Rα Y449 residue. Three proteins, Gimap4, FKBP51 and Bit1, have been previously associated with roles in survival and apoptosis and as such were validated by immunoblot analysis. These proteins will be further characterized with regards to their involvement in IL-7-mediated lymphoma development. In addition, our proteomic study of lymphoma-prone and –protected thymocyte progenitors has great relevance for studying changes in protein expression characteristic of progenitor cells destined to generate lymphoma. Identified proteins have the potential to be novel therapeutic targets for treating lymphomas, and will be evaluated for their ability to target T-cells specifically and leave other biological processes and functions unaffected.

## Table 2.1: Proteins differentially expressed in Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes

| Accession Number | Name | Tg IL-7 to Tg IL-7/IL-7Rα$^{449F}$ protein ratio | P value | EF | EF Range | Prec MW | Peptides Identified | Gene Symbol |
|---|---|---|---|---|---|---|---|---|
| **Upregulated in Tg IL-7/IL-7Rα$^{449F}$ DN Thymocytes** | | | | | | | | |
| Q64378 | FK506-binding protein 5 | 0.48 | 0.00 | 1.30 | 0.37-0.62 | 1304.72 | ALGLDSANEK | *Fkpb5* |
| | | | | | | 1394.75 | AWDIGVSTMK | |
| | | | | | | 2440.27 | DVVFVVGEGEDHDIPIGIDK | |
| | | | | | | 1924.95 | MQREEQCILYLGPR | |
| Q8R2Y8 | Bcl-2 inhibitor of transcription | 0.75 | 0.01 | 1.19 | 0.63-0.89 | 1952.09 | APDEDTLIQLLTHAK | *Ptrh2* |
| | | | | | | 2430.39 | TVLGIGPGPVELIDEVTGHLK | |
| **Downregulated in Tg IL-7/IL-7Rα$^{449F}$ DN Thymocytes** | | | | | | | | |
| Q99JY3 | GTPase, IMAP family member 4 isoform a | 11.32 | 0.00 | 1.88 | 6.02-21.3 | 1561.89 | MQLLTLVQSMVR | *Gimap4* |
| | | | | | | 1754.83 | FFQEVMHEFQNR | |
| | | | | | | 1921.97 | SSHELGNQDQGIPQLR | |
| P14824 | Annexin A6 | 2.38 | 0.00 | 1.19 | 2.0-2.83 | 2021.08 | DLMADLKSEISGDLAR | *Anxa6* |
| | | | | | | 2370.28 | EMSGDVKDAFVAIVQSVK | |
| | | | | | | 2039.11 | GFGSDKESILELITSR | |
| | | | | | | 1854.00 | GIGTDEATIIDIVTHR | |
| | | | | | | 2053.12 | LIVNLMRPLAYCDAK | |
| | | | | | | 1764.82 | SLEDALSSDTSGHFR | |
| | | | | | | 1028.62 | TLIEILATR | |
| | | | | | | 1371.79 | SEIDLLNIRR | |
| | | | | | | 1957.09 | LILGLMMPPAHYDAK | |
| P42225 | Signal Transducer and Activator of Transcription 1 | 2.34 | 0.00 | >2 | ND | 1596.86 | TELISVSEVHPSR | *Stat1* |
| | | | | | | 1887.12 | ALLKDQQPGTFLLR | |
| | | | | | | 1888.01 | FSLENNFLLQHNIR | |
| P607662 | Cell division control protein 42 | 1.31 | 0.00 | 1.14 | 1.15-1.49 | 2140.17 | NVFDEAILAALEPPEPK | *Cdc42* |
| | | | | | | 1511.85 | QKPITPETAEK | |

Accession numbers represents SWISS-PROT entries. Tg IL-7/IL-7RWT to Tg IL-7/IL-7RY449F protein ratio represents differential expression, as determined by 117/115 iTRAQ label ratio. P value represents a measure of significant differential protein expression levels. Prec MW represents the precursor molecular weight for the peptide sequence. Peptides identified represents the number of unique peptides identified for each protein. Confidence score (a measure of peptide identification certainty) for each unique peptide was >95%.

**Figure 2.1**

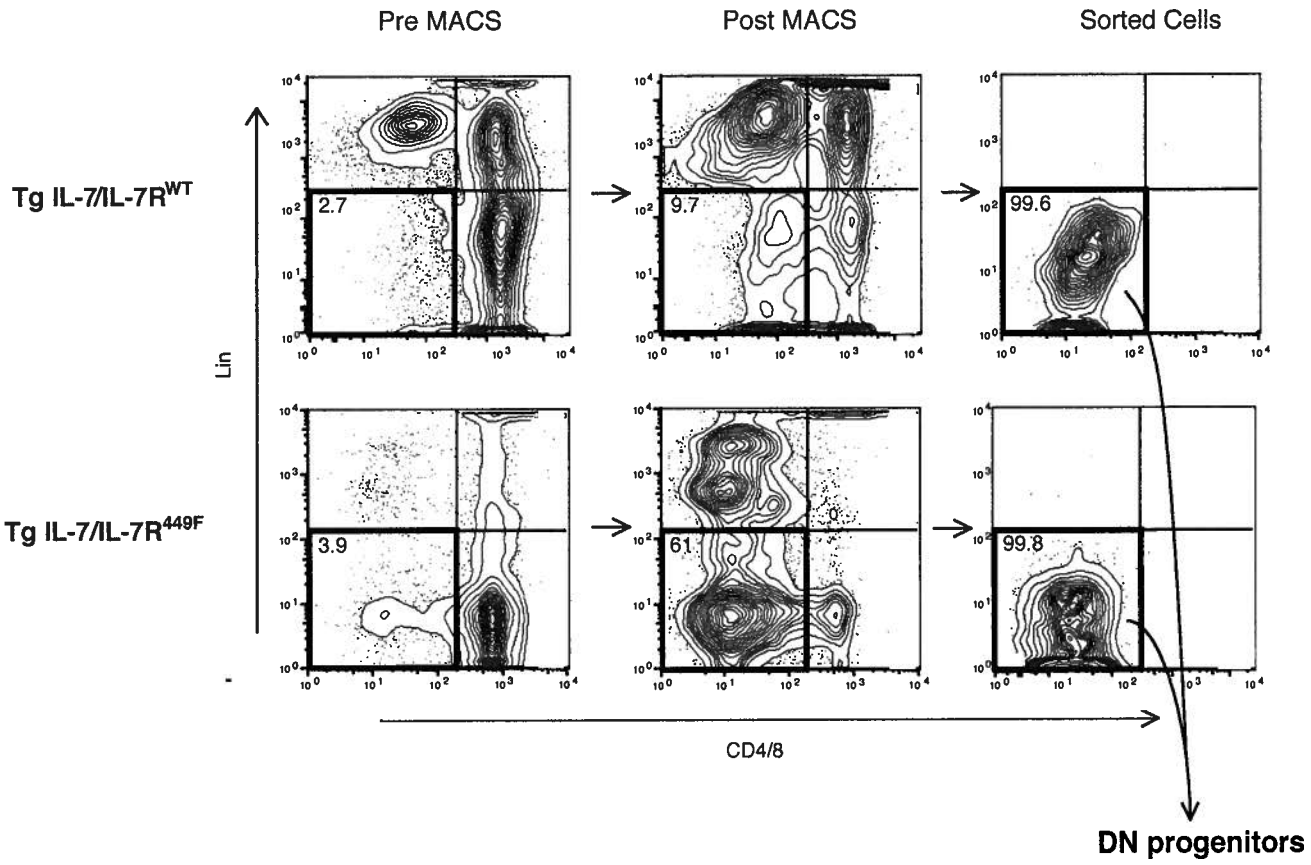


**Figure 2.1: Purification of αβ DN T cell progenitors.**
Representative cytometric flow cytometry analysis of total thymocytes, pre- and post-magnetic cell sorting (MACS) and post-flow cytometry cell sort from both Tg IL-7 and Tg IL-7/IL-7Rα$^{449F}$ mice at 8 weeks of age. Cells were stained with anti-CD4, anti-CD8 and Lin (anti-B220, anti-γδTCR, anti-TER119 and anti-Gr1). DN T cell progenitors are negative for all these markers.

Figure 2.2

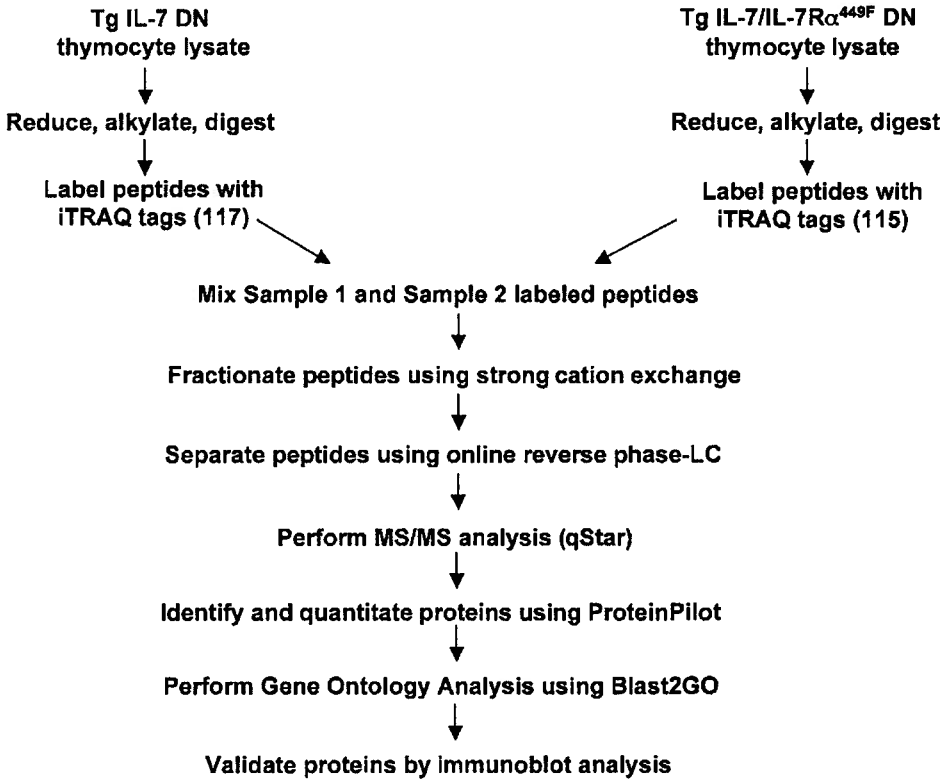


**Figure 2.2: Overview of experimental design.**
Flow chart showing the steps involved in determining quantitative differences between Tg IL- 7 and Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes and the analysis and comparison of these data.

**Figure 2.3**

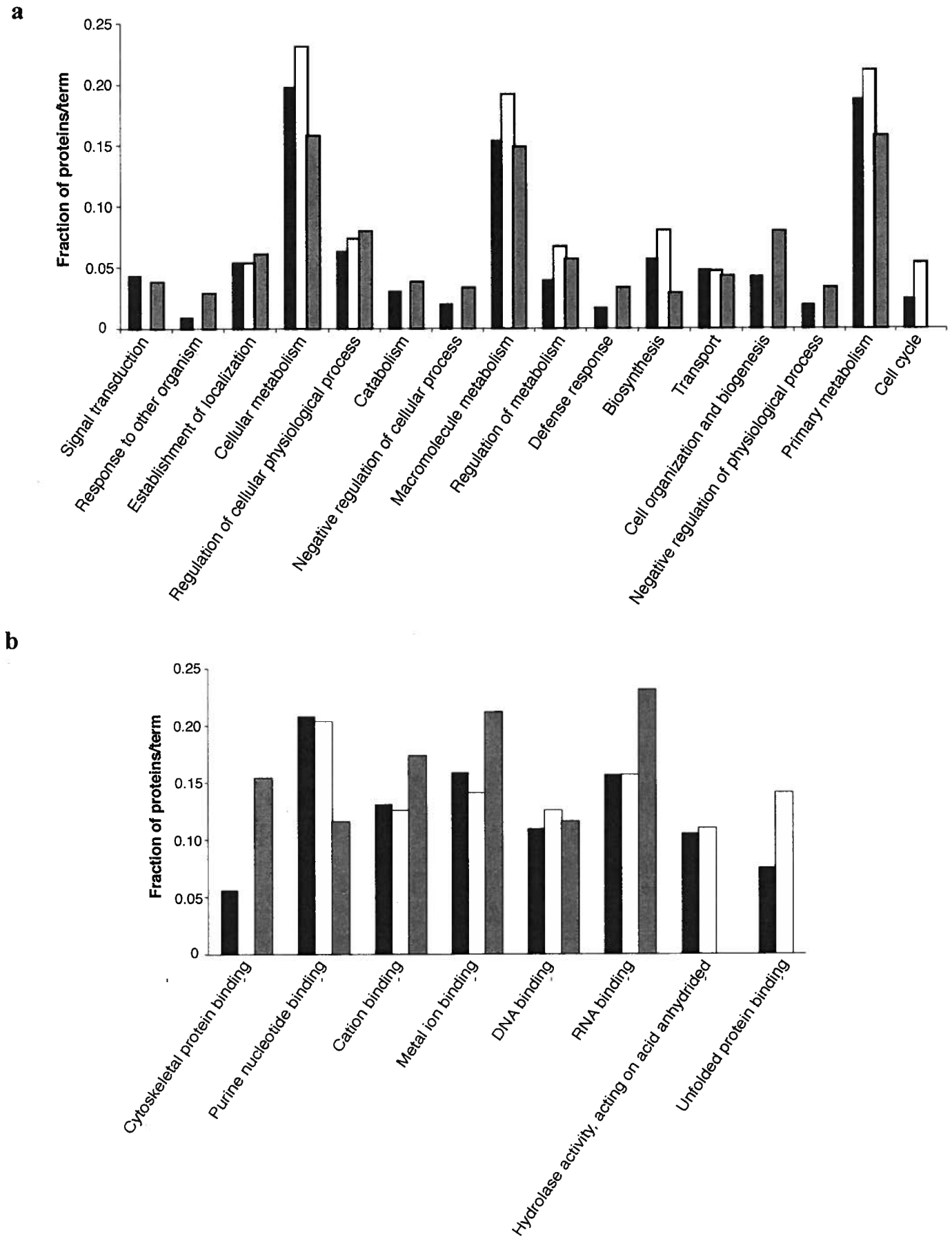
a
Fraction of proteins/term
0.25
0.20
0.15
0.10
0.05
0
Signal transduction
Response to other organism
Establishment of localization
Cellular metabolism
Regulation of cellular physiological process
Catabolism
Negative regulation of cellular process
Macromolecule metabolism
Regulation of metabolism
Defense response
Biosynthesis
Transport
Cell organization and biogenesis
Negative regulation of physiological process
Primary metabolism
Cell cycle
b
Fraction of proteins/term
0.25
0.20
0.15
0.10
0.05
0
Cytoskeletal protein binding
Purine nucleotide binding
Cation binding
Metal ion binding
DNA binding
RNA binding
Hydrolase activity, acting on acid anhydrides
Unfolded protein binding
Total proteins
Upregulated in Tg IL-7/IL-7Rα449F
Downregulated in Tg IL-7/IL-7Rα449F

**Figure 2.3: Protein categorization by gene ontology.**
a) Protein categorization by level four terms of a biological process directed acyclic graph, represented by percentage of proteins found under each term from total protein set (black), upregulated in Tg IL-7/IL-7Rα$^{449F}$ (white) and downregulated in Tg IL-7/IL-7Rα$^{449F}$ (grey).

b) Protein categorization by level four terms of a molecular function directed acyclic graph, represented by percentage of proteins found in each term from total protein set (black), upregulated in Tg IL-7/IL-7Rα$^{449F}$ (white) and downregulated in Tg IL-7/IL-7Rα$^{449F}$ (grey).

**Figure 2.4**

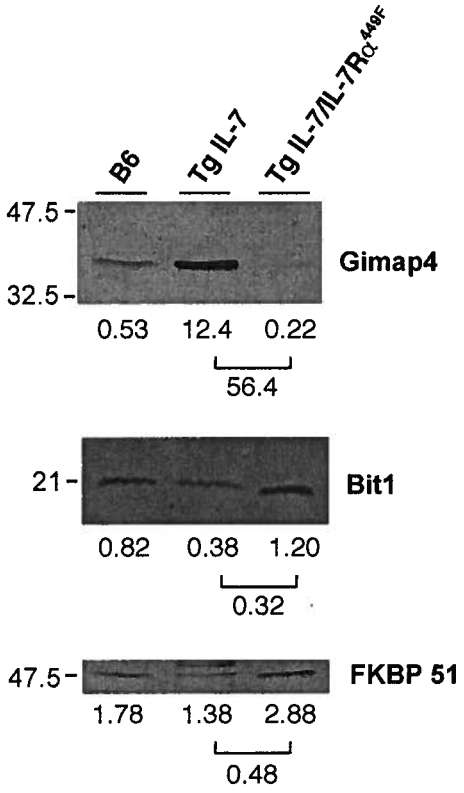


**Figure 2.4: Validation of Gimap4, Bit1 and FKBP 51.**
Double negative (DN) T cell progenitor lysates were prepared from C57BL/6 (B6), Tg IL-7, and Tg IL-7/IL-7Rα$^{449F}$ mice, 30 μg of protein were resolved by polyacrylamide gel electrophoresis and analyzed by western blot using rabbit polyclonal anti-Gimap4, anti-Bit1 and anti-FKBP 51 antibody. Quantification was performed using the LI-COR Odyssey 'Integrated Intensity' values in pixels/mm2. Numbers below figures represent normalized integrated intensity values averaged from 3 separate immunoblots of independently derived, pooled lysates. The ratio of expression of each protein in Tg IL-7 to Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes is shown below the bar. Equal loading was determined by coomassie staining of total protein loaded.

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## CHAPTER 3: General discussion

Interleukin(IL)-7 is an essential growth factor for both T and B cell precursors, exerting it's effects through the IL-7R. Given its nonredundant role in survival and development, it follows that excessive IL-7 signaling *in vivo* results in increased T and B cell proliferation leading to fatal immune cell tumor development, as seen in transgenic IL-7 mice (Tg IL-7). To assess the role of IL-7-induced signaling effectors in survival and proliferation of immune cells, we previously generated a mouse model where the Y449 residue on IL-7Rα is substituted with a phenylalanine (IL-7R$^{449F}$) to block all downstream signals initiated from the phosphorylated Y449 residue. IL-7R$^{449F}$mice were crossed with Tg IL7 mice (Tg IL-7/IL-7R$^{449F}$) to determine the involvement of Y449-dependent signaling pathways in lymphoma development. Strikingly, IL-7R$^{449F}$ mice overexpressing IL-7 are completely disease free, indicating that lymphoma development is entirely dependent on phosphorylation of Y449 in the IL-7Rα chain and subsequent downstream signaling pathways. Having observed that Tg IL-7/IL-7Rα$^{449F}$ mice are protected from lymphoma development, we aimed to characterize and analyze protein differences between Tg IL-7 and Tg IL-7/IL-7Rα$^{449F}$ T cell progenitors in order to better understand the molecular effects of this receptor mutation as well as possibly discover novel IL-7-induced signaling effectors.

### 3.1 Analysis of experimental approach

To identify changes in signaling effectors as a result of the Y449 receptor mutation we performed a global proteomic analysis on thymocyte progenitors from lymphoma-prone (Tg IL-7) and lymphoma-protected (Tg IL-7/IL-7Rα$^{449F}$) mice. This global approach was favored over more focused approaches such as a receptor pull down

as it allowed us to identify further downstream signaling events distal to the IL-7R, instead of being limited to immediate, early events proximal to the IL-7R. Through this approach we hoped to gain more information on the range of effector families affected. In addition, although collecting sufficient amounts of protein to perform this analysis was a large and time consuming undertaking, a proteomics approach has distinct advantages over other systems biology approaches such as microarray analysis in that it provides a very accurate account of protein level differences between samples at any one time. Microarray analysis, on the other hand, while attractive for requiring very little starting material does not necessarily reflect protein differences accurately; due to numerous mechanisms of translational regulation, mRNA levels can increase or decrease without the consequent protein product levels changing at all. We were able to eliminate these uncertainties by pursing an analysis of the two proteomes directly.

For the proteomic analysis, DN thymocyte progenitors were isolated from 7.5 week old mice and screened using CD4/CD8 profile analysis to ensure that samples were not clonally transformed cells and, hence, misrepresentative. DN thymocytes were targeted as there is a growing body of evidence indicating tumor forming cells may be similar to hematopoietic stem cells, more specifically a rare population with the unique ability to self renew making them capable of initiating and sustaining tumor growth (1, 2). Current therapeutic approaches targeting rapidly dividing cells may fail to eliminate these quiescent stem cell-like cells, leaving the potential for a new tumor to form and relapse to occur. This highlights the need to better understand the molecular properties of precursor cells as potential tumor forming cells in order to design more effective treatment approaches. For this reason, DN thymocytes were studied as they are the

precursors of mature T cells and as such are the most likely population for initiating and sustaining T cell lymphoma growth.

One potential caveat with this approach is that the DN population consists of four subsets (DN I-IV) characterized by the expression of CD44 and CD25, where each subset slightly differs in developmental potential and level of T cell commitment. Upon investigation it was found the DN I-IV subsets between Tg IL-7 and Tg IL-7/IL-7R$\alpha^{449F}$ mice differ in cell numbers and frequency, with roughly half of the DN compartment in Tg IL-7 mice made up of DN I stage cells, as compared to WT and Tg IL-7/IL-7R$\alpha^{449F}$ mice where the majority of DN cells are typically in the DN III and IV stages. Thus, proteins normally expressed in the DN III and IV subsets may appear elevated in Tg IL-7/IL-7R$\alpha^{449F}$ lysates. Nonetheless, the proteins of interest we focused on had greater fold differences than subset cell number differences. Further characterization of their DN subset expression would clarify their differential regulation by IL-7R Y449.

The proteomic analysis to identify quantitative differences between the two protein samples was done using iTRAQ technology and tandem mass spectrometry (3). iTRAQ technology allows two samples to be tagged with different isotope peptide tags so they can be simultaneously run through mass spectrometry, eliminating the possibility of variation between mass spectrometry runs. In addition, this technology tags all primary amines of peptides, ensuring greater proteome coverage and higher confidence in peptide identification compared to older cysteine labeling methods. The analysis was done on whole cell lysates which are typically very complex and have a high dynamic range, thus one could argue that separating cytoplasmic and membrane fractions or enriching for phosphorylated proteins would result in a more focused data set. We chose instead to

cast a wide net by analyzing the entire cell lysate, ensuring a very rich data set and allowing us to remain completely unbiased.

### 3.2 Gene ontology analysis of biological process and molecular function

The proteomic analysis of the two lysates resulted in a large dataset, with over 700 proteins identified and about 120 proteins found to be significantly up- or downregulated as compared to the Tg IL-7 control. To get an idea of general trends taking place in the two proteomes, we performed gene ontology (GO) analysis comparing all proteins identified to those found to be either up- or downregulated in Tg IL-7/IL-7R$\alpha^{449F}$ DN thymocytes as compared to Tg IL-7 DN thymocytes. The GO software assigns putative functions to identified proteins and generates directed acyclic graphs (DAG's) to visualize the results. DAG's group GO terms that have highly specific meanings under terms with broader meanings within two ontologies, biological process and molecular function.

Looking at biological process analysis, five processes were most obviously affected by the IL-7R$\alpha^{449F}$ knock-in mutation (Fig 2.3a). The analysis showed that there were a significantly higher number of upregulated proteins over downregulated proteins in the categories of cellular metabolism, macromolecule metabolism, biosynthesis, primary metabolism and cell cycle. This was unexpected as the upregulation of proteins involved in cell cycle and metabolism indicates increased cell growth and proliferation in the lymphoma-protected DN thymocytes. Interestingly, when comparing cell proliferation between Tg IL-7 and Tg IL-7/IL-7R$\alpha^{449F}$ T cells as measured by expression of the nuclear protein Ki67 it was found that Tg IL-7/IL-7R$\alpha^{449F}$ had a higher frequency of Ki67$^{+}$ DN thymocytes. These are paradoxical findings given that you would expect

increased cell growth and proliferation to contribute to tumor formation rather then tumor protection as seen in these mice. However, this lymphoma protection may be due to a failure to induce survival proteins, causing these rapidly dividing cell to die before they can establish tumors. Overall these findings highlight the complex homeostatic mechanisms regulating the DN progenitor pool.

Some general trends were also apparent in the analysis of molecular function (Fig 3.2b). One of the most significant changes observed was a number of downregulated proteins in Tg IL-7/IL-7R$\alpha^{449F}$ DN thymocytes involved in cytoskeletal protein binding, with no upregulated proteins found (Fig 3.2b). Closer analysis of the data set revealed that a large number of cytoskeletal proteins such as gelsolin, plastin, actin-related protein 2/3 complex subunits 3,4 and 5 and gamma-actin were downregulated in Tg IL-7/IL-7R$\alpha^{449F}$ DN thymocytes, with iTRAQ ratios ranging from 4.8 fold to 1.6 fold lower (Table 3.1). This decrease in cytoskeleton proteins could indicate a deregulation of processes such as adhesion and motility. As a tumor cell's capacity for migration and invasion is closely associated with changes in the cytoskeleton, deregulation of cytoskeletal components could be playing a major role in allowing cancer invasion and lymphoma development in Tg IL-7 mice.

In addition, downregulation of proteins normally involved in interacting selectively with components of the cytoskeleton could affect other processes such as immune synapse formation. Actin polymerization is a fundamental process in the polarization of thymocytes and the formation of an immune synapse, both of which are required for T cell activation and an efficient immune response to occur. The downregulation of a number of cytoskeleton proteins involved in initiating and mediating

actin polymerization in the Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes could indicate an inability to form an adequate immune synapse, having great implications for T cell function and immune responses in these mice. This would be consistent with observed CD4 acute response defects and impaired TCR induced proliferation in IL-7R$^{Y449}$ mice and is something that should be investigated further (4).

### 3.3 Identification and validation of survival/apoptosis proteins

After gene ontology analysis, we aimed to identify specific proteins previously associated with either survival, proliferation or apoptosis regulation that quantitatively differed between Tg IL-7 and Tg IL-7/IL-7Rα$^{449F}$ DN's. In addition to studying differences in global trends, we wanted to identify specific proteins deregulated by the Y449 knock-in mutation that may be involved in lymphoma development and further investigate them as such. Of those with significant changes in expression levels, we selected six proteins of initial interest that appeared, from previous studies, most likely to be involved in these processes (Table 1). We selected three proteins, Gimap4, FKBP51 and Bit1, previously implicated in mediating cell survival or apoptosis that were significantly quantitatively different between Tg IL-7 and Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes to validate by immunoblot analysis.

Gimap4 is a small GTPase which had, on average, 56.1 fold lower expression in Tg IL-7/IL-7Rα$^{449F}$ DN's by immunoblot analysis quantification indicating Gimap4 expression levels could be regulated through phosphorylation of the IL-7Rα Y449 residue (Fig 2.4). As such, the loss of Y449-mediated Gimap4 induction may play a major role in lymphoma protection from IL-7 overexpression. Investigation of Gimap4's role in the immune system found that both mice and rats deficient in Gimap4 are largely

unimpaired in lymphocyte development and function (5, 6). Both, however, showed a delayed apoptosis phenotype in lymphcytes where the transition in cells undergoing apoptosis to death is unusually prolonged, indicating under normal circumstances Gimap4 may be involved in accelerating T cell death (5, 6). This would be paradoxical to the lymphoma development we observe in Tg IL-7 mice and requires further investigation into the role of this protein in tumor growth. While known IL-7 signaling effectors activated by Y449 such as STAT5 or PI3 kinase are involved in numerous different signaling pathways, targeting Gimap4 may confer a high therapeutic index for treating or preventing lymphomas given that mice deficient in Gimap4 have normal, unaffected lymphocytes (6). To our knowledge, this is the first time a connection has been shown between Gimap4 and IL-7.

Two proteins of interest were selected from those with increased expression in the Tg IL-7/IL-7R$\alpha^{449F}$ DN's: Bit1 and FKBP51, with an average fold increase of 2.6 and 2.0 respectively (Fig 2.4). Bit1 is a mitochondrial-associated protein which upon the loss of integrin-mediated cell adhesion is released from the mitochondria and functions in a pro-apoptotic manner (7). Both proper cell adhesion and the anti-apoptotic proteins Bcl-2 and Bcl-xL appear to prevent the translocation of Bit1 (8). A recent study looking at Bit1 deficient mice found loss of Bit1 expression resulted in increased Erk phosphorylation, suggesting Bit1 negatively regulates Erk activation (9). Erk had been shown to activate a number of pro-survival proteins including transcription factors c-Myc and STAT5 and its increased activity has been shown to contribute to a number of different cancers including leukemia (10). Increased Bit1 levels in Tg IL-7/IL-7R$\alpha^{449F}$ DN's may contribute to lymphoma protection through inhibition of Erk activation, preventing

subsequent increased cell growth. Until now Bit1 has not been shown to be regulated by IL-7. Our results suggest that Bit1 is normally negatively regulated in thymocytes by IL-7Rα Y449-dependent signals and thus upon overexpression of IL-7, inhibition of this pro-apoptotic protein may permit increased cell survival.

FKBP51 is a T cell specific immunophilin which can bind to the immunosuppressive drugs FK506 or rapamycin (11). The immunophilin-drug complex mediates its effects by inhibiting calcineurin, a key signaling molecule in T cell activation (11). There is evidence that FKBP51 is required for IκBα degradation and consequently NFκB activation (12). Furthermore, FKBP51 overexpression has been shown to lead to NFκB activation (13). Such activation of NFκB may induce pro-apoptotic pathways, depending on the cellular context, and render Tg IL7/IL-7Rα$^{449F}$ thymocyte progenitors unable to support lymphoma development (14, 15). Whether IL-7 stimulation of normal, non-Tg thymocytes can induce Gimap4, NFκB activation via FKBP51 and suppress Bit1 is currently under investigation.

### 3.4 Future directions

We were able to identify three proteins, Gimap4, FKBP51 and Bit1, which have been previously associated with roles in survival and apoptosis and whose expression appears to be regulated by or dependent on the IL-7Rα Y449 residue. Further characterization of the roles these proteins play in normal thymocyte development is required to understand how their deregulation may contribute to transformation. This could be done by performing both gain of function complementation studies and loss of function experiments *in vivo* to determine the effect of overexpression or downregulation on T cell development and, potentially, lymphoma progression. These experiments could

be done in several genetic contexts, namely IL-7R$^{-/-}$, Tg IL-7, Tg IL-7/IL-7R$\alpha^{449F}$ and wild type primary HSC's, in order to fully evaluate the combinatorial effects of these candidate proteins with other IL-7R effectors. Such proteins could become novel, specific targets for preventing or blocking lymphoma development. In addition, given that Gimap4 expression levels showed the most dramatic dependence on the IL-7R$\alpha$ chain Y449 residue, abrogation of Gimap4 resulting from loss of Y449-dependent signals may play a major role in lymphoma protection from IL-7 overexpression. This could be evaluated by crossing Gimap4$^{-/-}$ deficient mice (6) to Tg IL-7 mice to determine the requirement of Gimap4 in IL-7-mediated lymphomas, assessing for survival and susceptibility to lymphoma development as compared to Tg IL-7 and wild type mice.

### 3.5 Candidate screening for other IL-7-induced survival effectors

In addition to studying novel effectors identified in our screen, we also investigated two Bcl-2 family members, Mcl-1 and Noxa, to determine their association with IL-7 and the IL-7R Y449 residue. The Bcl-2 family of cell survival/death proteins is a major group of proteins involved in mediating the downstream pro-survival effects of IL-7: the balance of pro-apoptotic-to-anti-apoptotic members determines the survival versus death decision. Mcl-1 is an anti-apoptotic Bcl-2 family member potentially responsible for mediating IL-7's effects, as mice deficient in Mcl-1 present similar characteristics to those deficient in IL-7 signaling: these mice show decreased numbers of T and B cells along with increased apoptosis T and B lymphocyte progenitors. Indeed, we observed that IL-7 stimulation of WT splenic T cells resulted in roughly a 2.3 fold increase in Mcl-1 levels (Appendix Fig. 1a), indicating in an acute stimulation setting IL-7 positively regulates Mcl-1. Interestingly we observed a smaller change in IL-7R$\alpha^{449F}$ T

cells when stimulated with IL-7, which resulted in a 1.7 fold increase, suggesting Mcl-1 regulation may be partially dependent on Y449 (Appendix Fig. 1a). While these results indicate Mcl-1 is positively regulated by IL-7, partially through Y449, in an acute setting, levels of Mcl-1 detected in total splenocytes directly *ex-vivo* showed very little difference between WT and either Tg IL-7 or Tg IL-7/IL-$7^{449F}$ (Appendix Fig. 1b). This indicates that with chronic IL-7 stimulation, Mcl-1 levels remain fairly stable. In addition, while levels of Noxa, an antiapoptotic member which negatively regulates Mcl-1, did show some differential regulation comparing WT, Tg IL-7 and Tg IL-7/IL-$7^{449F}$ splenocytes, there was no obvious pattern to these differences (Appendix Fig. 1b). It is of importance to note, however, that Mcl-1 and Noxa levels were detected here in lysate from total splenocytes, thus many different cell types could be contributing significantly. Examining these proteins in purified T cells would give a more accurate idea of its involvement in downstream IL-7 signaling pathways.

### 3.6 Conclusion

A global proteomic analysis was used to identify novel IL-7-induced signaling effectors. We were able to identify a number of proteins that are specifically dependent on the IL-7Rα Y449 residue. Three proteins, Gimap4, FKBP51 and Bit1, have been previously associated with roles in survival and apoptosis and as such were validated by immunoblot analysis. Bcl-2 family survival proteins Mcl-1 and Noxa were also investigated with regards to their regulation by the IL7R. These proteins are being further characterized with regards to their involvement in IL-7-mediated lymphoma development. In addition, our proteomic study of lymphoma-prone and –protected thymocyte progenitors provides insight into protein expression changes characteristic of

progenitor cells destined to generate lymphoma, leading to a better understanding of the biology of lymphomagenesis. Proteins identified as a result of this screen have the potential to be novel therapeutic targets for treating or preventing lymphomas, especially considering targeting them may affect T-cells specifically and leave other biological processes and functions unaffected.

## Table 3.1: Cytoskeleton proteins differentially expressed in Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes

| Accession Number | Name | Tg IL-7 to Tg IL-7/IL-7Rα$^{449F}$ protein ratio | P value | EF | EF Range | Gene Symbol |
|---|---|---|---|---|---|---|
| P13020 | Gelsolin precursor | 4.79 | 0.00 | 1.46 | 3.28-6.99 | *Gsn* |
| Q9JM76 | Actin-related protein 2/3 complex subunit 3 | 2.29 | 0.00 | 1.10 | 2.08-2.52 | *Arpc3* |
| Q61233 | Plastin-2 | 2.23 | 0.00 | 1.12 | 1.99-2.50 | *Lcp1* |
| P26041 | Moesin | 2.08 | 0.00 | 1.38 | 1.51-2.87 | *Msn* |
| P63260 | Actin, cytoplasmic 2 | 1.82 | 0.00 | 1.07 | 1.70-1.95 | *Actg1* |
| Q9CPW4 | Actin-related protein 2/3 complex subunit 5 | 1.77 | 0.01 | 1.46 | 1.21-2.58 | *Arpc5* |
| P59999 | Actin-related protein 2/3 complex subunit 4 | 1.62 | 0.00 | 1.23 | 1.32-1.99 | *Arpc4* |
| Q8VDD5 | Myosin-9 | 1.61 | 0.00 | 1.12 | 1.44-1.80 | *Myh9* |
| Q60605-1 | Myosin light polypeptide 6 | 1.6 | 0.01 | 1.38 | 1.16-2.21 | *My16* |
| P26039 | Talin-1 | 1.41 | 0.00 | 1.17 | 1.21-1.65 | *Tln1* |
| Q9JHU4 | Cytoplasmic dynein heavy chain | 1.30 | 0.00 | 1.14 | 1.14-1.48 | *Dync1h1* |

Table 1 lists all cytoskeleton proteins found in our proteomic screen to have a fold decrease greater than 1.3 as a result of the IL-7R Y449 mutation. Accession numbers represents SWISS-PROT entries. Tg IL-7 to Tg IL-7/IL-7Rα$^{449F}$ protein ratio represents differential expression, as determined by 117/115 iTRAQ label ratio. P value represents a measure of significant differential protein expression levels. EF (Error Factor) represents a measure of the error in the 117/115 ratio. EF Range represents (iTRAQ ratio/EF) to (iTRAQ ratio*EF). Confidence score (a measure of peptide identification certainty) for each unique peptide was >95%.

**Figure 3.1**

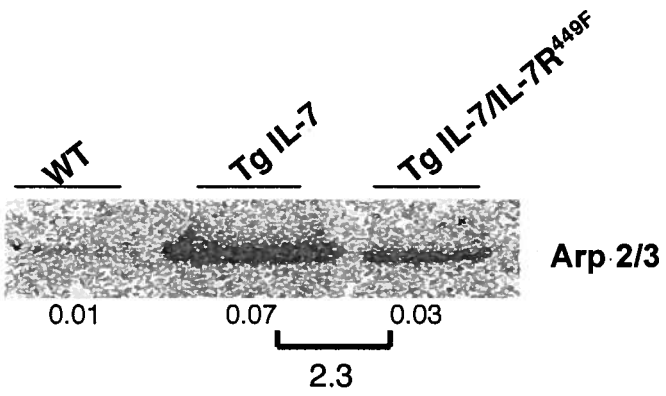


**Figure 3.1: Validation of Arp2/3.**
Figure 3.1 shows a representative immunoblot confirming Arp 2/3 quantitative differences between Tg IL-7 and Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes using fresh DN lysates. 30 ug of protein were resolved by polyacrylamide gel electrophoresis and analyzed by western blot using anti-Arp2/3 antibody. Quantification was performed using the LI-COR Odyssey 'Integrated Intensity' values in pixels/mm$^2$. Numbers below figures represent normalized integrated intensity values averaged from 3 separate immunoblots of independently derived, pooled lysates. The ratio of expression of each protein in Tg IL-7 to Tg IL-7/IL-7Rα$^{449F}$ DN thymocytes is shown below the bar. Equal loading was determined by coomassie staining of total protein loaded.

**Figure 3.2**

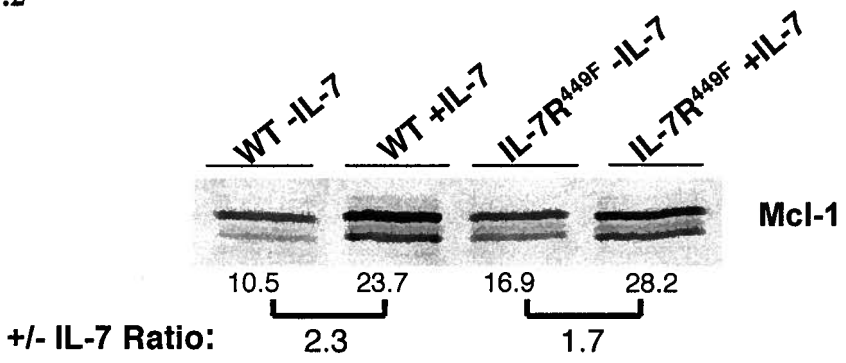


**Figure 3.2: Mcl-1 regulation by IL-7Rα Y449 following acute IL-7 stimulation.**
Figure 3.2 shows Mcl-1 protein level changes after 20 hours IL-7 stimulation in WT and IL-7R$^{449F}$ T cells. T cell's were isolated from B6 and IL-7R$^{449F}$ mice by magnetically depleting splenocytes of B cells and granulocytes. T cells were cytokine stripped and cultured in RPMI + 1% FBS for four hours at 2E6 cells/ml. IL-7 was added at 25 ng/ml to all +IL-7 samples and stimulated for 20 hours, at which point cells were lysed in RIPA lysis buffer and stored at -20°C. 30 ug of protein was resolved by polyacrylamide gel electrophoresis and analyzed by western blot using rabbit polyclonal anti-Mcl-1 antibody. Protein levels were quantified and normalized to total protein loaded by coomassie blue staining using a LI-COR Odyssey infra-red imager and represent 'Integrated Intensity" values.

Figure 3.3

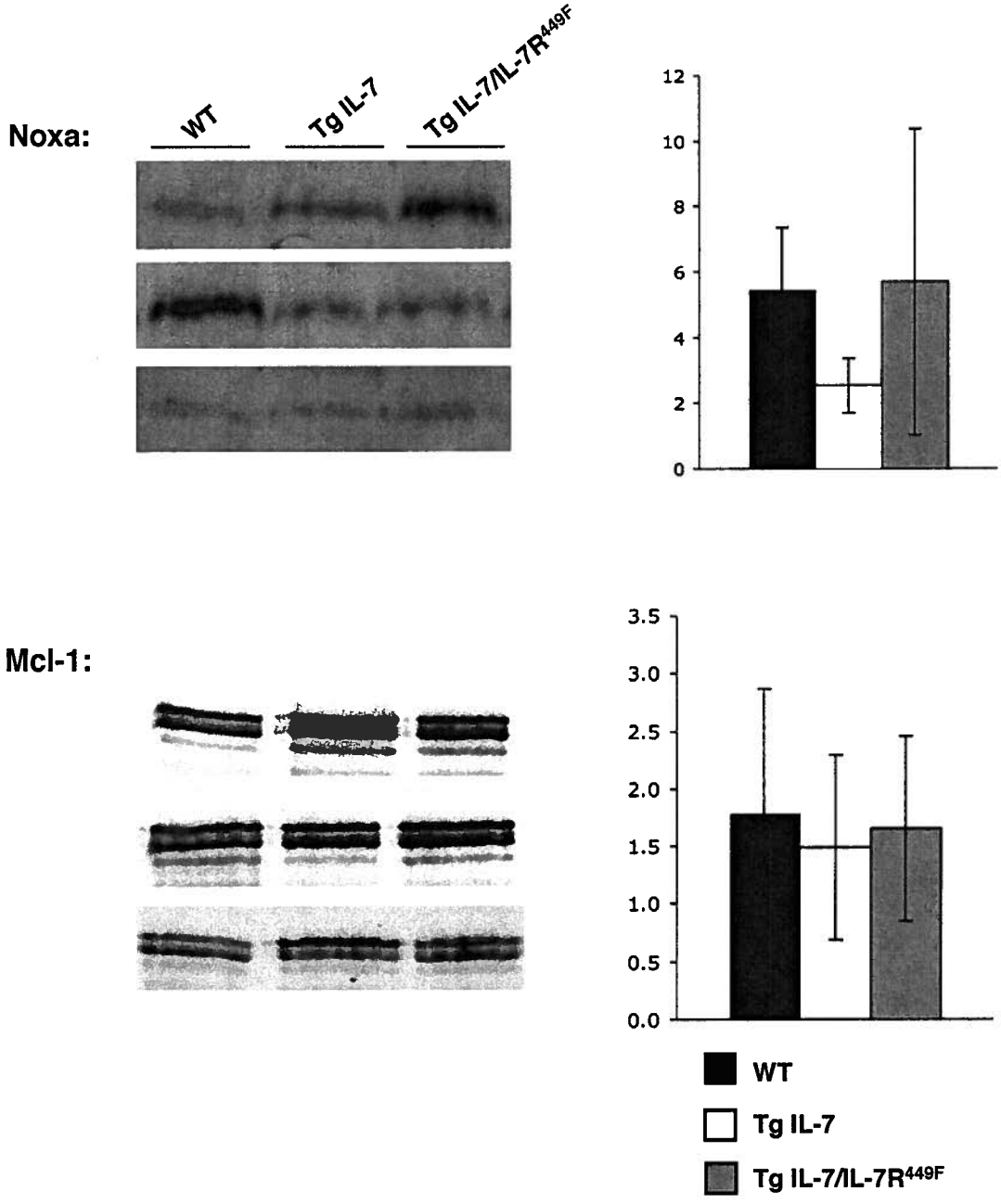


**Figure 3.3: Mcl-1 and Noxa regulation by IL-7Rα Y449 in a steady state setting.** Figure 1b shows chronic Mcl-1 and Noxa levels in total splenocyte lysate from WT, Tg IL-7 and Tg IL-7/ IL-7R$^{449F}$ mice . Total splenocytes were isolated from 12 week old mice, lysed in RIPA lysis buffer and stored at -20°C. 30 ug of protein was resolved by polyacrylamide gel electrophoresis and analyzed by western blot using rabbit polyclonal anti-Mcl-1 antibody or mouse monoclonal anti-Noxa antibody. Protein levels were quantified and normalized to total protein loaded by coomassie blue staining using a LI-COR Odyssey infra-red imager and represent 'Integrated Intensity" values.

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# APPENDIX

THE UNIVERSITY OF BRITISH COLUMBIA

# ANIMAL CARE CERTIFICATE

**Application Number:** A07-0417

**Investigator or Course Director:** Ninan Abraham

**Department:** Microbiology & Immunology

**Animals:**

| |
|---|
| Mice IL-7R knockout 48 |
| Mice OT-II 210 |
| Mice IL-7R449F knock-in 206 |
| Mice STAT5 knockout 36 |
| Mice C57BL/6 260 |
| Mice Tg Myc 172 |
| Mice BoyJ 116 |
| Mice OT-I 210 |
| Mice Thy1.1 60 |
| Mice Tg IL-7 72 |
| Mice p110 delta knock-in 105 |

**Start Date:** August 1, 2007 **Approval Date:** April 14, 2008

**Funding Sources:**

| | |
|---|---|
| **Funding Agency:** | Canadian Institutes of Health Research (CIHR) |
| **Funding Title:** | IL-7 Signaling in Normal and Aberrant T cell Development and Function |
| **Funding Agency:** | Canada Foundation for Innovation |
| **Funding Title:** | Laboratory of Immune System Functional Genomics |
| **Funding Agency:** | Canada Foundation for Innovation |
| **Funding Title:** | CFI Infrastructure Operating Funds |
| **Funding Agency:** | Michael Smith Foundation for Health Research |
| **Funding Title:** | IL-7 Singaling in normal and aberrant T cell development and function |
| **Funding Agency:** | Cancer Research Society |
| **Funding Title:** | Functional genomic and proteomic approaches to identify novel survival effectors induced by IL-7 |
| **Unfunded title:** | n/a |

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

**A copy of this certificate must be displayed in your animal facility.**

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

The University of British Columbia

# Biohazard Approval Certificate

PROTOCOL NUMBER: **H06-0113**

INVESTIGATOR OR COURSE DIRECTOR: **Abraham, Ninan**

DEPARTMENT: **Microbiology & Immunology**

PROJECT OR COURSE TITLE: **IL-7 Signalling in Normal and aberrant T cell Development and Function**

APPROVAL DATE: **08-08-08**

APPROVED CONTAINMENT LEVEL: **2**

FUNDING AGENCY: **Canadian Institutes of Health Research (CIHR)**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

[redacted]

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required.

**A copy of this certificate must be displayed in your facility.**

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