TYPE I INTERFERON SIGNALING PATHWAY MAY BE INVOLVED IN OPTIMAL INTERLEUKIN-2 PRODUCTION IN $CD4^+$ T CELLS

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DEDICATION

To my friends & family,
who were by my side before, during, and after.

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by

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KRISTAN ANDREA HAGAN

The University of Texas Southwestern Medical Center at Dallas, 2012

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Previous studies demonstrate that interferon alpha (IFN-α) promotes human T helper 1 memory development by positively regulating interleukin-2 (IL-2) expression, a hallmark cytokine of central memory $CD4^+$ T cells. The present work seeks to delineate how signaling through the interferon alpha/beta receptor promotes IL-2 secretion in murine $CD4^+$ T cell.

IL-2 provides an essential growth signal to naïve and memory T cells. Examining naïve OT-II interferon alpha-receptor-2 (IFNAR2) knockout $CD4^+$ T

cells *in vitro*, we observed conflicting results in secreted IL-2 protein at 72 hours post-stimulation and intracellular IL-2 protein expression at 48 hours post-stimulation. Subsequent studies suggest this was not due to lack of proliferation or an inherit defect in IL-2 secretion. In response to an *in vivo* vesicular stomatitis virus infection, OT-II IFNAR2 knockout $CD4^+$ T cells displayed defective expansion at seven days post-infection.

Signaling through the interferon alpha-receptor may enhance IL-2 expression in $CD4^+$ T cells, indicating a possible role for downstream transcription factors in sustaining IL-2 expression. Interestingly, $CD4^+$ T cells with a carboxy-terminal substitution in the signal transducer of activator of transcription-2 (STAT2) protein may display a similar defect in T cell receptor mediated IL-2 expression. IFNAR2 knockout and STAT2 knock-in splenocytes may display a defect in IL-2 mRNA expression as early as twelve hours post-stimulation. However, IL-2 promoter activity in OT-II IFNAR2 knockout $CD4^+$ T cells is insignificantly increased compared to OT-II wild-type cells, suggesting analysis of IL-2 mRNA stability be examined more closely.

Preliminary data suggests STAT2 may translocate to the nucleus in response to TCR stimulation in $CD4^+$ T cells and could potentially bind to a putative interferon stimulated response element within the IL-2 promoter. Our findings provide insight into how the type I IFN signaling pathway may play a role regulate IL-2 expression in $CD4^+$ T cells.

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1. Davis, A. M., K. A. Hagan, L. A. Matthews, G. Bajwa, M. A. Gill, M. Gale, Jr., and J. D. Farrar (2008). Blockade of virus infection by human CD4+ T cells via a cytokine relay network. Journal of Immunology 180: 6923-6932.

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LIST OF ABBREVIATIONS

Ag - antigen

AP-1 – activator protein-1

APCs – antigen presenting cells

AREs – AU-rich elements

ARRE-1/2 – antigen receptor-responsive elements

bp – base pair

CBP – CREB binding protein

CCR – chemokine (C-C motif) receptor

CCL – (C-C motif) ligand

CD – cluster of differentiation

CD28RR – CD28-responsive element

CFSE – carboxyfluorescein diacetate, succinimidyl ester

cDMEM – complete Dulbecco's Modified Eagle Medium

cIMDM – complete Iscove Modified Dulbecco Medium

CTL – cytolytic T lymphocytes

CXCR – chemokine (C-X-C motif) receptor

DBD – DNA binding domain

DCs – dendritic cells

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

ERK – extracellular signal-regulated kinases

FOXP3 – fork head box P3

GFP – green fluorescent protein

HCV – Hepatitis C virus

IFN – interferon

IFNAR – interferon α receptor

IFNGR – interferon-γ receptor

IκBα – inhibitor of κBα

IKK – inhibitor of nuclear factor κB kinase β

IL – interleukin

IPS-I – interferon beta promoter stimulator-I

IRF – interferon regulatory factor

ISGs – interferon stimulated genes

ISGF3 – interferon stimulated gene factor-3

ISRE – interferon stimulated response element

JAK – Janus kinase

Kb - kilobases

Knock-in (KI)

LCMV – lymphocytic choriomeningitis virus

LT – lymphotoxin

LTR – long terminal repeats

MAP – mitogen activated protein

MAPK – mitogen activated protein kinase

MCM5 - minichromosome maintenance complex component 5

MDA-5 – melanoma differentiated-associated gene-5

MEFs – mouse embryo fibroblasts

MHC II – major histocompatibility complex II

MyD88 - myeloid differentiation factor

NF90 – nuclear factor 90

NFAT – nuclear factor of activated T cell

NF-κB – nuclear factor κB

NS3/4A – nonstructural protein 3/4A

NS5A – non-structural protein 5A

OVA – ovalbumin

PAMPs – pathogen-associated molecular patterns

PBMC – peripheral blood mononuclear cells

pDCs – plasmacytoid dendritic cells

PKC – protein kinase C

PRD – positive regulatory domains

p-MHC II – peptide class II MHC complex

PRR – pattern recognition receptors

RIG-I – retinoic acid inducible gene-I

RNA – ribonucleic acid

RORγt – retinoic-acid-receptor-related orphan receptor γt

SCLC – small cell lung cancer

SDF-1α – stromal cell-derived factor-1α

SLE – systemic lupus erythematosus

SLP-76 – src homology 2 domain-containing leukocyte protein of 76 kDa

STAT – signal transducer and activator of T cells

T-bet – T box expressed in T cells

TAD – transcriptional activation domain

TCR – T cell receptor

T_H – T helper

TGF-β – transforming growth factor –β

TLR – toll like receptor

TNF-α – tumor necrosis factor-α

T_{reg} – T regulatory

TRAF3 – TNF receptor associated factor

TRIF – TIR-domain-containing adaptor-inducing interferon-β

TSS – transcription start site

TTP - tristetraprolin

TYK – tyrosine kinase

VSV – vesicular stomatitis virus

ZAP-70 – zeta-chain-associated protein kinase-70

CHAPTER I

INTRODUCTION

Pathogens elicit an innate and adaptive immune response.

During an immune response to an intracellular pathogen, antigen-presenting cells (APCs) recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) (1). Toll like receptor 3 (TLR3), TLR7, TLR8 and TLR9 are PRRs that detect ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) in endoplasmic compartments (2-4). While TLR1, TLR2, TLR4 and TLR6 recognize a diverse range of PAMPs that range from viral proteins to bacterial lipopeptides (1). TLR5 and TLR11 recognize flagellin from *Salmonella typhimurium* and profilins from *Toxoplasma gondii* respectively. TLRs signal through downstream proteins, myeloid differentiation factor (MyD88) or TIR-domain-containing adaptor-inducing interferon-β (TRIF) to activate NF-κB, MAPK, interferon regulatory factor 3 (IRF3) and IRF7. Activation of these transcription factors leads to increased expression of interferon (IFN) and pro-inflammatory cytokines.

In the cytoplasm retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated-gene-5 (MDA 5) detect either viral RNA or DNA. These proteins signal via the adaptor protein interferon beta promoter stimulator-1 (IPS-1) to downstream transcription factors interferon regulatory factor-3 (IRF3) or nuclear factor kappa B (NF-κB) to regulate IFN and pro-inflammatory cytokine expression (5-9).

Interferon regulation

There are three types of IFNs that signal through distinct signaling pathways. Type I IFN (IFN-α, β, ω, ε and κ) binds the high affinity IFN receptor (IFNAR2). This recruits IFNAR1, to form the type I IFN signaling complex. This complex activates and transphosphorylates Tyk2 and JAK1 which phosphorylate the cytoplasmic tail of IFNAR1 at tyrosine 466. STAT2, that is suggested to be associated with IFNAR2, can then bind tyrosine 466, and in turn is phosphorylated at tyroine 690, providing a docking site for STAT1. STAT1 is phosphorylated at tyrosine 701 in response to type I IFN signaling. STAT1 and STAT2 heterodimerize and bind IRF9 to form ISGF3 then translocate to the nucleus to bind interferon stimulated response elements (ISRE) within gene promoters (**Figure 1.1**) (10).

Type I IFNs are highly expressed during viral infections by plasmacytoid dendritic cells (pDCs) and virally infected cells (10). Ito and colleagues demonstrated that in response to herpes simplex virus infection, pDCs secrete type I IFN by 8 hours and protein levels peak by 12 hours post-infection (11). Buller and colleagues suggest in response to T cell receptor stimulation (TCR), T cells express 1/200 the mRNA level of type I IFN (12). It is suggested weak type I IFN may allow for efficient TCR activation in response to TCR stimulation (10). This study will examine how type I IFN affects TCR induced IL-2 expression in cluster of differentiation 4^+ ($CD4^+$) T cells.

T cell receptor signaling

In naïve $CD4^+$ T cells, TCR engagement through p-MHC II and CD28 co-stimulation results in activation of downstream transcription factors that play a role in increased expression of TCR regulated genes (13). In response to TCR stimulation NFAT is de-phosphorylated and translocates to the nucleus in response to an increase in intracellular calcium. Protein kinase C θ (PKC θ) is activated by diacylglycerol (DAG) in response to the hydrolysis of PIP2 into two second messengers, DAG and IP3. Activated PKC θ leads to phosphorylation of inhibitor of nuclear factor κB kinase β (IKKβ) that in turn phosphorylates inhibitor of κBα (IκBα) at serine residues 32 and 36 (14-16). IκBα is bound to

NF-κB in the cytoplasm until phosphorylated and subsequently ubiquitinated when it then becomes rapidly degraded and releases NF-κB to translocate to the nucleus to bind the promoters of TCR regulated genes (15, 16). Activator protein 1 (AP-1) and Oct1 translocate to the nucleus in response to DAG activation of the mitogen-activated protein kinase (MAPK) pathway (**Figure 1.2**).

Differentiation of CD4$^+$ T helper subsets

APCs secrete a wide range of cytokines that can drive differentiation of naïve CD4$^+$ T cells into specific T helper (T_H) subsets (17). Two main types of APCs present foreign peptide through the major histocompatibility complex II (MHC II) to CD4$^+$ T cells. Dendritic cells (DCs) present foreign peptide to naïve CD4$^+$ T cells and secrete interleukin 12 (IL-12), IL-6, IL-23, and type I IFN, amongst other cytokines (18, 19). While macrophages, the second type of APC that can present to CD4$^+$ T cells, secrete a variety of cytokines including IL-12, IL-18, tumor necrosis factor–α (TNF-α), transforming growth factor (TGF-β), and IL-6 (19, 20).

In response to TGF-β and IL-2, CD4$^+$ T cells differentiate into T regulatory (T_{reg}) cells. TGF-β signaling induces expression of the transcription factor fork head box P3 (FOXP3). T_{reg} cells play a role in immunosuppression and tolerance through secretion of IL-10 and TGF-β (21-23).

$CD4^+$ T cells that encounter IL-4 and whose TCR is engaged differentiate into T_H2 cells and play a role in immune responses to extracellular parasites. IL-4 signaling activates STAT6, which leads to expression of *Gata3*. Gata3 regulates expression of T_H2 cytokines IL-4, IL-5, and IL-13, which lead to activation of B cells, macrophages, and eosinophils (24). $CD4^+$ T cells differentiate into T_H1 cells in response to IL-12 and IFN-γ, and play an important role in the adaptive immune response. IFN-γ activates STAT1, which leads to the increased expression of the transcription factor *T-bet*. T-bet plays an important role in $CD4^+$ T cell differentiation and is considered the master regulator of T_H1 differentiation. IL-12Rβ2 expression in induced in response to TCR and IFN-γ signaling, and pairs with the IL-12Rβ1 to form the IL-12 receptor. T_H1 cells respond to IL-12 from APCs to induce a positive feedback loop by inducing expression of T-bet IFN-γ (22).

In response to IL-6 and TGF-β, naïve $CD4^+$ T cells differentiate into T_H17 cells. IL-6 activation of STAT3, leads to increased expression of retinoic-acid-receptor-related orphan receptor γt (RORγt). RORγt regulates expression of T_H17 cytokine genes *il21* and *il17*. IL-21 secretion creates a positive feedback loop that activates STAT3, leading to IL-23R expression. This allows newly differentiated T_H17 cells to respond to IL-23, secreted by APCs, and activate STAT3, which leads to decreased expression of IL-10 and increased expression of IL-22.

Aberrant activation of T_H17 cells occurs in autoimmune disorders such as psoriasis and rheumatoid arthritis (**Figure 1.3**) (22).

Type I IFN is highly expressed by pDC and infected cells in response to a pathogen infection. While type I IFN was not observed to play a role in $CD4^+$ differentiation, there was a role identified for type 1 IFN in enhancing IL-2 secretion in a central memory population of human T_H1 $CD4^+$ T cells. IL-2 is a potent growth factor, and is required for proliferation and expansion of $CD4^+$ and $CD8^+$ T cells.

Role of $CD4^+$ T_H1 cells in the adaptive immune response.

Naïve $CD4^+$ T cells can differentiate into multiple T helper (T_H) subsets in response to T cell receptor (TCR) stimulation and specific cytokine stimuli (17). Naïve $CD4^+$ T cells have high C-C chemokine receptor-7 (CCR7) expression and respond to ligands chemokine (C-C) motif ligand-21 (CCL21) and CCL19 (25). This cell type can migrate to lymph nodes in response to these two ligands, where they can potentially respond to mature DCs presenting foreign peptide through p-MHC II (25, 26). Nuclear factor of activated T cells (NFAT) is activated in response to TCR and co-stimulatory signals. Activation of NFAT regulates the expression of cytokines, growth factors and proliferation in $CD4^+$ T cells. In response to IL-12 and TCR stimulation through p-MHC II, T-bet expression is

up-regulated and naïve $CD4^+$ T cells differentiate into T_H1 cells with the ability to secrete IFN-γ (27, 28). IFN-γ binds to the IFN-γ receptor (IFNGR), activating Janus kinase-2 (JAK2) which subsequently activates JAK1. STAT1 binds to phosphorylated portions of the IFNGR, and is in turn phosphorylated leading to homodimerization of activated STAT1 and nuclear translocation (29). IFN-γ feeds back on T_H1 cells, leading to increased IL-12Rβ2 expression, creating a positive feedback loop for cells to respond to IL-12 (30).

T_H1 cells play an important role in an adaptive immune response by secreting a number of cytokines that instruct immune cells. IFN-γ secreted by T_H1 cells not only act on $CD4^+$ T cells, but also instructs macrophages to increase phagocytosis and microbiocidal activity to aid in killing foreign microbes (31). Secreted IFNγ, TNF-α and lymphotoxin (LT/TGF-β) from T_H1 cells activate neutrophils and instruct this cell type to also increase microbicidal activity (32).

IFN-α and IL-12 polarized human $CD4^+$ T cells differentially express IL-2

Two cytokines that are highly expressed in response to infections are IL-12 and IFN-α. A recent study from Davis and colleagues examined human $CD4^+$ T cells polarized with both IL-12 and IFN-α indicated that IL-2 secretion was differentially expressed, when compared to IFN-γ and TNF-α (33). It was

suggested that polarization with IFN-α and IL-12 drive the differentiation a central memory T_H1 $CD4^+$ T cell (33). There are three types of T_H1 cells, one of which is the effector memory $CD4^+$ T cell. These cells have high chemokine (C-X-C motif) receptor 3 (CXCR3) and low CCR7 receptor expression, which allows the cells to migrate to peripheral tissues and induce rapid effector function upon re-stimulation. Effector memory T_H1 $CD4^+$ T cells induce high levels of IFN-γ and TNF-α and provide the body with immediate protection during re-infections (26, 34).

The other two CD4+ T cells are capable of secreting high levels of IL-2, naïve and central memory T_H1 $CD4^+$ T cells (33). Cells expressing $CCR7^{hi}$ and CD45RA- have been accepted as central memory $CD4^+$ T_H1 cells. Central memory $CD4^+$ T cells, have acquired the ability to respond quickly to re-stimulation, resulting in rapid proliferation and subsequent effector cell function (34). Most importantly, central memory cells are associated with long-term immunological memory, with the ability to protect the host during many subsequent infections. Understanding how to drive differentiation of T_H1 $CD4^+$ central memory T cells would allow scientists and doctors to re-evaluate how to immunize patients to most effectively deliver a vaccine that would provide the most robust induction of central memory T_H1 $CD4^+$ T cells. Davis et al. examined chemokine receptor expression on the population of IL-2 secreting T_H1

$CD4^+$ T cells, and found the cells expressed high CCR7 and low CD45RA receptor expression, characteristic of central memory $CD4^+$ T cells (33, 34).

Role of IL-2 in T cell proliferation

IL-2 was first identified as T cell growth factor that provided a signal to T cells to proliferate and expand (35). The four transcription factors, NFAT, NF-κB, AP-1 and Oct1 bind to the IL-2 promoter with additional transcriptional machinery and form a nucleosome near -300bp upstream of the *il2* gene start site (36). Co-stimulation through CD28, by B7-1 and B7-2 on APCs is thought to boost activation of phosphoinositide-3-kinase that helps to provide a stronger signal for activation (13).

Studies have suggested that the kinetics of IL-2 expression is delayed in naïve $CD4^+$ T cells, when compared to re-stimulated cells (37). The thought behind this is the modification of specific regions of the IL-2 promoter that allow for more easily accessible regions for transcription factors and transcriptional enhancers during a re-stimulation (38). When proteins involved in histone modifications are absent, there was a clear effect on IL-2 promoter activity (13). IL-2 expression has two phases of regulation, with the first phase independent of de novo protein expression, while the second phase is dependent on de novo

protein expression and histone modification (36, 37). Contrary, by blocking new protein synthesis, there is an enhancement in IL-2 expression (39, 40). This indicates there is a regulatory mechanism induced post TCR stimulation to ensure IL-2 expression is not left uncontrolled (38, 41, 42).

Three chains comprise the IL-2 signaling complex, IL-2Rα, β and γ; prior to activation the high-affinity chain, IL-2Rα, is not present on the cell surface. In response to TCR stimulation expression of the high-affinity IL-2 chain, IL-2Rα, is increased allowing activated $CD4^+$ T cells to respond to IL-2 (43, 44). IL-2Rβ and IL-2Rγ, are constitutively expressed on the cell surface, and signal downstream in response to IL-2 ligand binding. After ligand binding, Jak1 and Jak3 are auto-phosphorylated and proceed to phosphorylate and activate STAT5, which then translocates to the nucleus as a homodimer (45). STAT5 regulates genes involved in proliferation and expansion of $CD4^+$ T cells (46). Blocking IL-2Rα results in a defect in proliferation in $CD4^+$ T cells, thus sustained IL-2 is necessary to proliferate and expand (**Figure 1.4)** (47).

$CD4^+$ T cells provide help to $CD8^+$ T cells

$CD8^+$ cytolytic T lymphocytes (CTL) play an important role in inducing apoptosis in infected target cells. Naïve $CD8^+$ T cells recognize foreign peptides

on MHC class I and receive multiple "signals" that differentiate naïve cells into effector CTLs. The first signal is recognition of foreign antigen through TCR-p-MHC I interaction. The second signal is help from $CD4^+$ T cells, in providing IL-2. The third signal is cytokines, such as IL-12 and IFN-α secreted from antigen presenting cells that drive CTL differentiation into effector and memory $CD8^+$ T cells (48).

It has been observed that $CD8^+$ T cells need "help" and it was speculated that IL-2 was essential for this secondary signal (49). Multiple groups have since shown IL-2 and $CD4^+$ T cells are essential in $CD8^+$ effector responses (50, 51). Not until recently did Wilson and colleagues show IL-2 deficient $CD4^+$ T cells failed to induce a primary immune effector response from $CD8^+$ T cells, in response to peptide stimulation (52). Although, *in vivo* studies examining infections with LCMV indicate that primary expansion of effector CTLs is intact, but IL-2 is essential for effector memory expansion during a re-infection (50, 51). Learning how to increase the differentiation of central memory IL-2 secreting $CD4^+$ T cells is essential in providing help to memory $CD8^+$ T cells.

Concluding Remarks

Previous reports emphasize the significance of the type I IFN signaling pathway, not only for its role in crosstalk with the type II IFN signaling pathway but also in enhanced IL-2 expression from central memory $CD4^+$ T_H1 cells. These results suggest an involvement of the type I IFN signaling pathway in enhancing a TCR mediated event. To further investigate the involvement of the type I IFN signaling pathway in $CD4^+$ T cells, it is necessary to utilize a murine model that contains a knockout of a component of the type I IFN receptor complex (53).

Aforementioned there are two populations that secrete a high concentration of IL-2 upon stimulation, central memory as well as naïve $CD4^+$ T cells. Previous work from Davis and colleagues examined the effect of IFN-α and IL-12 polarization on naïve human $CD4^+$ T cells that were re-stimulated. There was an observed enhancement of IL-2 secretion from re-stimulated $CD4^+$ T cells that were polarized with IFN-α and IL-12 (33). This observation begs the question of what the effect of IFN-α treatment is on naïve $CD4^+$ T cells. It also questions whether there are other cytokines or chemokines that can influence IL-2 expression, through receptor cross talk. Subsequent chapters will address the possibility of type I IFN receptor crosstalk with the TCR, to induce a specific

downstream signaling event (54, 55). While, IL-2 expression was not examined in these studies, it suggests IFN-α treatment and TCR stimulation can produce an additive effect when examining downstream TCR signaling events.

The mechanism of action behind IFN-α treatment and enhanced IL-2 secretion remains unknown. Investigating downstream transcription factors of the type I IFN signaling pathway may elucidate a possible mechanism. Again, taking advantage of murine models will allow manipulation of specific proteins of interest to more clearly determine how the type I IFN signaling pathway plays a role in enhanced IL-2 expression.

This study will address the role of the type I IFN receptor in IL-2 expression and begin examining the role of downstream signaling protein, STAT2, in response to TCR stimulation and sustained IL-2 expression. Preliminary results examining the IL-2 promoter suggest a putative interferon stimulated response element (ISRE) sequence just outside the enhanceosome region of the IL-2 promoter. These findings move the field forward to further understand the role of the type I IFN signaling pathway in sustained IL-2 expression.

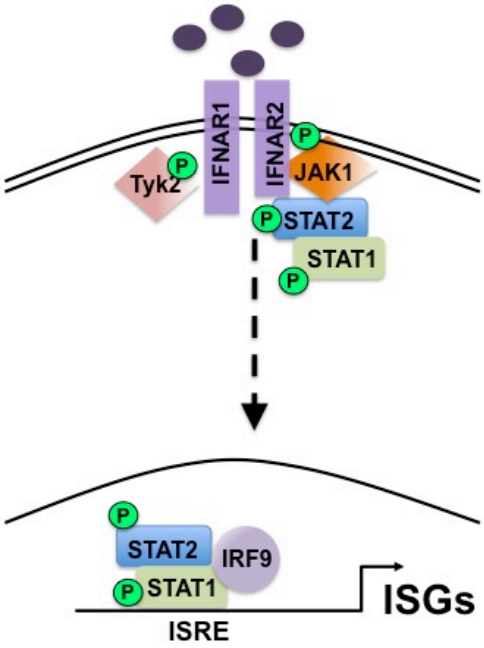

Figure 1.1 Type I IFN signaling results in upregulation of interferon stimulated genes (ISGs)

Type I IFN (IFN-α or IFN-β) binds the high affinity IFN receptor (IFNAR2). This recruits IFNAR2, to form the type I IFN signaling complex. This complex activates and transphosphorylates Tyk2 and JAK1 which phosphorylate the cytoplasmic tail of IFNAR1 at tyrosine 466. STAT2, that is suggested to be associated with IFNAR2, can then bind tyrosine 466, and in turn is phosphorylated at tyroine 690, providing a docking site for STAT1. STAT1 is phosphorylated at tyrosine 701 in response to type I IFN signaling. STAT1 and STAT2 heterodimerize and bind IRF9 to form ISGF3 then translocate to the nucleus to bind interferon stimulated response elements (ISRE) within gene promoters.

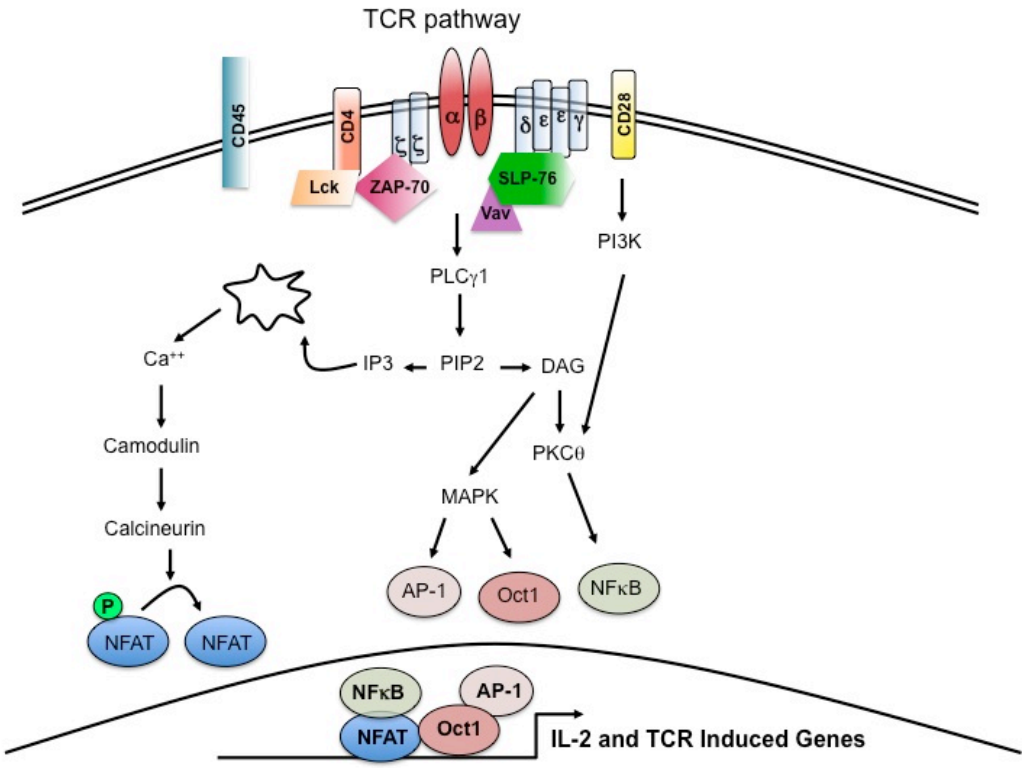

Figure 1.2 T cell receptor stimulation drives IL-2 and TCR induced gene expression through activation of essential upstream transcription factors

Engagement of the T cell receptor (TCR) with foreign peptide, presented by antigen presenting cells results in downstream activation of phospholipase C g1 (PLCγ1). PLCγ1 cleaves phosphatidylinositol 4,5 biphosphate (PIP2) into inositol triphosphate (IP3) and diacyl glycerol (DAG), second messengers capable of activating downstream transcription factors. DAG activates nuclear factor κB (NF-κB), activator protein 1 (AP-1) and Oct1. While IP3 results in release of calcium stores from endoplasmic reticulum and downstream activation of nuclear factor of activated T-cells (NFAT). AP-1, Oct1, NF-κB and NFAT translocate to the nucleus upon activation and bind to promoters that regulate expression of IL-2 and TCR induced genes.

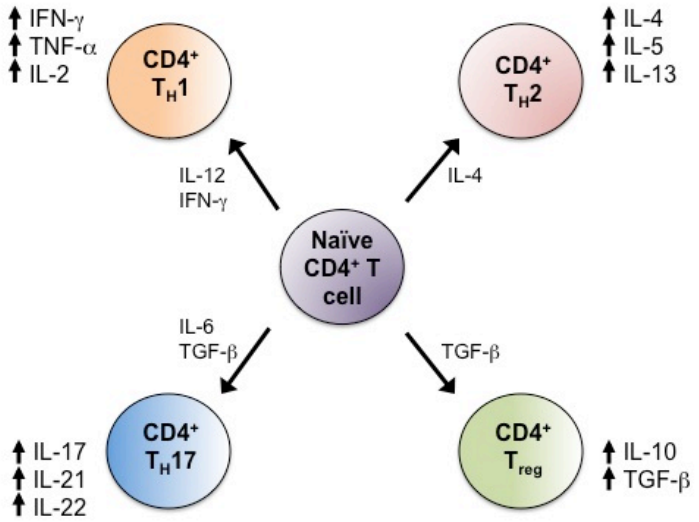

Figure 1.3 Differentiation of naïve human $CD4^+$ T cells

The type of cytokines that a naïve $CD4^+$ T cells encounters drives the differentiation of T helper cell subsets. $CD4^+$ T cells that encounter IL-12 and IFN-γ differentiate into $CD4^+$ T_H1 cells that highly express IL-2, IFN-γ, and TNF-α during intracellular pathogen infections. In response to IL-4 cells differentiate into $CD4^+$ T_H2 cells that secrete high levels of IL-4, IL-5, and IL-13 in response to a parasitic or allergic response. Cells that play a role in dampening an immune response are differentiated in response to TGF-β and are known as $CD4^+$ T regulatory cells. T_{reg} cells highly express IL-10 and TGF-β. A more recently identified T helper subset, T_H17 cells, are differentiated in response to IL-6 and TGF-β. These cells play an important role in inflammatory responses through the secretion of IL-17, IL-21 and IL22.

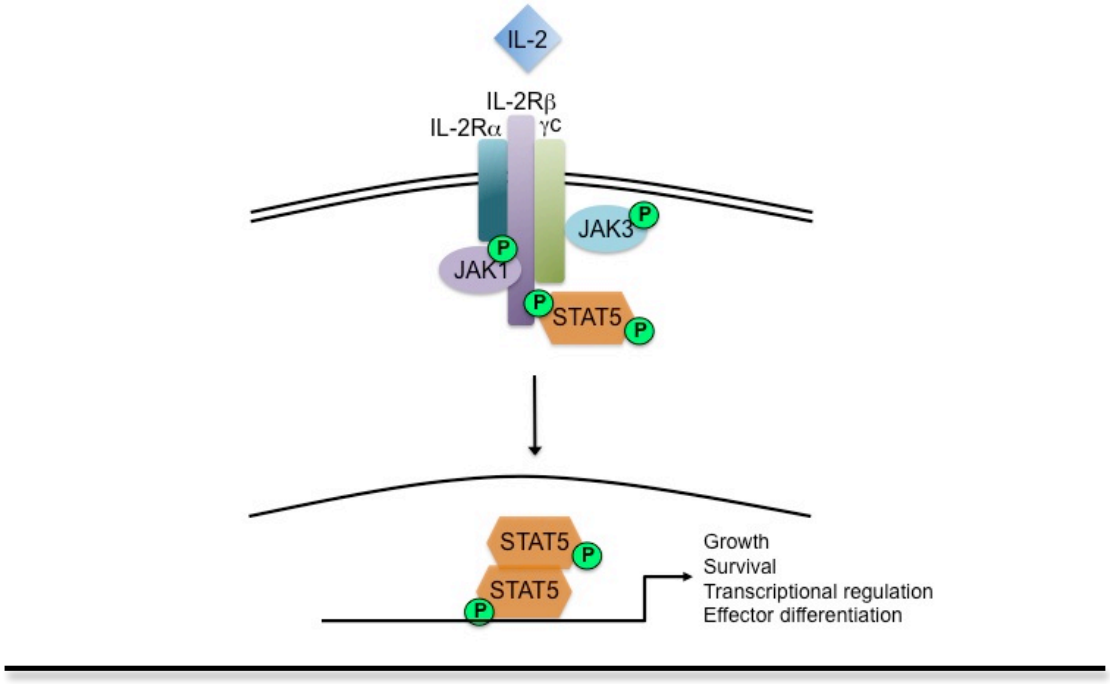

Figure 1.4 IL-2 receptor signaling

In response to IL-2 binding, the high affinity IL-2 receptor (IL-2Rα), IL-2Rβ, and γc activate downstream signaling proteins. JAK1 and JAK3 are autophosphorylated, which then proceed to phosphorylate the cytoplasmic tail of IL-2Rβ. STAT5 binds to the phosphorylated tail of IL-2Rβ, leading to phosphorylation of STAT5, and subsequent dimerization and nuclear translocation. STAT5 is involved in regulation expression of cell proliferation, survival, transcriptional regulation and effector differentiation.

CHAPTER II

MATERIALS AND METHODS

Mice

WT, IFNAR2-/- (gift from M. Diamond, Washington University, St. Louis, MO), and m/h STAT2 knock-in mice on a C57BL/6 background were crossed onto the OT-II background and housed in specific pathogen-free conditions in accordance with guidelines established by the Institutional Animal Care and Use Committee (University of Texas Southwestern Medical Center) (56). Mice were monitored daily for illness and experiments were approved and performed in accordance with the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center, Dallas, TX.

Purification of murine $CD4^+$ T cells

Naïve murine $CD4^+$ T cells were purified from C57BL/6 OT-II transgenic WT, IFNAR2-/-, m/h STAT2 KI that recognize ovalbumin (OVA) peptide 323-339 (56). Spleen and lymph nodes were harvested from mice (age 8 – 14 weeks) and

organs homogenized and placed on a nylon wool column for 1 hour to eliminate B cells and antigen-presenting cells. Unattached cells were rinsed off the column using complete Iscove modified Dulbecco medium (cIMDM) and used for subsequent studies. $CD4^+$ T cells were enriched using the Invitrogen murine $CD4^+$ T cell negative isolation kit (Invitrogen).

Polarizing culture conditions

Whole splenocytes and lymph nodes (5×10^6 cells/ml) were cultured under T_H1 polarizing conditions (anti-IL4 (11B11; 10 mg/ml), rmIL-12 (10 ng/ml), rmIFNγ (5 ng/ml), rhIL-2 (50 U/ml) and in the presence or absence of universal type 1 interferon (500 U/ml). Unpolarized cells (5×10^6 cells/ml) were cultured under neutralized conditions (anti-IL-4 (11B11; 10 μg/ml), anti-IL-12 (Tosh; 10 μg/ml), anti-IFNγ (R46A2), and rhIL-2 (50 U/ml). Cultures were activated with .5 μM OVA peptide for 3 days and split (1:10) into medium containing rhIL-2 (50 U/ml) and cultured until day 7. Cells were washing and re-stimulated (1×10^6 cells/ml) with 1.5 μg/ml plate bound anti-CD3 (2C11; eBioscience).

Cell stimulation

$CD4^+$ T cells (1 x 10^6 cells/ml) were either stimulated with plate bound anti-CD3 (1.5 μg/ml; clone 145-2C11, gift from Nicolai van Oers, Ph.D. UTSW Medical Center, Dallas, TX or purchased from R&D Systems) and anti-CD28 (1.5 μg/ml; clone 37.51, gift from Nicolai van Oers, Ph.D. UTSW Medical Center, Dallas, TX or purchased from eBioscience) or whole splenocytes (5 x 10^6 cells/ml) treated with .5 μM OVA peptide (GenScript) for the indicated time. To pharmacologically stimulate cells, 1 mM Ionomycin (Sigma) and 50 ng/ml phorbol-12-myristate-13 acetate (PMA) (A.G. Scientific, Inc) were added to $CD4^+$ T cell cultures for indicated time.

Cytokines, Abs, and reagents

Anti-IFNAR2 (MAB1083) and isotype (MAB006) control antibodies was purchased through R&D Systems. Recombinant mouse IL-12 and IFN-γ were purchased through R&D Systems. Universal type 1 interferon was purchased through PBL Interferon Source. FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse IL-2 were purchased eBioscience. Actinomycin D was purchased from Sigma. Polyclonal rabbit anti-mouse STAT2 was purchased from

Santa Cruz Biotechnology. Anti-rabbit AlexaFluor 488 was purchased from Invitrogen.

Intracellular cytokine staining

For intracellular cytokine staining, whole splenocytes (5 x 10^6 cells/ml) were cultured in 48 well flat bottom plates, and stimulated with .5 μM OVA peptide for the indicated time. Monensin (1X, BioLegend) was added to cultures during the last 4 hours of stimulation to prevent secretion of intracellular proteins. Cells were harvested, fixed in 2.5% Formalin, permeabilized with .2% saponin in 1X PBS/ .5% BSA, and stained with fluorochrome conjugated IL-2 and CD4.

Analysis of STAT2 translocation in $CD4^+$ T cells

Spleen and lymph nodes were harvested from OT-II mice and stimulated at 5 x 10^6 cells/ml in 12 well plates with .5 μM OVA peptide or 1000 U/ml universal type I IFN for the indicated time. Cells were harvested and transferred to pre-chilled 1.5 ml eppendorf tubes and washed with ice cold 1X PBS to remove excess media. Cells were stained for PE anti-mouse CD4 in 1X PBS/ .5% BSA then washed with 1X PBS and fixed with 4% PFA (Sigma) for 12 minutes. Next, fixed cells were permeabilized with .1% TritonX-100 in 1X PBS/ .5% BSA for 10

minutes are room temperature. Cells were stained for anti-mouse STAT2 (sc-950) followed by secondary detection of AF488 in .1% TritonX-100 in 1X PBS/ .5% BSA for 30 minutes are room temperature. DAPI stain was added to samples and analyzed using ImageStream imaging flow cytometer (Raymond Kong, Ph.D., Amnis Corporation, Seattle, WA). $CD4^+$ T cells were gated on, and compared for co-localization of DAPI to STAT2-AF488 between unstimulated and each stimulated time point.

Proliferation assay using CFSE

Purified naïve murine $CD4^+$ T cells were stained with 1 μM 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 minutes, which was subsequently quenched using supplemented IMDM, with 20% FBS. $CD4^+$ T cells were cultured with irradiated B6 splenocytes (at a ratio of 1:10) and stimulated with .3 μM OVA peptide or 1.5 μg/ml anti-CD3 for the indicated time.

Alternatively, purified $CD4^+$ T cells that were stained with CFSE were stimulated with plate bound anti-CD3 and anti-CD28 in the presence of drift, neutralized or polarizing conditions. Cells were stained for CD4 and analyzed by flow cytometry at 48 or 72 hours post-stimulation.

Quantitative real-time (qPCR) analysis

Whole splenocyte (5 x 10^6 cells/ml) cultures were stimulated with .5 μM OVA peptide for indicated time. Cells were harvested with Trizol (Invitrogen) and total RNA extracted following manufacturer's instructions. 750 ng of RNA was used for reverse transcription using ABI High Capacity RT-PCR kit (Applied Biosystems). IL-2 and GAPDH mRNA expression was measured using Taqman primers (Applied Biosystems) on an ABI7300 real-time thermocycler (Applied Biosytems). Murine GAPDH was used as a reference. Relative changes in mRNA expression were calculated using a method previously described and were referenced to strain specific unstimulated controls (57).

IL-2 and IFN-γ Enzyme-linked immunosorbent assay (ELISA)

Cells (1 x 10^6 cells/ml) were stimulated for the indicated time and supernatants collected and IL-2 and IFN-γ protein measured by ELISA. IL-2 ELISA (Biolegend) was performed according to manufacturers' protocol. IFN-γ ELISA was performed as previously described (56). Nunc Maxisorp plates were coated overnight with purified mAb R4-6A2 (3 μg/ml; BD Biosciences), and IFN-γ protein was detected with biotin-conjugated mAb XMG1.2 (.5 μg/ml; Biolegend).

Samples were incubated with streptavidin-HRP, and the presence of IFN-γ was determined by the addition of 3',3',5,5'–tetramethylbenzidine (TMB) substrate.

Retroviral transduction of naïve murine $CD4^+$ T cells and measurement of Renilla luciferase activity

Phoenix ecotropic packaging cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) and transfected with retroviral reporter constructs driven by the IL-2 promoter or an empty promoter (58). The *Renilla* luciferase promoter construct expresses GFP under the murine stem cell virus long terminal repeat (LTR), and is used to identify cells transduced with the retroviral construct. *Renilla* luciferase is driven by 1.9 kilobases of the murine IL-2 promoter or an empty promoter.

Viral supernatants from transfected packaging cells were collected and used to transduce activated (.5 μM OVA peptide for 36 hours) splenocytes from OT-II and OT-II/IFNAR2-/- mice on day 1 and 2. Cell cultures were centrifuged for 45 minutes at 1800 revolutions per minute (rpm) at room temperature in the presence of polybrene and incubated at 37 Celsius with 5% CO_2. Cells were split on day 3 and rested until day 7. On day 7 cells were stained for CD4 and sorted on BD Biosciences FACS Aria for CD4 and GFP double positive cells. Cells were re-

stimulated for 3 days with .5 μM OVA peptide and 50 U/ml rh-IL-2 in the presence of irradiated (2700 RAD) C57BL/6 splenocytes. On day 3, cells were split and rested until day 7. Cells were re-stimulated on day 7 with plate bound anti-CD3 for the indicated time and lysates harvested. Lysates were prepared with the *Renilla* Luciferase Assay System (Promega) and duplicate samples read for 3.0 seconds on a Perkin Elmer Plate Reader for *Renilla* luciferase activity.

In vivo infections

Male C57BL/6 LY5.1 mice received OT-II or OT-II/IFNAR2-/- purified $CD4^+$ T cells (1×10^5) by tail vein injection on day 0, and allowed to rest for 24 hours. On day 1, select cohorts received an infection of 1×10^6 pfu of VSV-OVA by tail vein injection. On day 7 mice were either sedated with isoflurane to obtain a blood sample by retro-orbital bleed or sacrificed by CO_2 asphyxiation and lymph nodes and spleen harvested. Samples were fixed with 2.5% formalin and stained with PE anti-mouse CD4 (Biolegend) and Alexa Fluor 700 anti-mouse CD45.2 (Biolegend). Cells were analyzed on the BD Biosciences LSR II flow cytometer.

Actinomycin D treatment of murine splenocytes

OT-II, OT-II/IFNAR2-/- and OT-II/ m/h STAT2 KI splenocytes (5 x 10^6 cells/ml) were activated with .5 μM OVA peptide for 4 or 5 hours. Then cell cultures were treated with 10 μg/ml actinomycin D or an equal volume of dimethyl sulfoxide (DMSO). Cells were harvested and total RNA extracted every hour for the following 2-3 hours. RNA was prepared for analysis by qPCR as described above.

TRANFAC analysis of ISRE consensus sequences

ISRE consensus sequences were identified by searching the TRANSFAC database (*http://www.gene-regulation.com/pub/databases.html*) for human IFIT1 (ISG56) promoter elements. Subsequent hits were identified by a database of similar ISRE consensus sequences. These results were directly compared to the putative site identified by J. David Farrar, Ph.D..

Statistical significance

Statistical significance was determined by Student's t test. $p < .05$ was determined to be significant.

CHAPTER III

ROLE OF THE TYPE I INTERFERON SIGNALING PATHWAY IN REGULATING IL-2 EXPRESSION

Introduction

Two types of $CD4^+$ T cells secrete high levels of IL-2, naïve and central memory $CD4^+$ T cells (34). IL-2 is an essential growth factor that provides a signal to $CD4^+$ and $CD8^+$ T cells to proliferate. Although much is known about how IL-2 is regulated at the promoter level, little is known about the role cytokines or chemokines play in enhancing IL-2 expression (36).

Multiple cytokines and chemokines have demonstrated their involvement in receptor crosstalk with the TCR in $CD4^+$ T cells. Kumar and colleagues suggest treatment of stimulated human peripheral blood mononuclear cells (PBMCs) with stromal cell-derived factor-1α (SDF-1α) enhances IL-2 expression (59). Bone marrow stromal cells constitutively express SDF-1α and it has been suggested SDF-1α is also expressed by DCs and endothelial cells (60, 61). Pablos and colleagues demonstrate that during pro-inflammatory conditions,

dermal fibroblasts are capable of expressing SDF-1α, possibly to recruit lymphocytes and mononuclear cells to sites of inflammation (60). SDF-1α binds to CXCR4, commonly found on T cells and mononuclear cells and enhances expression of genes associated with adhesion, chemotaxis, cell-cycle progression and lymphocyte development (59). Kumar and colleagues provide evidence that CXCR4 co-localizes with the TCR and utilizes zeta-chain-associated protein kinase-70 (ZAP-70) and src homology 2 domain-containing leukocyte protein of 76 (SLP-76) (59). Recently it was suggested CXCR4 interaction with the TCR also occurs in endoplasmic compartments within the cell (62). Kumar and colleagues suggest signaling via CXCR4 utilizes TCR signaling components and enhances and prolongs extracellular signal-regulated kinases (ERK) activation, leading to increased expression of AP-1 dependent genes (59). This is suspected to be AP-1 specific, as transcriptional activity of NFAT and NF-κB is not enhanced in the presence of SDF-1α (59). The ability of CXCR4 to crosstalk with the TCR is not exclusive, as the type I IFN receptor complex has also been implicated in receptor crosstalk with the TCR.

The type 1 IFN receptor-signaling complex has been demonstrated to utilize non-conical downstream signaling proteins that are associated with T cell receptor signaling (54, 55, 63). Petricoin and colleagues suggest that the anti-proliferative actions of type 1 IFN require downstream signaling components that associate with the TCR signaling complex (54). In response to IFN-α signaling

ZAP-70, Lck, and CD45 associate with the type 1 IFN receptor in Jurkat cells and primary human peripheral blood lymphocytes. In the absence of TCR signaling proteins, anti-proliferative actions of IFN-α were not observed in Jurkat cells. IFN signaling and activation of the JAK/STAT pathway leading to formation of the ISGF3 complex was retained in the absence of TCR signaling proteins (54). Stevens and colleagues suggest the type 1 IFN receptor utilizes SLP-76, Vav1 and Lck to activate the MAPK pathway (55). Only in cells in which Lck is present does the signaling through the type I IFN receptor activate the MAPK pathway (55, 64). Primary $CD4^+$ T cells stimulated with either IFN-α or through the TCR displayed similar phosphorylation levels of SLP76 at 5 minutes post-stimulation (55). NFAT promoter activity was measured in Jurkat cells after stimulation with both anti-CD3 and IFN-α. There was a slight, but not significant, increase in NFAT promoter activity during early points following stimulation as measured by relative luciferase activity (55). The study by Stevens and colleagues suggests that NFAT is not differentially regulated by a combination of TCR and IFN-α stimulation (55). However, downstream genes specific of TCR or IFN-α stimulation were not examined for differential expression in response to both stimuli.

Davis and colleagues recently described a differential role for IFN-α and TCR stimulus in primary human $CD4^+$ T cells (33). Naïve human $CD4^+$ T cells that are treated with IFN-α and IL-12 under stimulatory conditions secrete

enhanced levels of IL-2 in response to a re-stimulation. These cells also show characteristics of central memory T_H1 $CD4^+$ T cells, indicated by increased IL-2 secretion and surface markers of $CCR7^{hi}$ and $CD45^{lo}$ (33, 34). However, the mechanism of IFN-α in enhancing IL-2 expression has not yet been determined. It would be interesting to determine based on previous findings, if IFN-α synergizes with TCR stimulation to enhance the MAPK pathway, leading to increased AP-1 activation. AP-1 is a critical transcription factor in regulating IL-2 expression at the promoter level in $CD4^+$ T cells.

Havenar-Daughton and colleagues described a role for the type 1 IFN receptor in murine $CD4^+$ T cells (65). In the absence of the type 1 IFN receptor in $CD4^+$ T cells, cells fail to expand in response to an *in vivo* acute infection with lymphocytic choriomeningitis virus (LCMV) infection. However, despite their inability to respond to a viral infection, murine $CD4^+$ T cells do expand in response to a bacterial *Listeria monocytogenes* infection.

It is suggested that $CD4^+$ T cells mount unique responses to differentially expressed cytokines from viral versus bacterial infections. IFN-α is more highly expressed compared to IL-12 in response to a LCMV infection, while the opposite is true during a *Listeria monocytogenes* infection (66). IL-12 has been shown to play a role in $CD8^+$ T cell survival in cells that have previously responded to antigen (Ag), and Havenar-Daughton et al. speculate this may be true for $CD4^+$ T cells as well (65, 67, 68). IFN-α has been shown to induce an anti-proliferative

and pro-survival effect in a variety of cells, including $CD4^+$ T cells (65, 69-72). The anti-proliferative effect of IFN-α may be required to ensure lymphocyte survival, although this has not directly been shown. Although IL-2 is essential for $CD4^+$ T cell proliferation, Havenar-Daughton and colleagues did not measure IFNAR-/- $CD4^+$ T cells ability to secrete this specific cytokine.

Type 1 IFN signaling plays an important but undefined role in sustained IL-2 expression in human $CD4^+$ T cells. While it has been observed in re-stimulated human $CD4^+$ T cells that IFN-α and IL-12 enhance IL-2 expression, this has not been tested in naïve murine $CD4^+$ T cells (33). This study investigates whether blocking the type 1 IFN receptor disrupts IL-2 expression in OT-II $CD4^+$ T cells. If blocking IFNAR2 disrupts IL-2 expression, it will not conclude whether it is disrupting type 1 IFN signaling or recruitment to the TCR immunological synapse (IS). Signaling through CXCR4 and TCR results in enhanced IL-2 expression and recruitment of CXCR4 to the TCR immunological synapse and to internal endosomal compartments (62). Interestingly, blocking CXCR4 in TCR stimulated cells results in disrupted localization to the TCR IS, and a slight decrease in IL-2 secretion (73, 74). Jurkat cells are not known to secrete SDF-1α, thus blocking the receptors' ability to locate to the TCR IS may disrupt the cell's ability to enhance IL-2 expression.

Xie and colleagues recently demonstrated that $CD4^+$ T cells from TNF receptor associated factor-3 (TRAF3)-/- mice display defective IL-2 secretion

(75). The defect in IL-2 secretion does not appear to be specific, as there is an observed decrease in IFN-γ, TNF-α and IL-4 secretion at 2 days post-stimulation with anti-CD3/anti-CD28 when compared to WT cells. This could suggest TRAF3 plays a larger role in regulating $CD4^+$ T cell activation or T cell development. Despite this, they provided evidence that TRAF3 may localize the immunological synapse as early as 3 minutes post anti-CD3/anti-CD28 stimulation, implicating TRAF3 in an undesignated role in TCR signaling (75). However, Xie and colleagues did not examine downstream activation of proteins, such as IRF3. TRAF3 plays an important role in type 1 IFN expression, however whether signaling through TRAF3 is important for regulating type I IFN in $CD4^+$ T cells is unknown.

IFN-α and IFN-β signal through the type 1 IFN signaling complex composed of IFNAR1 and IFNAR2 to regulate expression of interferon stimulated genes (ISGs). In response to ligand binding, JAK1 and TYK1 are auto-phosphorylated, resulting in subsequent phosphorylation of STAT1 and STAT2. These transcription factors heterodimerize and bind to IRF-9 to form ISGF3. This complex translocates to the nucleus to bind to interferon stimulated response elements (ISRE) within promoters and regulate gene expression. While STAT2 has not been documented to play a role in TCR stimulation, STAT1 has.

Transcription factors involved in type I and II IFN signaling had been suggested to be activated in response to TCR stimulation (76). Gamero and

colleagues find that STAT1, a component of the type 1 and type II signaling complex is phosphorylated in response to anti-CD3 stimulation by 30 minutes post-stimulation (76). They find STAT1 is phosphorylated at serine residue 727 in response to anti-CD3 stimulation, as well as IFN-α stimulation. In response to both anti-CD3 and IFN-α stimulation there is an observed enhancement in IFN-α driven promoter activity measured by luciferase activity (76). Uddin and colleagues suggest Serine 727 phosphorylation of STAT1 is mediated by PKC-δ in response to IFN-α stimulation (77). However it has not been determined what mediates STAT1 Ser-727 phosphorylation in response to anti-CD3 stimulation.

The role of PKC-δ in response to TCR stimulation in lymphocytes is controversial. PKC-δ is speculated to play a role in negative regulation of IL-2 in lymphocytes, as there is enhanced IL-2 in blood plasma from PKC-δ -/- mice injected with anti-CD3 (78). Conversely, Gorelik and colleagues provide data to support human patients with systemic lupus erythematosus (SLE) display decreased PKC-δ phosphorylation and decreased ERK activity (79). Miyamoto and colleagues observed that in mice defective in PKC-δ activity or expression there is a high incidence of lupus (80, 81). Human SLE and murine lupus subjects are observed to have decreased IL-2 secretion from $CD4^+$ T cells, which is opposite of what Gruber and colleagues observed (78). It remains unclear how STAT1 is phosphorylated in response to anti-CD3 stimulation, and what role it plays in lymphocytes. How and if PKC-δ plays a role in STAT1 phosphorylation

in response to TCR stimulation in $CD4^+$ T cells has not been determined. STAT1 and STAT2 form a heterodimer in response to IFN-α stimulation and translocate to the nucleus as ISGF3. The localization of STAT1 has been investigated in response to TCR stimulation. Maldonado and colleagues investigated the localization of the type II IFN receptor and determined in response to TCR stimulation, the receptor complex localized to the TCR IS by 30 minutes post-stimulation. Phosphorylated STAT1 at S727 co-localized with nuclear proteins in murine $CD4^+$ T cells in response to 30 minutes of TCR stimulation. This event was independent of direct type II IFN signaling, which indicated that STAT1 activation was TCR dependent (82). Currently there are no previous studies has STAT2 been investigated in response in response to TCR stimulation. This study will begin the initial studies investigating STAT2 nuclear translocation in response to TCR stimulation in $CD4^+$ T cells.

This study investigates the role of STAT2 in murine $CD4^+$ T cells by utilizing a mouse with a unique STAT2 C-terminus. The C-terminus of the murine STAT2 has been replaced with the C-terminus of human STAT2, creating a chimeric STAT2 molecule (56). The m/h STAT2 KI mouse was originally created to determine if this chimeric STAT2 molecule could activate murine STAT4 in response to type 1 IFN in murine $CD4^+$ T cells (56). The minisatellite insertion at the C-terminus of murine STAT2 was speculated to disrupt activation of STAT4 (56, 83). In human cells, this mini-satellite insertion is not present and

STAT2 is able to activate STAT4 (83). Swapping the murine STAT2 C-terminus for the human STAT2 C-terminus was hypothesized to restore activation of STAT4 in murine $CD4^+$ T cells (83). Persky and colleagues determined the chimeric STAT2 molecule was unable to restore STAT4 activation, but the role of STAT2 in regulating ISGs remained intact similar to WT cells (56, 84).

While the N-terminus of murine and human STAT2 is very similar, the C-terminus of STAT2 is species specific that may provide interactions with unique sets of proteins (85). If STAT2 plays a role regulating IL-2 expression, the chimeric STAT2 molecule may disrupt the ability of STAT2 to bind specific proteins to enhance IL-2 expression.

Murine STAT2 interacts with a variety of proteins, such as IRF-9, STAT1, CBP/p300, and minichromosome maintenance complex component 5 (MCM5). The coil-coiled domain of STAT2 plays an important role in interactions with IRF-9 (**Figure 3.1**) (86, 87). The DNA binding domain (DBD) of STAT2 is unique in that it does not provide the ability to bind to DNA within the nucleus, this interaction is instead facilitated by IRF9 and STAT1. The SH2 domain is an essential portion of STAT2 that plays an important role in binding to phosphorylated tyrosine residues as well as forming a heterodimer with the STAT1 molecule (88). The transcriptional activation domain (TAD) domain in the C-terminal domain of STAT2 is involved in interactions with p300/ CREB binding protein (CBP). It is speculated that the TAD of murine STAT2 maintains

species-specific interactions with other murine proteins. These interactions may be disrupted if the C-terminal portion of murine STAT2 is altered (85). This study will investigate whether IL-2 expression is augmented in the presence of an altered murine STAT2 molecule.

It has been suggested that there are two phases of IL-2 expression that are regulated at the transcriptional level (37). McKarns and colleagues suggest the primary phase is rapid and independent of de novo protein synthesis (37). The secondary phase is thought to be dependent on de novo protein synthesis and histone modification (36). Based on previous studies that demonstrate proliferation is observed in OT-II/IFNAR2-/- $CD4^+$ T cells, we hypothesize the initial phase is not disrupted (65). $CD4^+$ T cells require IL-2 as a growth signal, and sustained signaling is required for expansion or cells undergo apoptosis (65). TNFR-/- $CD4^+$ T cells are defective in a secondary burst of IL-2 expression, which McKarns and colleagues suggest requires signaling via the TNFR to activate c-rel (37). To address these possibilities, one must exam the kinetics of IL-2 expression in OT-II/IFNAR2-/- and OT-II/m/h STAT2 KI $CD4^+$ T cells compared to OT-II $CD4^+$ T cells. This would help determine not only what phase of IL-2 expression is defective but also the timeline of when STAT2 may be activated and translocate to the nucleus in OT-II $CD4^+$ T cells.

The decrease in IL-2 expression could also be due to a lack of mRNA stability. STAT2 could be indirectly involved in sustaining IL-2 expression by

regulating proteins that are necessary in stabilizing IL-2 mRNA transcripts. Nuclear factor 90 (NF90) is one example of a protein that binds the 3' untranslated regions of IL-2 mRNA in AU-rich elements (AREs) (89, 90). AREs regions of mRNA promote rapid degradation of transcripts unless stabilized by AU-binding proteins (91). NF90 is phosphorylated in response to T cell stimulation and exported from the nucleus, where it binds AREs sites in the 3' untranslated region of IL-2 mRNA to stabilize the transcript (89, 92, 93). NF90 is phosphorylated in response to AKT activation, downstream of CD28 co-stimulation (89).

Type 1 IFN signaling has been suggested to upregulate tristetraprolin (TTP) that destabilizes IL-2 mRNA transcripts. Ogilvie and colleagues suggest TTP plays a role in destabilizing IL-2 mRNA transcripts as early as 6 hours post-stimulation in splenocyte cultures (94). This is contradictory to what is expected, since in the present study IL-2 expression is decreased in OT-II/IFNAR2-/- $CD4^+$ T cells. In the absence of type I IFN signaling, this would imply the absence of elevated TTP expression. In T cells from TTP-/- mice, IL-2 expression is increased due to a lack of negative regulation of IL-2 mRNA transcripts (94). This study will examine IL-2 mRNA stability, giving us insight into whether the decrease in IL-2 expression in OT-II/IFNAR2-/- $CD4^+$ T cells is due to mRNA stability.

ISGF3 binds to ISRE consensus sequences within gene promoters to regulate expression. There has not been an ISRE consensus sequence identified within the IL-2 promoter. A 100% identical consensus sequence is not necessary, as small nucleotide differences may slightly decrease the efficiency of the promoter region to enhance IL-2 transcription. Recent evidence suggests small degrees of variability between consensus sequences, as seen in the IFN-β promoter, can result in decreased efficiency but still enables transcription factors to bind (95). IRF-3 binds to a series of adjacent positive regulatory domains (PRD) within the IFN-β promoter. Escalante and colleagues set out to optimize IFN-β expression but mutating or deleting nucleotides of the IRF binding sites to consensus like sequences. IRF-3 binds to consensus sequences, most of which possess a 2 base pair (bp) separation between promoter regions. However, there are two adjacent IRF binding sites that are separated by 3bp. This 1 bp difference shifts the transcription factor binding site, providing a non-consensus and suboptimal binding site for IRF-3. After deleting and mutating specific nucleotides to provide optimal consensus sequences, IFN-β promoter activity is enhanced. Identifying a similar, but not identical, ISRE consensus sequence may allow ISGF3 to bind but display a reduced ability to enhance IL-2 promoter activity compared to an identical consensus sequence.

The IL-2 promoter is regulated in a cell and stimulation specific manner. The four essential transcription factors that regulate IL-2 expression are Oct1, NF-

κB, NFAT, and AP-1. In the absence of one of the above transcription factors, IL-2 expression is severely affected. While not one of these transcription factors is T cell specific, it is suggested that a combination of these factors activated by specific TCR stimulation results in the placement of these factors at specific sites within the IL-2 promoter. These proteins bind to specific consensus sites located throughout the IL-2 promoter, particularly in the -300bp region known as the IL-2 enhancesome (**Figure 3.2)**. NFAT1 and NFAT2 play a cooperative role with AP-1, Oct1 and NF-κB in regulating *il-2 gene* expression. Specific regions in the IL-2 promoter where these proteins bind are the CD28-responsive region (CD28RR), antigen receptor-responsive elements (ARRE-1 and ARRE-2) and the NFIL-2B region. While the proximal region of the IL-2 promoter has been mapped out fairly extensively, the region beyond -400bp has remained elusive (36).

More recent research has begun to examine the distal region of the *il-2 gene* promoter in regulating IL-2 expression (41). Further research in this area will allow us to understand the role of additional transcription factors that may play a role in regulating IL-2 expression at the promoter level. This study will begin investigating ISGF3 consensus sites within the IL-2 promoter.

Results

Naïve OT-II/IFNAR2-/- CD4$^+$ T cells stimulated with anti-CD3/anti-CD28 display decreased IL-2 secretion.

In response to anti-CD3/anti-CD28 stimulation, naïve WT CD4$^+$ T cells secrete maximal IL-2 at approximately 72 hours post-stimulation (37). Sustained IL-2 secretion allows for proliferation and cell expansion and disruption of the cell's ability to respond to IL-2 results in decreased proliferation (96). The observation from Havenar-Daughton and colleagues indicates there may be a defect in naïve IFNAR-/- ability to secrete IL-2 to sustain proliferation and cellular expansion in response to an *in vivo* viral infection (65). However, the defect in CD4$^+$ T cell expansion appears to be specific to a viral infection as there is no observed defect in IFNAR-/- cells ability to expand in response to a bacterial infection. The study by Havenar-Daughton and colleagues suggests there may be a need for specific cytokine signaling to enhance IL-2 expression.

Previous studies in the Farrar lab showed that human CD4$^+$ T cells polarized with IL-12 and IFN-α display enhanced IL-2 secretion. Based on this observation I hypothesized that if type 1 IFN signaling is necessary to enhance IL-2 expression, there will be a decrease in IL-2 secretion in IFNAR2-/- CD4$^+$ T cells.

To investigate this, an *in vitro* stimulation model was developed to examine IL-2 secretion from naïve OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells. Naïve $CD4^+$ T cells from either OT-II or OT-II/IFNAR2-/-, were purified by negative selection from spleen and lymph nodes of healthy 8-14 week old mice. Negative selection of $CD4^+$ T cells allows for T cells to remain untouched. Cells were plated at the same density on an anti-CD3/anti-CD28 coated 96-well plate in the absence of cytokines and stimulated for 72 hours. As a positive control, cells were treated with PMA and Ionomycin, to pharmacologically induce IL-2 expression (97). PMA is known to activate PKCθ, while Ionomycin activates the NFAT pathway through increase in intracellular calcium (98).

Supernatants were collected at 72 hours and IL-2 levels measured by enzyme-linked immunosorbent assay (ELISA). There was significantly less ($p = .0003$) IL-2 secreted by OT-II/IFNAR2-/- $CD4^+$ T cells at 72 hours post-stimulation when compared to OT-II $CD4^+$ T cells (**Figure 3.3**). Although IL-2 secretion is defective in response to anti-CD3/anti-CD28 stimulation, PMA and Ionomycin induced IL-2 secretion appears to remain intact in OT-II/IFNAR2 -/- $CD4^+$ T cells, (**Figure 3.3**). This suggests there may not be an inherit defect in IL-2 expression in OT-II/IFNAR2-/- $CD4^+$ T cells. An inherit defect could be caused by an off target effect in *il-2 gene* expression when IFNAR2-/- mouse was originally generated (99).

Decreased intracellular IL-2 at 48hrs post-stimulation in naïve OT-II/IFNAR2-/- $CD4^+$ T cells after splenocyte activation with OVA-peptide.

OT-II/IFNAR2-/- $CD4^+$ T cells do not secrete sustained levels of IL-2 at 72 hours post-stimulation in response to anti-CD3/anti-CD28. To confirm this is not a defect in IL-2 secretion, intracellular IL-2 protein expression in both OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells was examined. Whole splenocyte cultures were prepared from OT-II and OT-II/IFNAR2-/- mice and stimulated with identical concentrations of OVA peptide for 48 hours. Monensin was added the last 4 hours of stimulation to block secretion of intracellular proteins. Intracellular IL-2 was examined in $CD4^+$ T cells in both OT-II and OT-II/IFNAR2-/- splenocyte cultures by flow cytometry. OT-II/IFNAR2-/- $CD4^+$ T (mean +/- SEM of unstimulated 8.38 +/- 3.52; .stimulated 24.85 +/- 2.22) display a significant defect ($p = .007$) in intracellular IL-2 protein expression when compared to OT-II $CD4^+$ T cells (mean +/- SEM of unstimulated 8.32 +/- 3.53; stimulated 36.88 +/- 2.03) (**Figure 3.5 A and B**). This data provides evidence that there is not a defect in IL-2 secretion. It will be necessary to examine IL-2 regulation at the molecular level mRNA to determine where the defect lies.

Naïve OT-II/IFNAR2-/- CD4$^+$ T cells display similar proliferation kinetics compared to OT-II CD4$^+$ T cells.

Cell proliferation can be examined in a multiple ways. One method that is now commonly used is to label CD4$^+$ T cells that were purified by negative selection with a fluorescent molecule, carboxyfluorescein diacetate, succinimidyl ester (CFSE) (100). This molecule binds to proteins within the cell and as the cell proliferates the protein is divided amongst itself and the daughter cell. Cells can be stained with an identifying marker, such as anti-CD4 or anti-CD8, and specific populations of cells can be examined for proliferation by flow cytometry. This allows the ability to use a mixed culture of irradiated splenocytes and negative purified CD4$^+$ T cells from either OT-II or OT-II/IFNAR2-/- mice. Splenocytes cultures contain APCs that allow stimulation with OVA peptide or soluble anti-CD3, a more physiologically relevant method of stimulating cells and examine CD4$^+$ T cell proliferation.

Naïve CD4$^+$ T cells from OT-II and OT-II/IFNAR2-/- were purified by negative selection, labeled with CFSE, and stimulated with irradiated splenocytes in the presence of OVA peptide for 48 hours. OT-II/IFNAR2-/- CD4$^+$ T cells were capable of proliferating at 72hrs post-stimulation. OT-II/IFNAR2-/- CD4$^+$ T cells had a similar ability to proliferate when compared to OT-II CD4$^+$ T cells (**Figure 3.8 A and B**). A small percent of unstimulated CD4$^+$ T cells display a

small shift in CFSE dilution at 72 hours. This may be due to activation or inflammation of $CD4^+$ T cells from irradiated APCs undergoing cell death. To ensure this ability to proliferate was not due to help from APCs, purified $CD4^+$ T cells from OT-II/IFNAR2-/- and OT-II were stimulated with plate bound anti-CD3/anti-CD28. Similar to what was seen with APCs, purified OT-II/IFNAR2-/- $CD4^+$ T cells were capable of proliferating similar to OT-II $CD4^+$ T cells (**Figure 3.8 C and D**). The peaks in $CD4^+$ cell division vary between stimulatory conditions (**Figure 3.8 A and B**). This may be due to differences in staining cells with CFSE or the strength of stimulation between plate bound anti-CD3/anti-CD28 and OVA peptide. This indicated that OT-II/IFNAR2-/- $CD4^+$ T cells might be able to secrete a small amount of IL-2 that allowed cells to initiate proliferation. Secreted IL-2 protein was not measured from cells in these experiments, however a pilot experiment measuring IL-2 protein from whole splenocyte cultures stimulated with OVA-peptide was performed. That pilot experiment (data not shown) suggests OVA-peptide stimulated splenocytes from OT-II/IFNAR2-/- mice display decreased IL-2 secretion when compared to OT-II. In future studies it will be interesting to examine the kinetics of IL-2 expression, to determine when this defect in IL-2 expression occurs.

OT-II/IFNAR2-/- CD4$^+$ T cells show defect in expansion in response to primary in vivo infection with VSV-OVA.

In vitro observations suggest OT-II/IFNAR2-/- CD4$^+$ T cells are capable of proliferating at 48 hours post-stimulation, which supports data that has been previously published (65). It was previously shown that expansion of OT-II/IFNAR2-/- CD4$^+$ T cells in response to an *in vivo* viral pathogen infection was defective (65). The current study was designed to repeat previous findings as well as determine if OT-II/IFNAR2-/- CD4$^+$ T cells display defective expansion in response to other viral pathogens *in vivo*.

Naïve CD4$^+$ T cells from OT-II or OT-II/IFNAR2-/- mice were purified by negative selection and transferred into healthy LY5.1 C57BL/6 mice. OT-II CD4$^+$ T cells express a transgenic TCR with specificity to a portion of the ovalbumin protein. Mice were infected with recombinant VSV, expressing ovalbumin, and infected mice were monitored until day 7 post-infection. Seven days post-infection mice where sacrificed and peripheral blood or spleens were prepared for analysis, as described in Materials and Methods. Cells were stained for an OT-II specific marker, CD45.2, and CD4 then further examined by flow cytometry for the presence of antigen specific T cells. At 7 days post-infection, OT-II/IFNAR2-/- CD4$^+$ T cells (mean +/- SEM of uninfected .007 +/- .001; VSV-OVA infected .063 +/- .013) were significantly defective ($p = .008$) in their ability

to expand in response to VSV-OVA when compared to OT-II $CD4^+$ T cells (mean +/- SEM uninfected .030 +/- .004; VSV-OVA infected .332 +/- .093) (**Figure 3.10; compare lane 5 to lane 6**). This result is similar to what was observed by Havenar-Daughton using LCMV (65). However, this observation is interesting in that when analyzed *in vitro*, OT-II/IFNAR2-/- $CD4^+$ T cells do not indicate they are defective in proliferation at 2 or 3 days post-stimulation (**Figure 3.8**).

It has not been reported if OT-II/IFNAR2-/- $CD4^+$ T cells are defective in IL-2 secretion *in vivo* and if this correlates with defective cell expansion. It will be necessary to study this more in depth, by examining intracellular IL-2 protein expression in OT-II/IFNAR2-/- $CD4^+$ T cells *ex vivo*. However, OT-II/IFNAR2-/- $CD4^+$ T cells may be capable of initial proliferation but undergo apoptosis due to un-sustained IL-2 secretion. Havenar-Daughton examined apoptosis and saw an increase in *ex vivo* IFNAR-/- $CD4^+$ T cells (65). The literature suggests that lymphocytes deprived of IL-2 undergo apoptosis, which supports observations made by Havenar-Daughton and colleagues (65, 101, 102). Preliminary results from this study (data not shown) suggest there is a slight increase in apoptotic cells when OT-II/IFNAR2-/- $CD4^+$ T cells are cultured *in vitro*. Further analysis will need to be performed to determine when following stimulation there is an increase in apoptosis in OT-II/IFNAR2-/- $CD4^+$ T cells when compared to OT-II $CD4^+$ T cells.

Blocking IFNAR2 in OT-II CD4$^+$ T cells abrogates IL-2 expression.

The observation of decreased IL-2 expression in OT-II/IFNAR2-/- CD4$^+$ T cells indicated IFN-α is not only capable of enhancing IL-2 expression, but the type 1 IFN receptor is involved in sustaining IL-2 expression. To support this initial finding, it was necessary to block the IFNAR2 receptor in OT-II CD4$^+$ T cells to determine if we could recapitulate the initial observation seen in IFNAR2-/- CD4$^+$ T cells. OT-II CD4$^+$ T cells were purified from spleen and lymph nodes of healthy mice and stimulated with plate bound anti-CD3/anti-CD28 for 72 hours in the presence or absence of anti-IFNAR2 or Rat IgG isotype control. Supernatants were collected and analyzed by ELISA for secreted IL-2 protein. Following treatment of OT-II CD4$^+$ T cells with an anti-IFNAR2 antibody, there is a significant reduction ($p = .015$) in IL-2 secretion after 72hrs of anti-CD3/CD28 stimulation when compared to isotype treated controls (**Figure 3.13**). The concentration of IL-2 secreted from purified CD4$^+$ T cells is nearly eight fold less in **Figure 3.13** than that observed in **Figure 3.3**. This could be due to variations in external factors that affect the cell's ability to secrete IL-2. Based on these results, the IFNAR2 plays a role in sustained IL-2 secretion in response to anti-CD3/anti-CD28 stimulation.

Enhanced IL-2 expression in naïve OT-II CD4$^+$ T cells treated with IFN-α

Previous reports in human CD4$^+$ T cells suggest IFN-α and IL-12 differentially regulate IL-2 expression when used in combination but not when used alone (33). The cells with enhanced IL-2 expression displayed an *in vitro* phenotype characteristic of central memory T_H1 CD4$^+$ T cells (33). Using a murine model, we wanted to determine what effect IFN-α, IL-12 and IFN-γ treatment had on OT-II CD4$^+$ T cells.

Naïve CD4$^+$ T cells were purified and stimulated with plate bound anti-CD3/anti-CD28 in the presence of either neutralized, IL-12, IFN-γ, IFN-α, or a combination of these cytokines. Cells were harvested at 48 hours post-stimulation and mRNA extracted for analysis by quantitative real time PCR. IL-2 mRNA was analyzed by determining the fold-increase over the unstimulated sample. In response to IL-12 treatment alone, there was no significant increase in IL-2 expression ($p = .751$) over the neutralized sample. However, when naïve CD4$^+$ T cells are treated with either IFN-α alone, or in combination with IL-12 and IFN-γ there was a significant increase ($p < .0001$) in IL-2 mRNA expression compared to neutralized control at 48 hours post-stimulation (**Figure 3.14**). This suggests IFN-α alone is capable of enhancing IL-2 expression, and treatment with IL-12 and IFN-γ further enhances the expression. An initial pilot experiment (results not shown) displayed increased IL-2 secretion in naïve purified OT-II CD4$^+$ T

cells treated with IFN-α and IL-12.

$CD4^+$ T cells from chimeric OT-II/ m/h STAT2 KI phenocopy OT-II/IFNAR2-/- and display decreased IL-2 expression.

To understand how the type 1 IFN signaling pathway is important in sustaining IL-2 expression, it is necessary to examine downstream transcription factors. STAT1 and STAT2 are two transcription factors downstream of the type 1 IFN signaling complex (103). I have access to a mouse with a unique STAT2 C-terminus. The C-terminal portion of mouse STAT2 is swapped with the C-terminus of human STAT2, creating a chimeric m/h STAT2 knock-in (KI) (**Figure 3.15**) (56). This allows a specific region of STAT2 to be examined to determine if it plays a role in sustaining IL-2 secretion. Splenocytes from OT-II, OT-II/ m/h STAT2 KI, and OT-II/IFNAR2 -/- were stimulated with OVA peptide and intracellular IL-2 was examined at 48 hours post-stimulation. Cells were stained for anti-CD4 and anti-IL-2, allowing a specific cell type to be examined by flow cytometry. Intracellular IL-2 in OT-II/ m/h STAT2 KI $CD4^+$ T cells at 48 hours post-stimulation was significantly less ($p = .0001$) than OT-II $CD4^+$ T cells (**Figure 3.16 A and B**). Interestingly OT-II/ m/h STAT2 KI $CD4^+$ T cells displayed an intermediate phenotype when compared to OT-II and OT-II/ IFNAR2-/- $CD4^+$ T cells. The total percent of intracellular IL-2 varied from

experiment to experiment (**Figure 3.17 and Figure 3.18**), but the same trend was observed.

IL-2 mRNA expression in OT-II/ m/h STAT2 KI $CD4^+$ at 8 hours post-stimulation with OVA peptide displays a very similar phenotype to that of OT-II/ IFNAR2-/- $CD4^+$ T cells. Expression of IL-2 mRNA is significantly decreased in both OT-II/ IFNAR2-/- ($p = .002$) and OT-II/ m/h STAT2 KI ($p = .016$) $CD4^+$ T cells when compared to OT-II $CD4^+$ T cells (**Figure 3.16 C**). The observation that IL-2 expression is not sustained in m/h STAT2 KI $CD4^+$ T cells indicates that not only is STAT2 involved, but that the C-terminus of STAT2 is essential in sustaining IL-2 expression.

It is necessary to determine if the defect in IL-2 expression is a specific defect in OT-II/ m/h STAT2 KI $CD4^+$ T cells. As seen in TRAF3-/- $CD4^+$ T cells, there appears to be a defect in the expression of cytokines IFN-γ, TNF-α, IL-4, and IL-2 (75). This defect in cytokine expression is not limited to IL-2, which could implicate TRAF3 in regulating a more global TCR response. Identifying a specific defect in IL-2 secretion could more clearly identify how IL-2 is being regulated.

$CD4^+$ T_H1 differentiation remains intact in chimeric OT-II/ m/h STAT2 KI.

It is important to determine whether the defect in cytokine secretion is specific to IL-2 or if T_H1 secreted cytokines, such as IFN-γ, are augmented in OT-II/ m/h STAT2 KI mice. Whole splenocytes from OT-II and OT-II/ m/h STAT2 KI mice were cultured in the presence of neutralized conditions, polarized to $CD4^+$ T_H1 conditions, IFN-α alone, or T_H1 conditions with IFN-α and stimulated with OVA peptide. Cultures were rested until day 7, followed by restimulation with anti-CD3 for 24 hours and IL-2 and IFN-γ protein examined by ELISA. As seen in naïve OT-II/ m/h STAT2 KI $CD4^+$ T cells, re-stimulated $CD4^+$ T cells displayed defective IL-2 secretion when compared to OT-II $CD4^+$ T cells (**Figure 3.19 A**). However, contrary to what was observed in TRAF3-/- $CD4^+$ T cells, OT-II/ m/h STAT2 KI $CD4^+$ T cells did not display defective IFN-γ secretion (**Figure 3.19 B**). It may be necessary to examine TNF-α, IL-4 and IL-5, TGF-β cytokine secretion, to determine if other $CD4^+$ T cell subsets are or are not affected by loss of the C-terminus of STAT2. In the present study where IL-2 and IFN-γ secretion were examined, the defect in OT-II/ m/h STAT2 KI is specific to IL-2 expression. Implicating STAT2 in regulating IL-2 expression either directly or indirectly.

Sustained IL-2 expression is defective in OT-II/IFNAR2-/- and OT-II/ m/h STAT2 KI splenocytes.

An initial analysis of IL-2 mRNA expression indicated there was a defect at 8 hours post-stimulation in OT-II/IFNAR2-/- and OT-II/ m/h STAT2 KI splenocytes (**Figure 3.20**). It has been suggested naïve $CD4^+$ T cells display two bursts of IL-2 expression (37). Understanding which burst is affected will help us delineate how STAT2 may be acting to sustain IL-2 expression. Splenocytes from OT-II were activated with OVA peptide, harvested at various time-points post-stimulation and total RNA extracted.

Surprisingly, IL-2 mRNA expression from OT-II/ m/h STAT2 KI splenocytes displayed a non-significant difference when compared to OT-II splenocytes at 6 hours post-stimulation. Indicating that OT-II/ m/h STAT2 KI retain the initial ability to express IL-2. However, sustained IL-2 expression appears defective as early as 6 hours in OT-II/IFNAR2-/- ($p < .0001$) and as early as 12 hours post-stimulation in OT-II/ m/h STAT2 splenocytes ($p = .018$) (**Figure 3.20**). Stimulation through CD28 on $CD4^+$ T cells is essential for enhanced IL-2 expression in WT cells (13, 37, 104-106). Histone acetylation and DNA demethylation occur only in response to CD28 stimulation, and without these modifications IL-2 expression is not sustained (13). If type 1 IFN signaling pathway crosstalks with downstream components of CD28 signaling, then a

mutation in STAT2 may disrupt signaling that provides sustained IL-2 expression. There is also the possibility the defect we are observing in IL-2 mRNA expression is due to lack of mRNA stability. The 3' UTR of IL-2 mRNA contains AREs that promote rapid degradation of mRNA.

IL-2 mRNA degradation not increased in OT-II/IFNAR2-/- or OT-II/ m/h STAT2 KI splenocytes.

The defect in OT-I/IFNAR2-/-and OT-II/ m/h STAT2 KI splenocytes occurs as early as 12 hours post-stimulation. To determine if RNA stability plays a role in decreased IL-2 expression, whole splenocyte cultures were examined for IL-2 mRNA transcripts as early as 4 hours post-stimulation with OVA-peptide.

Whole splenocyte cultures from OT-II, OT-II/IFNAR2-/- and OT-II/ m/h STAT2 KI were stimulated with OVA peptide for 4 hours and whole RNA harvested for a baseline reference point. Cells were then treated with actinomycin D, a chemical that halts transcription, at 4 hours post-stimulation and total RNA extracted from cultures every hour for 2 to 3 hours following treatment. IL-2 mRNA was normalized to GAPDH mRNA expression at indicated time points and fold change compared to the 4 or 5 hour time point was used for analysis. This allows us to directly compare the induction or suppression of *il-2* mRNA expression between strains to a point of reference.

Untreated OT-II splenocytes displayed initial IL-2 expression at 4 hours that continued until the last time point at 7 hours post-stimulation. Actinomycin D treated cells displayed initial expression at 4 hours, followed by steady IL-2 mRNA expression until 7 hours. Interestingly IL-2 mRNA from treated and untreated OT-II/IFNAR2-/- ($p < .003$) (**Figure 3.21 A**) and OT-II/ m/h STAT2 KI ($p < .02$) (**Figure 3.21 B**) splenocytes displayed a very similar expression pattern to that of actinomycin D treated OT-II cells at 6 and 7 hours, respectfully. This suggests there is not an increased rate of IL-2 mRNA degradation in OT-II/IFNAR2 -/- and OT-II/ m/h STAT2 KI splenocytes. The type I IFN signaling pathway, and more specifically STAT2, is most likely not involved in stabilizing IL-2 mRNA transcripts. If STAT2 were involved in stabilizing IL-2 mRNA, a gradual decrease in IL-2 mRNA expression would be expected over the course of actinomycin D treatment. Examining IL-2 promoter activity will help determine if there is a defect at the promoter level that STAT2 may play a role in.

IL-2 promoter activity augmented in OT-II/IFNAR2-/- $CD4^+$ T cells.

Results from examining IL-2 mRNA suggest there may not be a defect in mRNA stability in OT-II/IFNAR2-/- and OT-II/ m/h STAT2 KI splenocytes. Investigating the promoter activity of IL-2 will determine whether there lies a defect in IL-2 transcription that the type 1 IFN signaling pathway may play a part

in sustaining. To investigate the IL-2 promoter activity, an IL-2 luciferase reporter was utilized (58). This construct also expresses GFP in transduced cells that allows cells to be sorted and re-stimulated for further analysis (58). Whole splenocyte cultures were prepared from OT-II and OT-II/IFNAR2-/- mice and stimulated with OVA peptide. Splenocytes were transduced, as described in Material and Methods, and sorted 7 days post-stimulation. Cells were sorted based on GFP^+ and $CD4^+$ double positive cells, and re-stimulated with OVA peptide in the presence of irradiated BALB/C splenocytes for 7 days. $CD4^+$ T cells were re-stimulated with plate bound anti-CD3 for 7 hours and luciferase activity measured from cell lysates.

Interestingly OT-II/IFNAR2-/- $CD4^+$ T cells did not display a defect in IL-2 promoter activity when compared to OT-II $CD4^+$ T cells. Contrary to IL-2 protein and mRNA data presented in this study, there was an insignificant increase ($p = .352$) in IL-2 promoter activity in OT-II/IFNAR2-/- $CD4^+$ T cells (**Figure 3.22**).

Putative ISRE sequence identified within IL-2 promoter is similar to ISRE consensus sequence.

The IL-2 promoter has been heavily investigated. The enhanceosome present at 0 to -300 bp upstream of the transcription start site (TSS) contains

binding sites for NFAT, NF-κB, AP-1 and Oct1 that are required for IL-2 expression. The IL-2 enhanceosome is a regulatory site within the IL-2 promoter where enhanced chromatin remodeling is present. This allows for stabilization of essential transcription factors and RNA polymerase, that allow for sustained *il2 gene* expression (36). DNA demethylation and histone modifications are present farther upstream of the -300 bp region of the IL-2 promoter, but transcription factor binding sites in these regions has not been investigated.

Previous unpublished work from the Farrar lab identified a putative ISRE within the IL-2 promoter. The putative ISRE site is located at -450 bp upstream of the TSS on the IL-2 promoter and is identical between mice and human (**Figure 3.23**). I aligned the putative ISRE sequence found in the human IL-2 promoter to known ISRE consensus sequences found within human ISGs (**Table 3.1**). Using TRANSFAC it was possible to identify human genes that contain ISRE consensus sequences in promoter regions. Comparing the putative ISRE site in the human and mouse IL-2 promoter to ISRE consensus sequences within human ISG promoters, there appears to be a very similar but not identical ISRE consensus sequence within the IL-2 promoter at -450 bp. While the putative ISRE sequence within the murine and human IL-2 promoter is identical, analysis was not performed examining murine ISGs. Escalante and colleagues determined that a non-consensus IRF sequence still allowed for IRF binding, but enhancement was significantly less. Upon mutation of the non-consensus

sequence to an IRF consensus sequence, promoter activity increased (95). Although there is no evidence in the literature of a similar phenomenon that occurs with ISRE consensus sequences, it must be investigated within the IL-2 promoter. Deleting a nucleotide in the putative ISRE sequence to provide the 1 bp to 2 bp spacing seen within most ISRE consensus sequences will be interesting to determine if this enhances IL-2 promoter activity.

If STAT2 is binding to the putative ISRE sequence identified within the IL-2 promoter, then nuclear translocation of STAT2 must occur. Understanding and identifying the kinetics of STAT2 translocation in response to TCR stimulation, will allow future investigation into how this activation is occurring.

Preliminary data by ImageStream may indicate STAT2 nuclear translocation in OT-II CD4$^+$ T cells in response to TCR stimulation

Previous findings suggest that STAT1 is phosphorylated at S727 in response to TCR, IFN-α, and IFN-γ stimulation (76, 107). Maldonado and colleagues provide evidence that S727 phosphorylated STAT1 translocates to the nucleus by 30 minutes post TCR stimulation in CD4$^+$ T cells (82). This appears to be independent of IFN-γ and IFN-α treatment, as even in the absence of signaling translocation is present. It has not been determined how STAT1 is activated in response to TCR stimulation, or whether STAT1 forms homodimeric

or heterodimeric complexes that translocate to the nucleus. Recent results suggest STAT2 is involved in sustaining IL-2 expression. The defect appears to be specific to IL-2 expression in $CD4^+$ T cells, as IFN-γ secretion in re-stimulated cells does not appear affected by a mutated STAT2 molecule. A putative ISRE sequence within the IL-2 promoter provides substantial reason to pursue whether STAT2 is activated and translocates to the nucleus in response to TCR stimulation.

Whole splenocytes from OT-II mice were stimulated with OVA peptide or IFN-α, as a positive control, for the indicated time and immediately fixed and stained for CD4 and STAT2. Cells were shipped to Amnis Corporation in Seattle,WA for DAPI staining and analysis by ImageStream technologies. A value was determined based on the co-localization of STAT2 and DAPI in a $CD4^+$ specific population of cells. $CD4^+$ T cells from OT-II mice showed nuclear accumulation of STAT2 at 2.5 hours post-stimulation with .5μM of OVA peptide (**Figure 3.24 A**). It appeared, that accumulation gradually increased over the course of 2.5 hours, with a peak at 2.5 hours post-stimulation (**Figure 3.24 B**).

Discussion

This study suggests OT-II/IFNAR2-/- and $CD4^+$ T cells may display defective IL-2 secretion in response to *in vitro* stimulation through anti-CD3/anti-CD28 at 72 hours. Examining intracellular protein expression, OT-II/IFNAR2-/- and OT-II/m/h STAT2 KI $CD4^+$ T cells appear to display decreased IL-2 intracellular protein expression and *il-2* mRNA levels when compared to OT-II $CD4^+$ T cells. There was variation in the percent of $CD4^+$ T cells with intracellular IL-2 from experiment to experiment, however the trend remained the same. A difference in the magnitude may be due to exogenous effects on the splenocyte cultures such as cell stress or a difference in the lot of OVA peptide purchased.

Results suggest that treating OT-II $CD4^+$ T cells with exogenous IFN-α alone or in combination with IL-12 and IFN-γ may enhance *il-2* mRNA expression at 48 hours post-stimulation. Exogenous IFN-α treatment enhances IL-2 expression, thus it begs the question whether $CD4^+$ T cells secrete a ligand that binds to the type 1 IFN receptor to activate the downstream signaling pathway. In OT-II/IFNAR2-/- $CD4^+$ T cells this feedback would not be possible, as cells would be unable to respond to ligand, thus sustained IL-2 expression would not be observed. Previous work in the lab indicated human $CD4^+$ T cells do not express type 1 IFN (108). It is possible due to genetic differences that in a

murine system $CD4^+$ T cells may secrete type 1 IFNs in response to TCR signaling. Human and murine $CD4^+$ T cells can respond to alternative ligands that utilize the type 1 IFN receptor (109). It is essential for us to examine the possibility that $CD4^+$ T cells are secreting a ligand that is binding to the type 1 IFN receptor. Neutralizing IFN-α and IFN-β in naïve $CD4^+$ T cell cultures stimulated anti-CD3/anti-CD28, will allow us to determine if either of these type 1 IFNs play a role in feeding back on the type 1 IFN receptor to sustain IL-2 expression.

This study suggest by blocking the type 1 IFN receptor in OT-II $CD4^+$ T cells it is possible to recapitulate the results observed in OTI-II/ IFNAR2-/- $CD4^+$ T cells. However, it is unknown if this blocking antibody disrupts the signaling and the membrane translocation of IFNAR. Other groups have demonstrated CXCR4, CCR5, and IFNγR locate to the TCR signaling complex in response to stimulation (59, 74, 82). Blocking CXCR4 in lymphocytes disrupts localization to the TCR IS and a slight decrease in IL-2 secretion is observed in response to TCR signaling (74). CXCR4 is speculated to utilize components of the TCR signaling complex to signal downstream, which may be why it takes advantage of associating with the TCR IS (59).

Recent findings from Maldonado and colleagues provide evidence that the type II IFN receptor associates with the TCR IS as early as 30 minutes following TCR stimulation (82). Currently there is no evidence in the literature to suggest

why the type II IFN receptor associates with the TCR IS in response to anti-CD3 stimulation. It would be interesting, however, to determine if the type II IFN receptor utilizes proteins associated with the TCR signaling complex, seen similarly with the type I IFN signaling pathway. If TCR signaling and IFN signaling share downstream components, this may provide reason to locate within proximity to one another, and disrupting this interaction may affect IL-2 expression. It will be necessary to examine the localization of the type 1 IFN receptor complex in response to TCR signaling.

Previous reports suggest IFN-α signaling in lymphocytes utilizes ZAP-70 and SLP76, providing reason for why the type 1 IFN receptor may localize to the TCR signaling complex (54, 55, 63). Ahmed and colleagues describe two distinct staining patterns of downstream TCR signaling proteins, such as ZAP-70, in response to TCR signaling or IFN-α signaling in Jurkat cells (63). This hints at there may not be localization of IFNAR with the TCR signaling complex. However, the localization of ZAP-70 may be unique if examined in the presence of both anti-CD3/anti-CD28 and IFN-α stimulation. Alternatively, the localization of IFNAR with the TCR may be dynamic and capturing this interaction may provide difficulty is not examined closely. Further tests will need to be done to conclusively determine if IFNAR localizes to the TCR IS in primary $CD4^+$ T cells in response to TCR stimulation.

Examining the kinetics of *il-2* mRNA expression suggests that the defect may not be transient in OT-II/IFNAR2-/- and OT-II/ m/h STAT2 KI. OVA peptide stimulated OT-II/IFNAR2-/- and OT-II/ m/h STAT2 KI splenocytes, suggests there is a defect in sustained *il-2* mRNA expression as early as 12 hours post-stimulation. There is evidence that suggests initial IL-2 expression is dependent on the immediate upstream TCR signaling events, while sustained IL-2 expression is dependent on *de novo* protein synthesis and histone modification (36, 37). There are multiple mechanisms that can regulate the sustained IL-2 expression, such as mRNA stability, histone modifications, DNA demethylation, and decreased rate of transcription.

McKarns and colleagues suggest there are two bursts of IL-2 mRNA expression in response to TCR stimulation (37). However, in the present study this observation has not been clearly demonstrated. This observation may depend on the method of stimulation used, as in McKarns study $CD4^+$ T cells were stimulated with anti-TCRβ and anti-CD28 (37). In present study whole splenocyte cultures were stimulated with OVA-peptide. The kinetics and suggested bursts of IL-2 expression that may be observed could be dependent on the method of stimulation used.

This study suggests there may a defect in sustained IL-2 mRNA expression in OT-II/ IFNAR2-/- and OT-II/ m/hSTAT2 KI that occurs as early as 12 hours post-stimulation. While this study did not account for the number of

$CD4^+$ T cells present in splenocyte cultures, it has been published that m/hSTAT2 KI mice have similar numbers of $CD4^+$ T cells when compared to WT mice (56). However, this study suggests there may not be a decrease in IL-2 mRNA stability in either of these strains.

Sojka and colleagues suggest *in vivo* IL-2 expression is rapid, occurring by 6 hours post-stimulation in naïve $CD4^+$ T cells (110). It may be necessary to determine when IL-2 expression is dysregulated in OT-II/IFNAR2-/- $CD4^+$ T cells before examining this phenomenon *in vivo*. Understanding which phase is interrupted *in vitro*, may give clues of when it is best to examine $CD4^+$ T cell *ex vivo* to catch a glimpse of normal versus defective cytokine expression.

Havenar-Daughton observed a similar defect in expansion of antigen specific IFNAR-/- $CD4^+$ T cells in response to an *in vivo* viral infection (65). However, conversely they observed no defect in antigen specific IFNAR-/- $CD4^+$ T cells in response to bacterial infection (65). This current study did not investigate whether OT-II/IFNAR2-/- $CD4^+$ T cells responded in a similar manner to a bacterial infection. It has been observed (data not shown) that IFNAR-/- mice infected with *Listeria monocytogenes* show less pathology and increased resistance compared to WT mice. It is suggested that IFN-α increases lymphocyte sensitivity to lysis mediated from *Listeria monocytogenes* virulence factor listeriolysin O (111). It would be interesting to determine if p-MHC II engages the TCR differentially during a viral vs bacterial infection and if this

interaction dictates the ability and necessity for cells to respond to cytokines to mount a response. Grakoui and colleagues suggest that a stronger p-MHC II complex with the TCR drives the differentiation of T_H1 $CD4^+$ T cells, while a weaker p-MHC II interaction with the TCR induces a more T_H2 CD4+ T cell driven response (112). How this interaction influences the cytokines that APCs secrete or how the T cell responds to a specific stimulus has not been examined.

It would be interesting to investigate if it is possible to rescue expansion of OT-II/IFNAR2-/- $CD4^+$ T cells through delivery of exogenous IL-2. If IL-2 deficiency is the only factor driving apoptosis and decreased expansion of OT-II/IFNAR2-/- $CD4^+$ T cells, it should be possible to restore expansion and decrease the number of cells undergoing apoptosis. IL-2 is rapidly eliminated *in vivo*, with a half-life of minutes before it is metabolized (113). It has been suggested that use of an IL-2/anti-IL-2 complex provides a more sustained stimulation due to an increased half-life compared to IL-2 alone (114, 115).

$CD4^+$ T cells have been shown to respond to IL-2/anti-IL-2 complex and can drive the expansion of $CD4^+$ T regulatory (T_{reg}) cells that are functional and capable of suppressing clinical symptoms of experimental autoimmune myasthenia gravis in mice (116). $CD8^+$ T cells have also been reported to respond to IL-2/anti-IL-2 complex, enhancing expansion of antigen specific cells (117). IL-2/anti-IL-2 complex provides an efficient stimulus to $CD4^+$ and $CD8^+$ T cells that leads to expansion *in vivo* (114-117). One would hypothesize from these

observations that if deficient IL-2 signaling results in decreased expansion and apoptosis of OT-II/IFNAR2-/- $CD4^+$ T cells, IL-2/anti-IL-2 complex should restore expansion and cell survival.

These findings suggest the type 1 IFN receptor is necessary for sustained IL-2 signaling in $CD4^+$ T cells. However, it is unknown at this point if signaling through the type 1 IFN receptor is necessary for sustained or enhanced IL-2 signaling in naïve murine $CD4^+$ T cells. This study began investigating the need for downstream adaptor and signaling proteins, to investigate the role for IFN signaling pathway. Earlier reports examined the absence of downstream TCR signaling proteins in the anti-proliferative mechanisms of IFN-α, and determined signaling was abrogated. These groups did not examine the reciprocal effect, if deleting or mutating components of the type 1 IFN signaling complex disrupted TCR mediated signaling (54, 55, 63, 64).

The current study suggests that STAT2 may be necessary for sustained IL-2 expression. A specific defect in IL-2 expression is observed in OT-II/ m/h STAT2 $CD4^+$ T cells. STAT1, STAT2 and IRF9 form a heterodimeric complex that binds to ISRE sites within promoters to regulate gene expression. If the ISGF3 complex is activated in response to TCR stimulation in $CD4^+$ T cells, it will interesting to examine IL-2 expression in STAT1-/- and STAT2-/- mice. One would hypothesize that IL-2 expression would be defective in either of these knockouts if ISGF3 is responsible for sustained IL-2 expression.

The implication of STAT2 involvement in IL-2 expression also begs the question of STAT2 nuclear translocation to be addressed. In response to IFN-α stimulation, ISGF3 translocates to the nucleus by 15 to 30 minutes post-stimulation. It will be important to investigate whether STAT2 translocates in response to TCR stimulation. Using a sophisticated method of analysis will help determine if STAT2 translocates to the nucleus in response to TCR stimulation.

There was an observed increase in nuclear STAT2 accumulation in CD4+ T cells in response to TCR stimulation. The kinetics of STAT2 accumulation is interesting and results differ from the 30-minute nuclear accumulation of STAT1 in response to TCR stimulation (82). There is a difference in experimental set-up that could account for additional time to activate STAT2 and result in nuclear accumulation. Maldonado and colleagues stimulated cells with plate bound anti-CD3 and anti-CD28, to provide a direct stimulus to $CD4^+$ T cells (82). After the addition of OVA-peptide to murine splenocyte, the peptide must be taken up by APCs, interact with and present peptide to $CD4^+$ T cells in culture. With the additional step of peptide uptake by APCs, it could add time to the initiation of cell stimulation, leading to a false impression of when STAT2 nuclear translocation is occurring post TCR stimulation. It will be necessary to examine $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28, or preloaded APCs to determine the kinetics of STAT2 nuclear translocation.

It is only possible to speculate how STAT2 is activated until further

kinetic analysis is performed to determine when nuclear translocation occurs. If STAT2 shares similar kinetics to STAT1, activation could occur through direct TCR stimulation (82). Although, if a delay in STAT2 nuclear translocation occurs, it could indicate an alternative mechanism of activation for this particular molecule. This study provides us with a system that can examine STAT2 translocation in $CD4^+$ T cells in a physiologically relevant stimulation. If STAT2 translocates to the nucleus it may play a direct role in the regulation of IL-2 expression.

An interesting observation in this study was that IL-2 expression is defective in a mouse strain with a unique C-terminal mutation. It has been suggested the C-terminal portion of STAT2 is necessary for conserved interactions with murine proteins (85). I hypothesize this interaction is essential for either directly or indirectly sustaining IL-2 expression. Currently, there are no publications describing an ISRE site within the IL-2 promoter. The presence of one would suggest STAT2 might directly bind to the IL-2 promoter. It will be necessary to examine the IL-2 promoter more closely to determine if there is a putative ISRE site that may provide reason to examine if ISGF3 binds the promoter.

If direct regulation of IL-2 were occurring, this would place STAT2 at the IL-2 promoter. STAT2 could possibly be directly involved in enhancing IL-2 expression during the early or late stage of IL-2 expression observed in naïve

$CD4^+$ T cells. However, if STAT2 were indirectly involved in enhancing IL-2 expression, it could be acting to up-regulate expression of a protein that then binds to the IL-2 promoter to regulate expression or to that stabilizes IL-2 mRNA.

Examining IL-2 promoter activity, there does not appear to be a defect in transduced primary $CD4^+$ T cells. Interestingly, there may be an insignificant increase in IL-2 luciferase promoter expression in OT-II/IFNAR2-/- $CD4^+$ T cells. One caveat of using an IL-2 luciferase promoter construct to examine promoter activity is that the construct contains 1.9 kilobases (kb) of the IL-2 promoter. Using an artificial system that lacks the full *IL-2 gene* promoter may provide misleading results, as there may be negative or positive regulatory elements upstream that would alter results.

Other groups have speculated the IL-2 promoter contains up to 10 kb upstream of the IL-2 start site (36). If farther upstream of the IL-2 promoter contains a negative regulatory element that is no longer regulated in by utilizing the luciferase reporter; it would be expected that IL-2 expression would be augmented. Examining endogenous IL-2 promoter activity would allow more conclusions to be made on how IL-2 promoter activity differs between OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells. By examining secreted IL-2 protein from transduced cells, it would be possible to make an initial determination if the IL-2 reporter construct is falsely reporting promoter activity. There are alternative

mechanisms to measure promoter activity that may provide an unambiguous measure of endogenous IL-2 promoter activity.

One additional method to measure the rate of transcription initiation is a nuclear run-on assay (118). This method examines endogenous transcript initiation, allowing promoter activity and rate of transcription to be examined. The role of the type 1 IFN signaling pathway and STAT2 in sustained IL-2 expression has not been established. One hypothesis is that STAT2 acts directly on the IL-2 promoter by interacting with STAT1 and IRF9 to form ISGF3 and binding to an ISRE consensus sequence. Careful analysis of the IL-2 promoter will guide future investigations if an ISRE consensus sequence is identified.

Preliminary *in situ il-2 gene* promoter analysis provides evidence of a putative ISRE sequence located at -450 bp of the *il-2 gene* promoter. In comparing the putative site to ISRE consensus sequences found with ISGs, there appears to be a very similar but not identical sequence. The spacing between the first and second segment of the putative sequence contains 1 additional bp. The additional base pair may still allow for transcription factor binding, but not allow for full enhancement of the *il-2 gene* by ISGF3. Escalante and colleagues suggest substituting or deleting a nucleotide that mutates a non-consensus sequence to a consensus sequence greatly enhances the ability of a TF to enhance promoter activity (95). Although the putative ISRE sequence contains one additional bp, ISGF3 may still be able to bind, thus it is necessary to explore the ability to bind

the putative ISRE sequence within the *il-2 gene* promoter. The initial *in situ* analysis of the *il-2 gene* promoter along with preliminary data that suggests STAT2 nuclear translocation in response to TCR stimulation provides evidence to begin exploring the molecular mechanism of STAT2 in sustaining IL-2 expression. The kinetics of STAT2 translocation must be investigated further to determine if this is TCR induced or signaling through the type I IFN receptor induced activation of STAT2. Phosphorylated STAT1 translocates to the nucleus within 30-minutes post TCR stimulation (82). It is unknown how activation occurs, although this would be an important avenue to investigate if STAT1 and STAT2 are both activated in response to TCR stimulation.

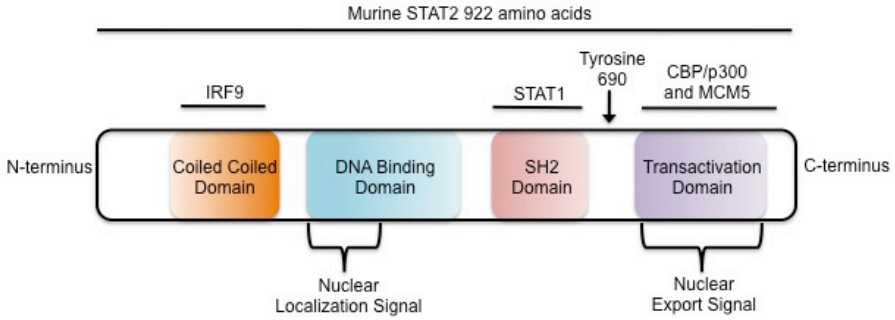

Figure 3.1 Structural and interaction domains of the murine STAT2 protein.

Murine STAT2 is a 922 amino acid protein that interacts with a variety of proteins, such as IRF-9, STAT1, CBP/p300, and MCM5. These interactions are facilitated through the coiled coiled, DNA binding, SH2 and transactivation domains.

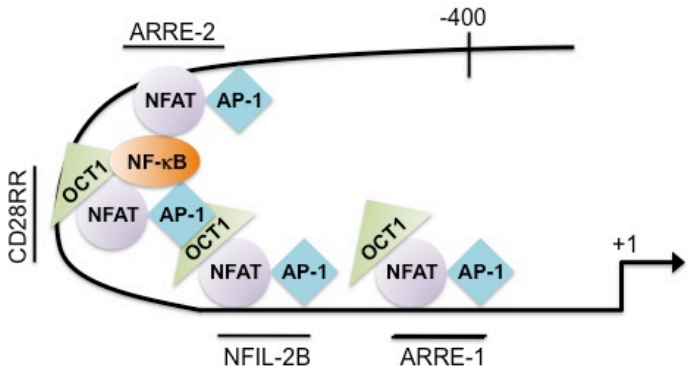

Figure 3.2 The IL-2 promoter contains multiple responsive elements where essential transcription factors bind to drive gene expression.

NF-κB, AP-1, Oct1, NFAT bind to specific consensus sites located throughout the IL-2 promoter, particularly in the -300bp region known as the IL-2 enhancesome. Specific regions in the IL-2 promoter where these proteins bind are the CD28-responsive region (CD28RR), antigen receptor responsive elements (ARRE-1 and ARRE-2) and the NFIL-2B region. The proximal region of the IL-2 promoter has been mapped out fairly extensively, however the region beyond -400bp has remains elusive (36).

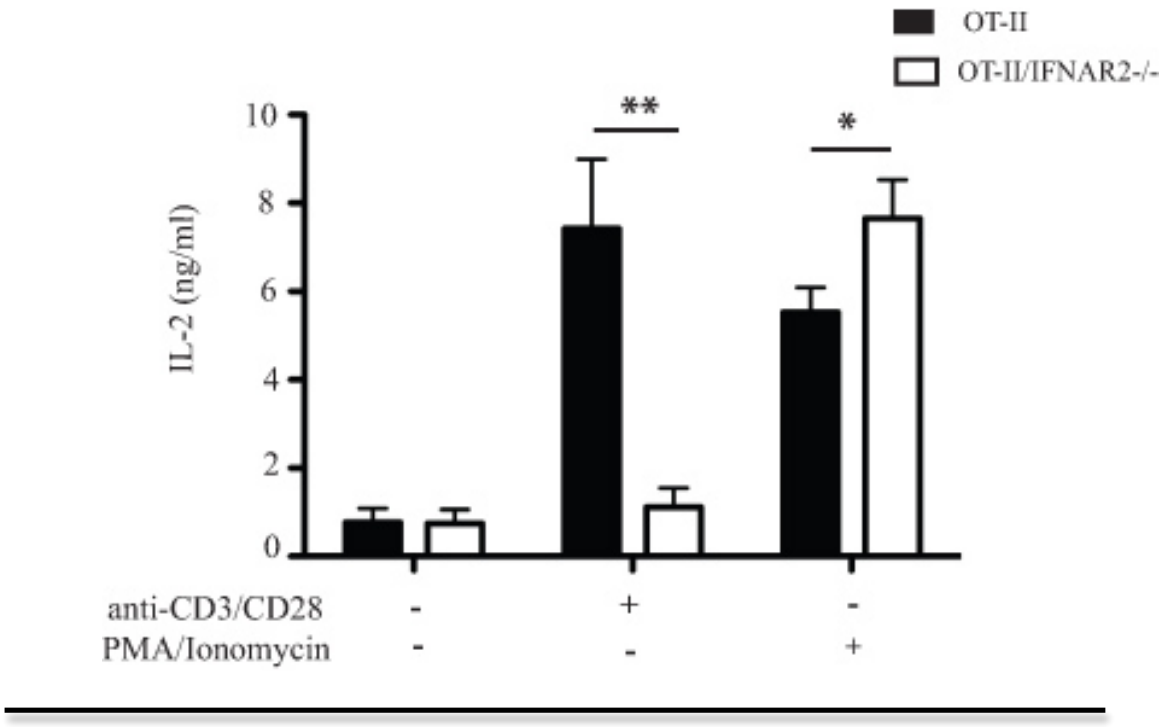

Figure 3.3 Naïve OTII/IFNAR2-/- CD4$^+$ T cells stimulated with anti-CD3/anti-CD28 display decreased IL-2 secretion at 72 hours post-stimulation.

Purified naïve CD4$^+$ T cells from OT-II and OT-II/IFNAR2-/- are stimulated with plate bound anti-CD3/anti-CD28 or PMA/Ionomycin for 72 hours and supernatants analyzed for secreted IL-2 protein by ELISA. Mean of eight independent experiments, sample size unstimulated: OT-II, n = 25 (mean 770.43 +/- 316.66); OT-II/IFNAR2-/-, n = 24 (mean 749.36 +/- 310.89). Stimulated with anti-CD3/anti-CD28: OT-II, n = 24 (mean 7420.98 +/- 1571.43); OT-II/IFNAR2-/-, n = 24 (mean 1112.66 +/- 434.59). Stimulated with PMA/Ionomycin: OT-II, n = 12 (mean 5529.52 +/- 556.72); OT-II/IFNAR2-/-, n = 12, (mean 7653 +/- 876.5). Error bars represent standard error of the mean. ** p = .0003 and * p = .0528 as determined by unpaired Student's t-test.

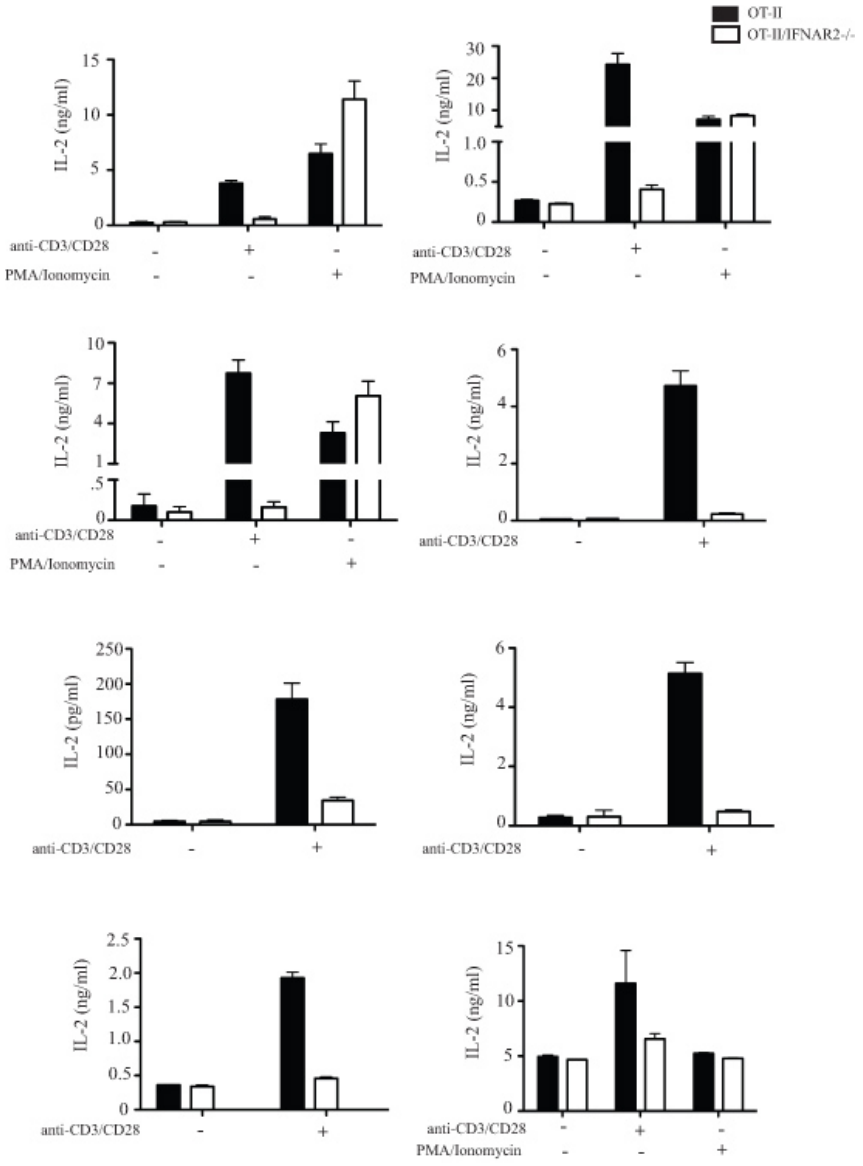

Figure 3.4 Eight representative experiments from Figure 3.3 examining secreted IL-2 protein from OT-II and OT-II/IFNAR2-/- at 72 hours post-stimulation with anti-CD3/anti-CD28.

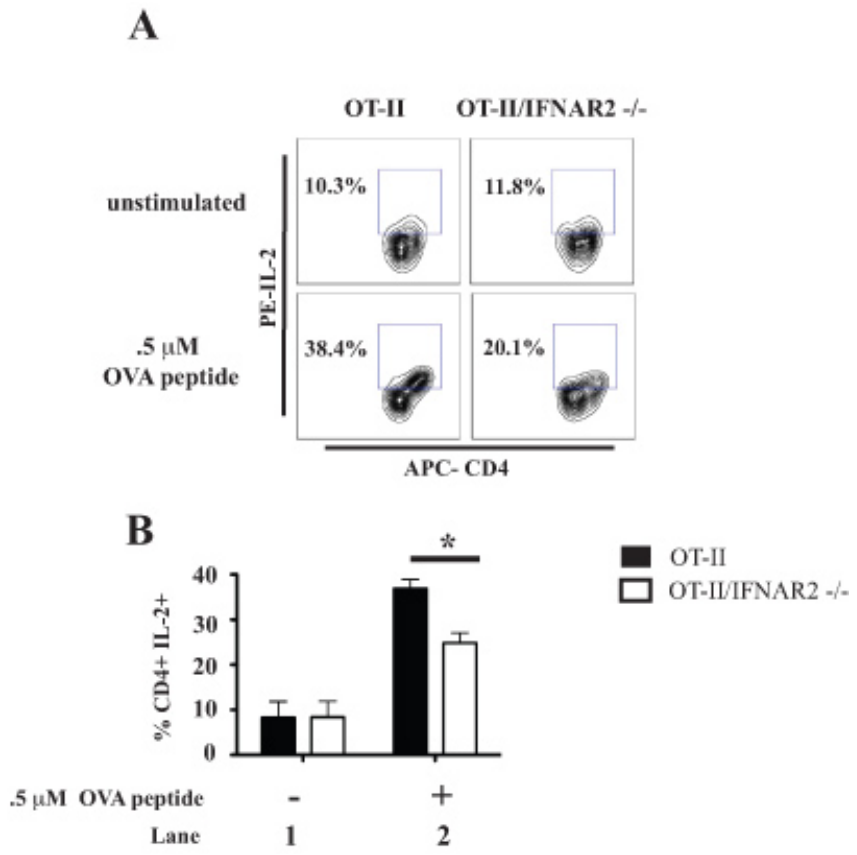

Figure 3.5 Decreased intracellular IL-2 at 48 hours post-stimulation in naïve OT-II/IFNAR2-/- CD4$^+$ T cells after OVA peptide stimulation.

(**A**) Representative FACS plots of intracellular IL-2 protein expression in OT-II and OT-II/IFNAR2-/- CD4$^+$ T cells (**B**) Percent of CD4$^+$ T cells that stain positive for intracellular IL-2. Mean of 2 independent experiments, sample size unstimulated: OT-II, n = 3 (mean 8.32; +/- 3.53); OT-II/IFNAR2-/-, n = 3 (mean 8.38; +/- 3.52). OVA peptide stimulated: OT-II, n = 4 (mean 36.88; +/- 2.03); OT-II/IFNAR2-/- n = 4 (mean 24.85; +/- 2.22). Error bar represents the standard error of the mean. * p = .007 as determined by unpaired Student's t-test.

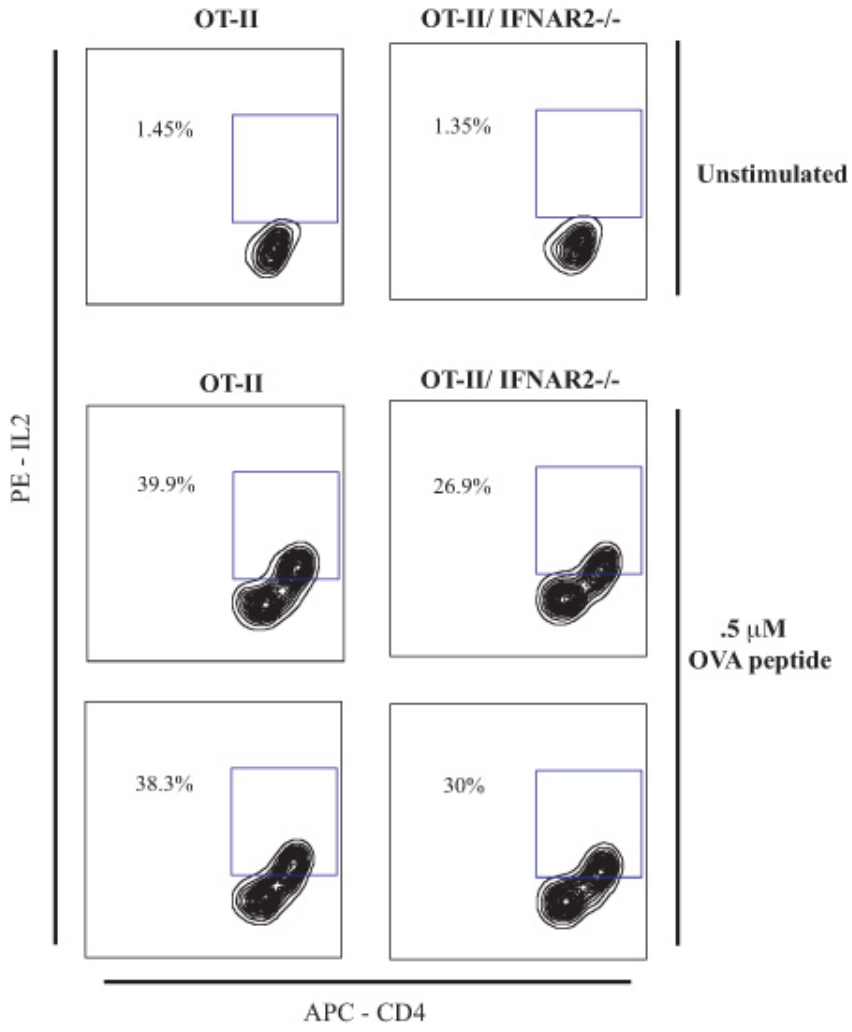

Figure 3.6 One representative experiment examining intracellular IL-2 expression in OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells at 48 hours post-stimulation.

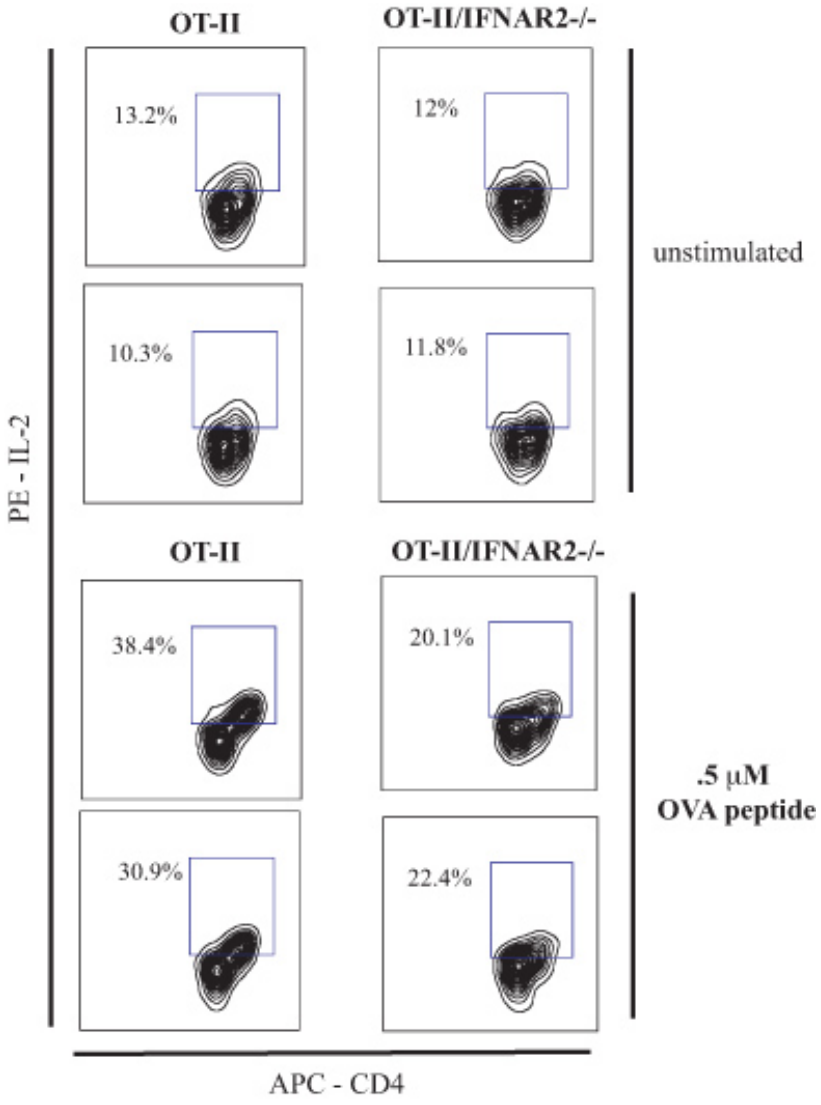

Figure 3.7 One representative experiment examining intracellular IL-2 expression in OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells at 48 hours post-stimulation.

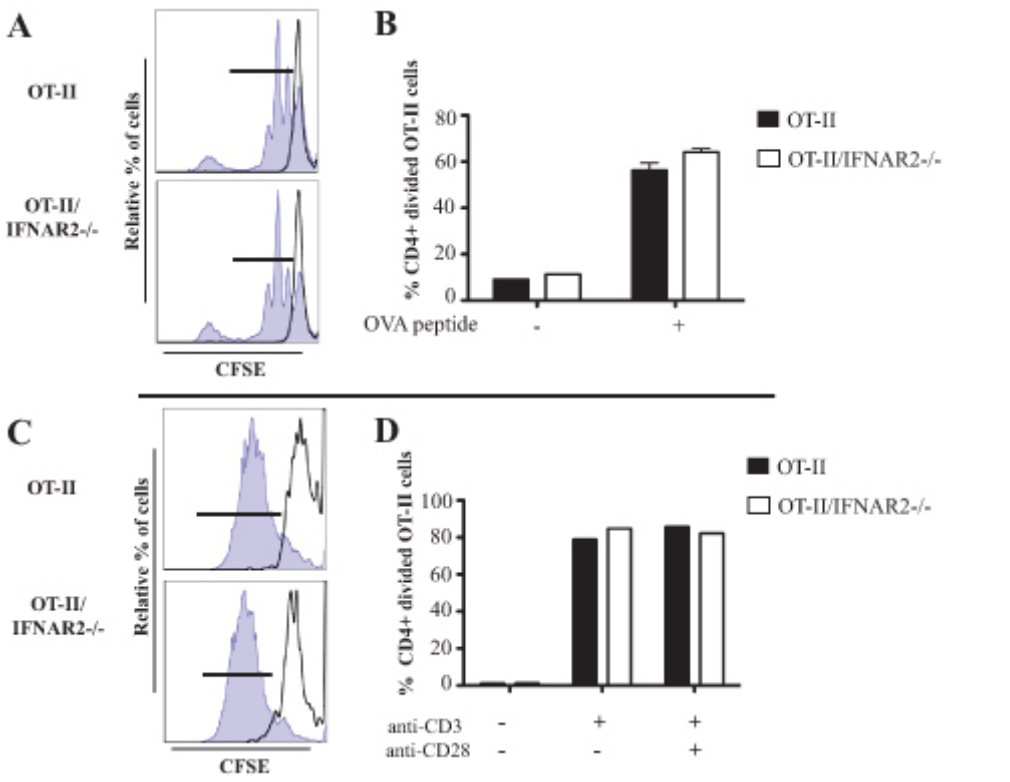

Figure 3.8 Naïve OT-II/IFNAR2-/- CD4$^+$ T cells display normal proliferation at 48 or 72 hours post-stimulation, despite decreased IL-2 secretion.

(**A and B**) Percent values of one independent experiment, sample size unstimulated: OT-II, n = 1 (9.04); OT-II/IFNAR2-/-, n = 1 (11.3). Stimulated: OT-II, n = 2 (mean 56.3 +/- 3.1); OT-II/IFNAR2-/-, n = 2 (mean 64.1 +/- 1.6). Purified OT-II/IFNAR2-/- and OT-II CD4$^+$ T cells, stained with CFSE and stimulated with .3 μM OVA peptide in the presence of irradiated splenocytes for 48 hours display similar proliferation kinetics. Error bars represent standard error of the mean. (**C and D**) Percent values of one independent experiment, sample size unstimulated: OT-II (1.16); OT-II/IFNAR2-/- (1.21). Anti-CD3 stimulated, OT-II (78.90); OT-II/IFNAR2-/- (84.80). Anti-CD3/anti-CD28, OT-II (85.70); OT-II/IFNAR2-/- (82.20). APCs do not appear to provide additional help, as similar proliferation kinetics are also observed when OT-II/IFNAR2-/- and OT-II CD4$^+$ T cells are stimulated with plate bound anti-CD3 or anti-CD3/anti-CD28 for 72 hours. CD4$^+$ T cells were gated on and CFSE dilution based on unstimulated control. (black line is unstimulated; blue is stimulated).

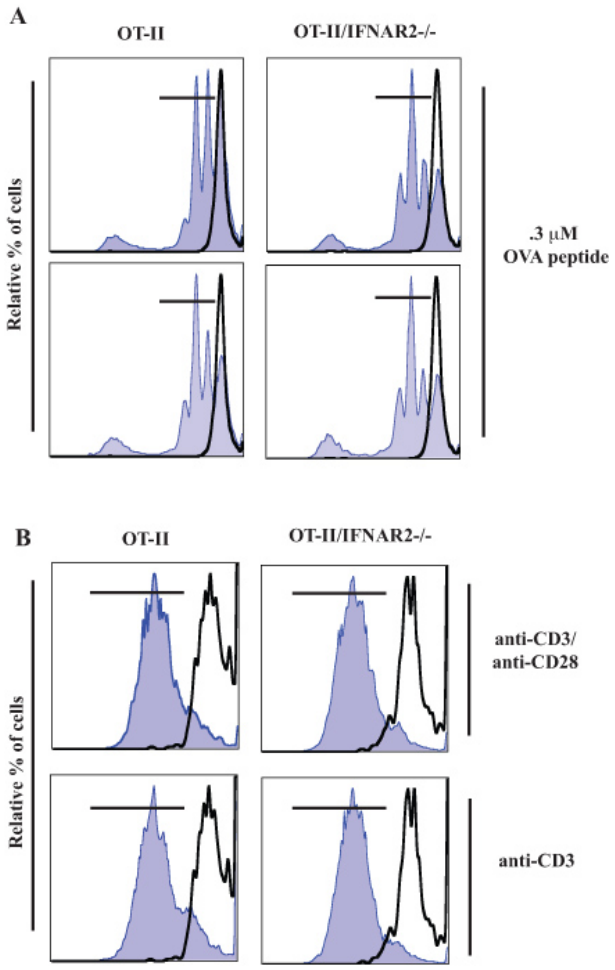

Figure 3.9 Representative experiments of data presented in Figure 3.8.

(**A**) Representative experiments of data presented in panel A and B of Figure 3.8. Purified $CD4^+$ T cells stained with CFSE were stimulated with .3 μM OVA peptide for 48 hours in the presence of irradiated splenocytes. Cells were analyzed for proliferation by flow cytometry. (**B**) Representative experiments of data presented in panels C and D of Figure 3.8. Purified $CD4^+$ T cells stained with CFSE were stimulated with plate bound anti-CD3/anti-CD28 for 72 hours, then analyzed for cell proliferation by flow cytometry.

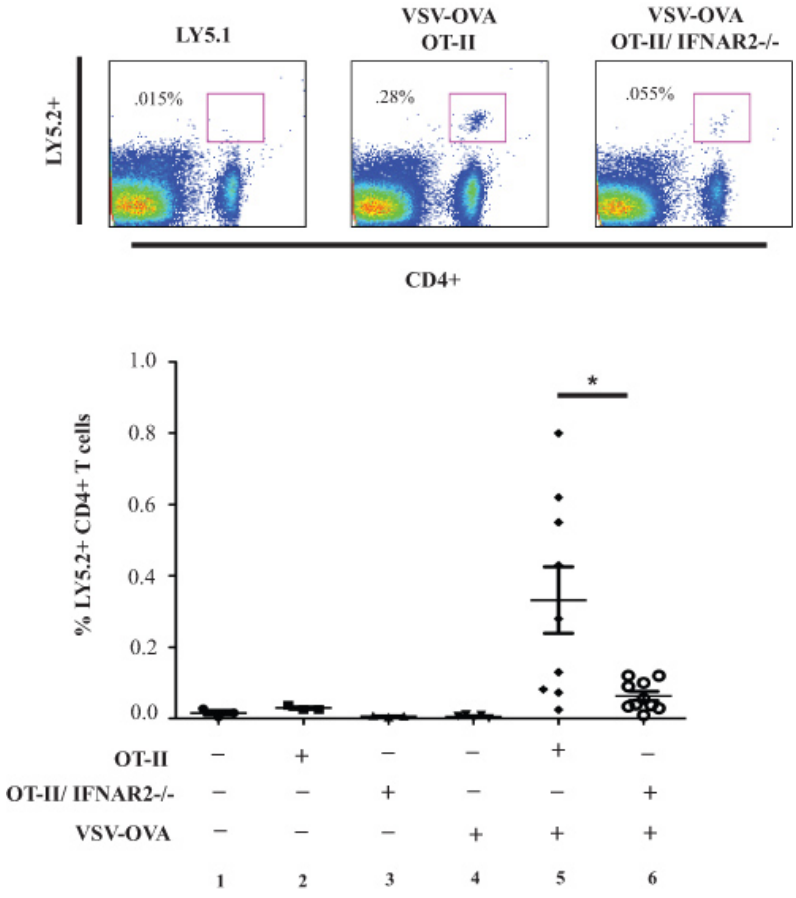

Figure 3.10 OT-II/IFNAR2-/- CD4$^+$ T cells display defect in expansion in response to primary *in vivo* infection with VSV-OVA.

(A and B) Naïve OT-II and OT-II/IFNAR2-/- CD4$^+$ T cells were transferred into healthy C57BL/6-LY5.1 mice and infected with VSV-OVA. Seven days post-infection with primary infection of VSV-OVA, peripheral blood was stained for CD4$^+$ and, congenic marker, LY5.2. Mean of one independent experiment, sample size: lane 1, n = 3 (mean .015 +/- .007); lane 2, n = 3 (mean. 030 +/- .004); lane 3, n = 3 (mean .007 +/- .001); lane 4, n = 5 (mean .005 +/- .002); lane 5, n = 9 (mean .332 +/- .093); lane 6, n = 10 (mean .063 +/- .013) * p = .008 as determined by unpaired Student's t-test, error bars represent standard error of the mean.

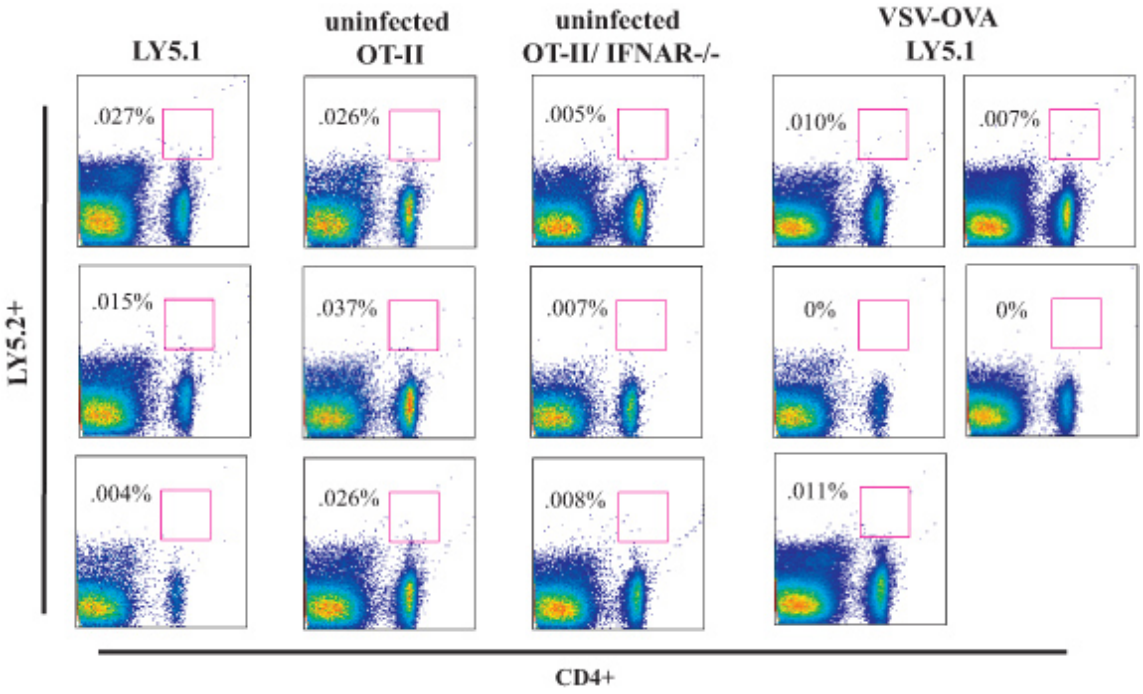

Figure 3.11 Representative FACS plots for data presented in Figure 3.10.

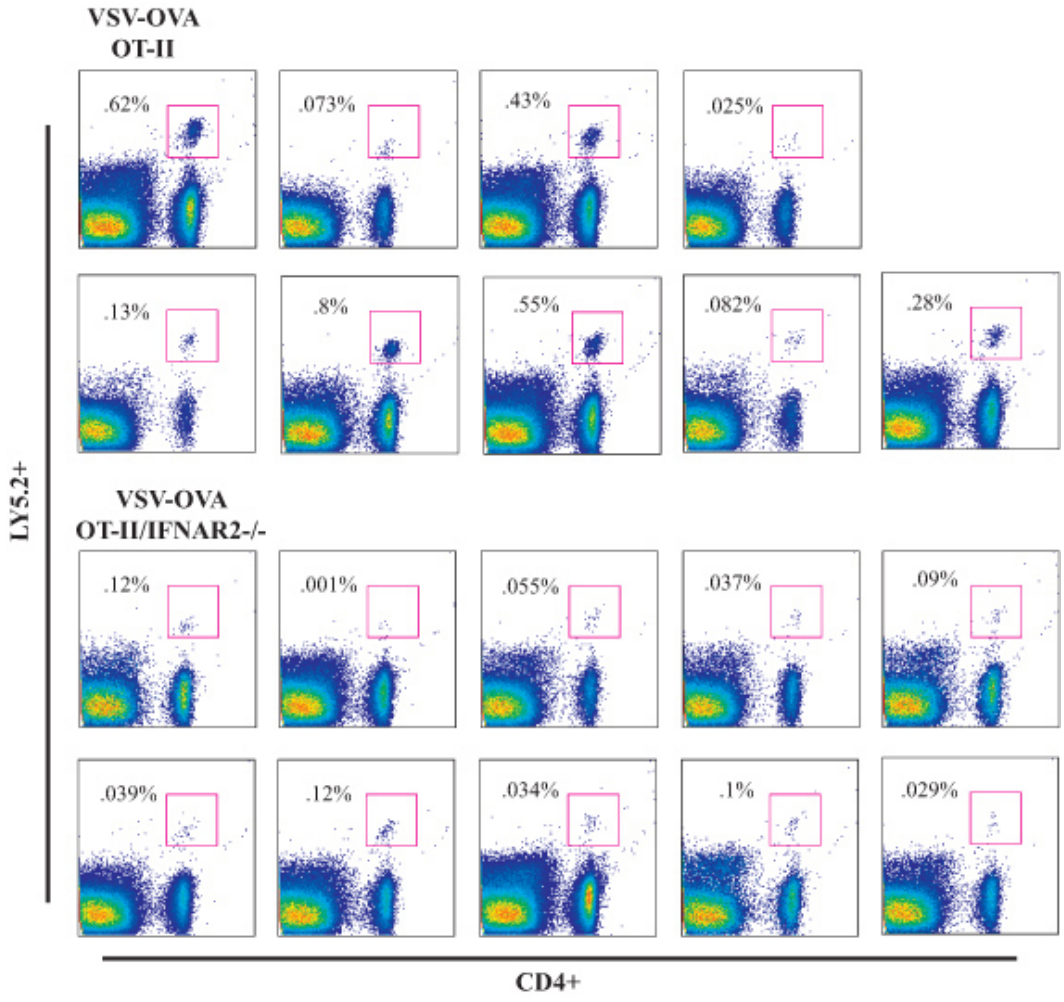

Figure 3.12 Representative FACS plots for data presented in Figure 3.10.

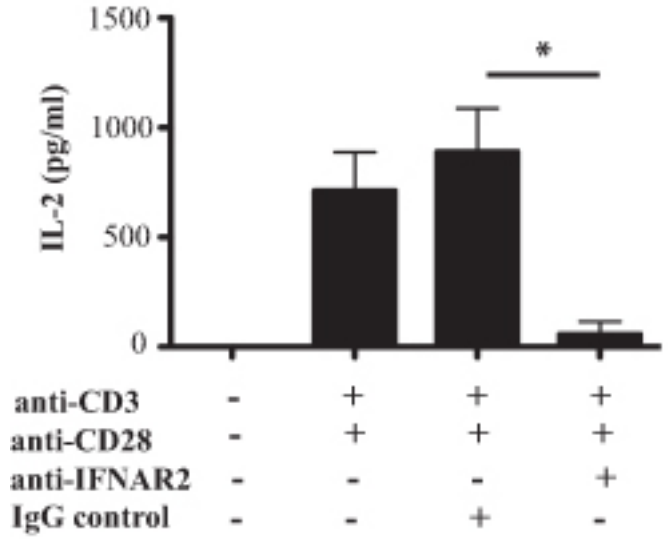

Figure 3.13 Blocking IFNAR2 on OT-II CD4$^+$ T cells abrogates IL-2 expression.

Purified CD4$^+$ T cells stimulated with plate bound anti-CD3/anti-CD28 in the presence of anti-IFNAR2 or IgG control, were harvest 72 hours post-stimulation and supernatants analyzed by ELISA. OT-II CD4$^+$ T cells displayed a significant decrease in IL-2 secretion when treated with anti-IFNAR2 when compared to untreated or IgG control treated. Mean of one independent experiment, sample size: unstimulated, n = 3 (mean 0.00); anti-CD3/anti-CD28, n = 3 (mean 712.67 +/- 175.52); anti-CD3/anti-CD28 + IgG control, n = 3 (mean 890.67 +/- 197.36); anti-CD3/anti-CD28 + anti-IFNAR2, n = 3 (mean 59.4 +/- 56.33) * $p < .015$ as determined by Student's t test, error bars represent standard error of the mean.

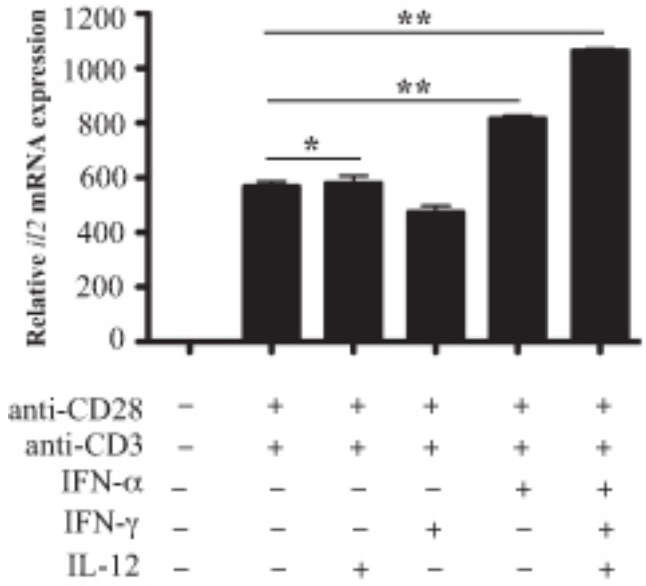

Figure 3.14 Enhanced IL-2 expression in naïve OT-II CD4$^+$ T cells following treatment with IFN-α.

Naïve OT-II CD4$^+$ T cells stimulated on plate bound anti-CD3/anti-CD28 while treated with IFN-α, IFN-γ, IL-12 alone or in combination were harvested for total RNA at 48 hours post-stimulation. *Il2* mRNA transcripts were examined by TaqMan gene expression assay and normalized to *gapdh* mRNA transcripts. Mean of one independent experiment, sample size unstimulated, n = 3 (mean 1.0 +/- .058); anti-CD3/anti-CD28 + neutralized conditions, n = 3 (mean 568.7 +/- 18.25); anti-CD3/anti-CD28 + IL-12, n = 3 (mean 580.03 +/- 27.84); anti-CD3/anti-CD28 + IFN-γ, n = 3 (475.37 +/- 21.10); anti-CD3/anti-CD28 + IFN-α, n = 3 (mean 816.63 +/- 9.93); anti-CD3/anti-CD28 + IL-12, IFN-γ and IFN-α, n = 3 (mean 1065.07 +/- 6.5). *p = .751; **p < .001) as determined by Student's t test, error bars represent standard error of the mean.

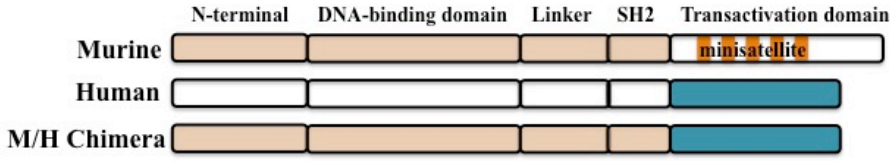

Figure 3.15 C-terminus of murine STAT2 is swapped for human STAT2, creating a M/H chimeric STAT2 molecule.

Murine STAT2 contains a minisatellite insertion at the c-terminus within the transactivation domain. Swapping the C-terminus of murine STAT2 for human STAT2 creates a chimeric STAT2 molecule. For the purpose of this study, this allows us to study the contribution of the C-terminus in regulating *il2 gene* expression.

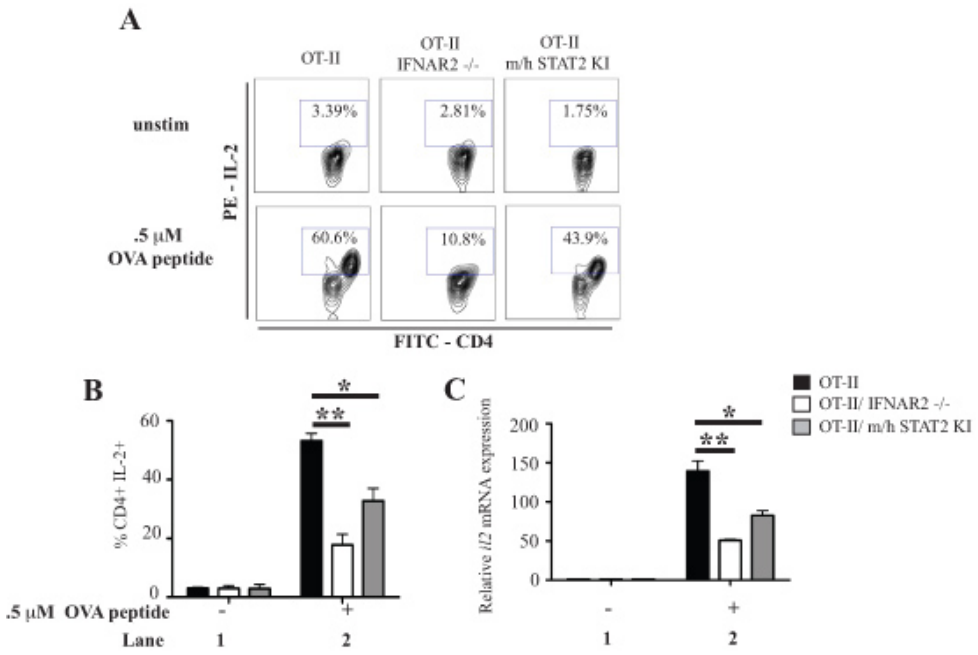

Figure 3.16 OT-II/m/h STAT2 KI CD4$^+$ T cells phenocopy OT-II/IFNAR2-/- CD4$^+$ T cells and display decreased IL-2 expression.

(**A and B**) Decreased intracellular IL-2 expression present in OT-II/IFNAR2-/- and OT-II/ m/h STAT2 KI CD4$^+$ T cells at 48 hours post-stimulation with OVA-peptide. Mean of two independent experiments, sample size unstimulated: OT-II, n = 3 (mean 3.02 +/- .34); OT-II/IFNAR2-/-, n = 3 (mean 2.99 +/- .922); OT-II/ m/h STAT2 KI, n = 3 (mean 2.05 +/- .543). Stimulated with .5 μM OVA peptide: OT-II, n = 6 (mean 53.17 +/- 2.58); OT-II/IFNAR2-/-, n = 6 (mean 17.8 +/- 3.64); OT-II/ m/h STAT2 KI, n = 6 (mean 32.78 +/- 4.22). (*p = .0001; **p < .002) determined by Student's t test, error bars represent standard error of the mean. (**C**) Decreased *il2* mRNA expression in OT-II/IFNAR2-/- and OT-II/m/h STAT2 KI splenocytes stimulated with OVA peptide for 8 hours. Mean of one independent experiment, sample size: Unstimulated; OT-II, n = 3 (mean 1.03 +/- .033); OT-II/IFNAR2-/-, n = 3 (mean 1.0 +/- .10); OT-II/ m/h STAT2 KI, n = 3 (mean 1.0 +/-.12). Stimulated .5 μM OVA peptide; OT-II, n = 3 (mean 139.57 +/- 12.81); OT-II/IFNAR2-/-, n = 3 (mean 50.73 +/- 1.33); OT-II/ m/h STAT2 KI, n = 3 (mean 82.60 +/- 5.9). *p = .016; **p = .002) as determined by Student's t test. Error bars represent standard error of the mean

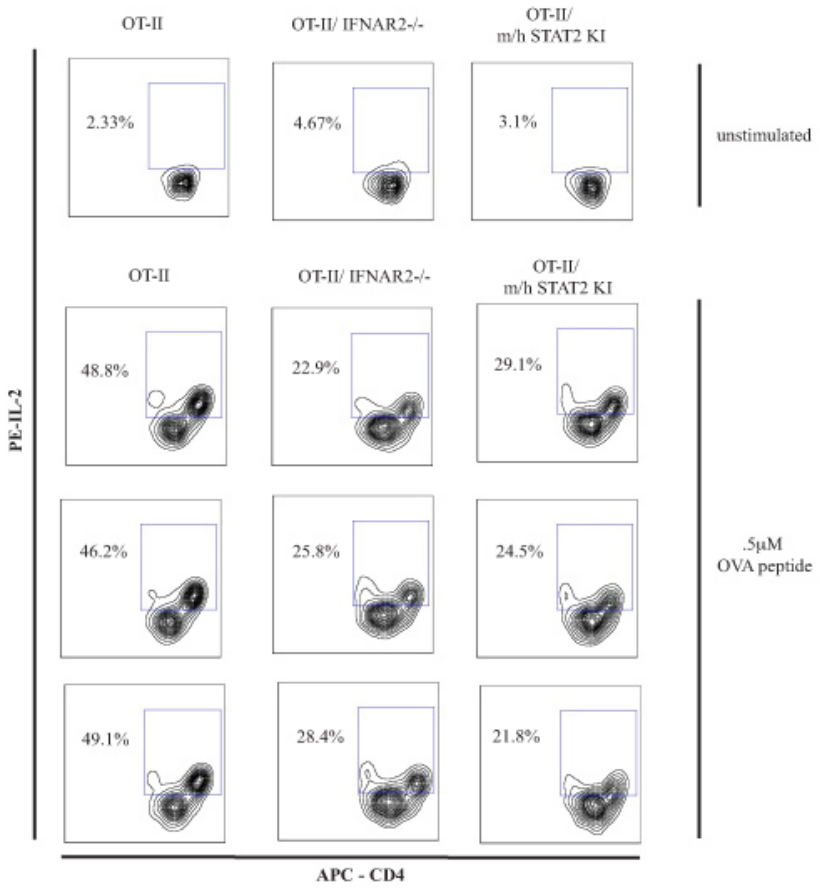

Figure 3.17 One representative experiment examining intracellular IL-2 expression in OT-II, OT-II/IFNAR2-/-, and OT-II/ m/h STAT2 KI at 48 hours post-stimulation.

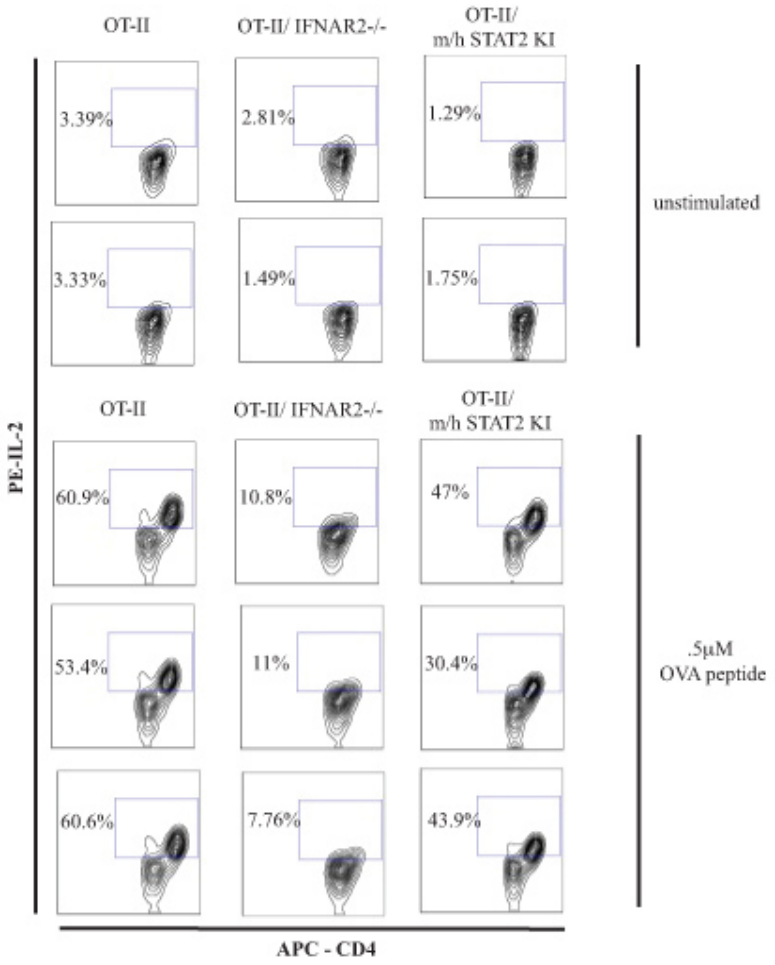

Figure 3.18 One representative experiment examining intracellular IL-2 expression in OT-II, OT-II/IFNAR2-/-, and OT-II/ m/h STAT2 KI at 48 hours post-stimulation.

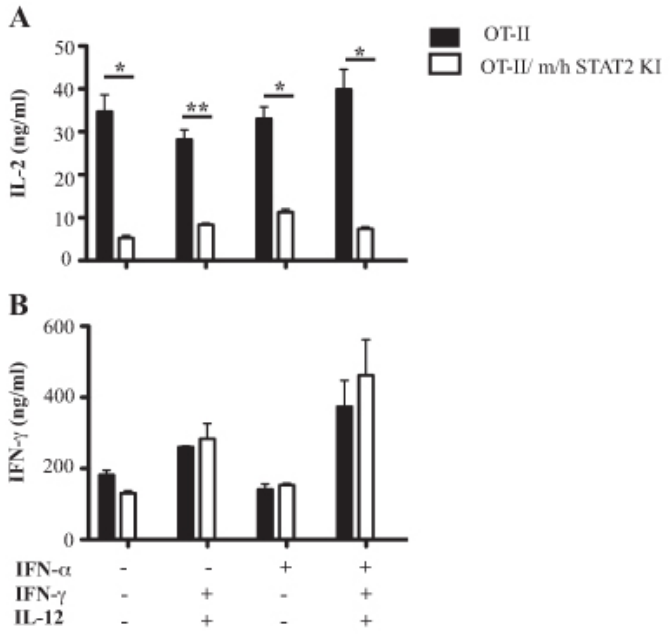

Figure 3.19 CD4$^+$ T_H1 differentiation remains intact in OT-II/ m/h STAT2 KI mice.

Splenocytes cultured for 3 days in indicated polarizing conditions with .5 μM OVA peptide, were re-stimulated on day 7 for 24 hours and supernatants harvested for analysis by ELISA. (**A**) Significant defect in IL-2 secretion when OT-II is compared to OT-II/ m/h STAT2 KI in each polarizing condition. Mean of one experiment, sample size: Neutralized condition; OT-II, n = 3 (mean 34.67 +/- 3.9); OT-II/ m/h STAT2 KI, n = 3 (mean 5.2 +/- .63). IFN-γ/IL-12 polarization; OT-II, n = 3 (mean 28.17 +/- 2.3); OT-II/ m/h STAT2 KI, n = 3 (mean 8.4 +/- .33). IFN-α polarization; OT-II, n = 3 (mean 33.03 +/- 2.7); OT-II/ m/h STAT2 KI, n = 3 (mean 11.28 +/- .65). IL-12/IFN-γ/IFN-α polarization; OT-II, n = 3 (mean 39.86 +/- 4.7); OT-II/ m/h STAT2 KI, n = 3 (mean 7.4 +/- .42) (* p = .002; **p = .001) as determined by unpaired Student's t-test (**B**) IFN-γ secretion in CD4$^+$ T_H1 cells, is not affected. Mean of one experiment, sample size: Neutralized condition; OT-II, n = 3 (mean 181.75 +/- 12.89); OT-II/ m/h STAT2 KI, n = 3 (mean 130.4 +/- 7.6). IL-12/IFN-γ polarization; OT-II, n = 3 (mean 260.2 +/- 3.07); OT-II/ m/h STAT2 KI, n = 3 (mean 283.04 +/- 43.7). IFN-α polarization; OT-II, n = 3 (mean 140.84 +/- 15.6); OT-II/ m/h STAT2 KI, n = 3 (mean 153.08 +/- 5.11). IL-12/IFN-g/IFN-a polarization; OT-II, n = 3 (mean 373.77 +/- 73.55); OT-II/ m/h STAT2 KI (mean 461.89 +/- 99.7). Error bars represent standard error of the mean.

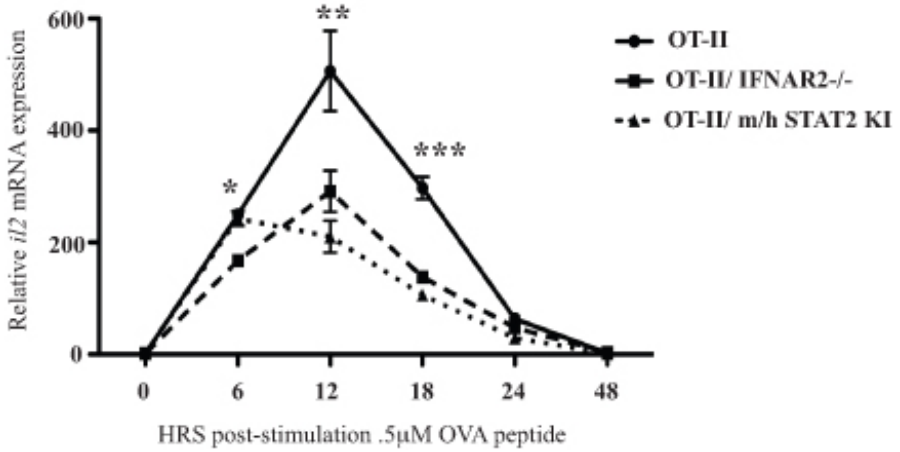

Figure 3.20 Sustained IL-2 expression is defective in OT-II/ IFNAR2-/- and OT-II/ m/h STAT2 KI splenocytes.

Whole splenocytes from OT-II, OT-II/ IFNAR2-/-, and OT-II/ m/h STAT2 KI were activated with .5μM OVA peptide for the indicated time and cells harvested and total RNA extracted. IL-2 mRNA was normalized to GAPDH mRNA is relative to each strain's unstimulated sample. Mean of one independent experiment, sample size: Unstimulated (0 hours): OT-II, n = 3 (mean 1.0 +/- .058); OT-II/IFNAR2-/-, n = 3 (mean 1.033 +/- .09); OT-II/ m/h STAT2 KI, n = 3 (mean 1.0 +/- .06). Six hours post-stimulation: OT-II, n = 3 (mean 249.07 +/- 4.5); OT-II/IFNAR2-/-, n = 3 (mean 167.37 +/- 2.67); OT-II/ m/h STAT2 KI, n = 3 (mean 242.87 +/- 13.12). Twelve hours post-stimulation: OT-II, n = 3 (mean 506.47 +/- 71.81); OT-II/IFNAR2-/-, n = 3 (mean 291.4 +/- 36.75); OT-II/ m/h STAT2 KI, n = 3 (mean 210.17 +/- 28.65). Eighteen hours post-stimulation: OT-II, n = 3 (mean 297.4 +/- 20.04); OT-II/IFNAR2-/-, n = 3 (mean 138.2 +/- 2.21); OT-II/ m/h STAT2 KI, n = 3 (mean 106.23 +/- 7.68). Twenty four hours post-stimulation: OT-II, n = 3 (mean 63.34 +/- 5.03); OT-II/IFNAR2-/-, n = 3 (mean 47.4 +/- .76); OT-II/ m/h STAT2 KI, n = 3 (29.17 +/- 1.8). Forty eight hours post-stimulation: OT-II, n = 3 (mean 1.5 +/- .1); OT-II/IFNAR2-/-, n = 3 (3.17 +/- .22); OT-II/ m/h STAT2 KI, n = 3 (mean 1.03 +/- .09). Compared to OT-II *p = .678 (OT-II/ m/h STAT2 KI), *p < .0001 (OT-II/ IFNAR2-/-); Compared to OT-II **p = .056 (OT-II/ IFNAR2-/-), **p = .018 (OT-II/ m/h STAT2 KI); ***p = .019 (OT-II/ IFNAR2-/-), *** p = .001 (OT-II/ m/h STAT2 KI) as determined by Student's t test. Error bars represent standard error of the mean.

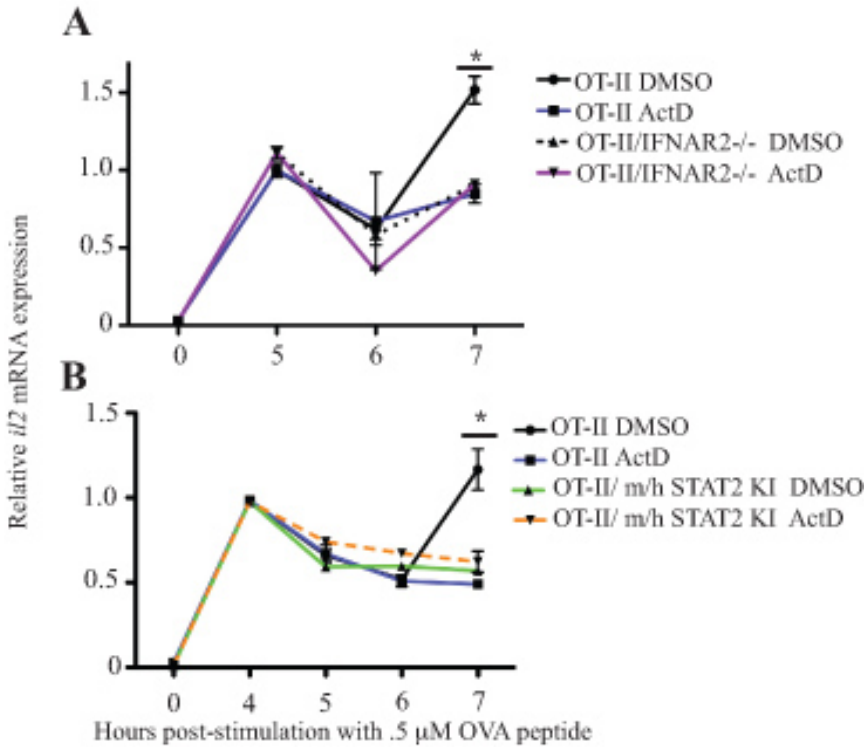

Figure 3.21 IL-2 mRNA degradation not increased in OT-II/ IFNAR2-/- or OT-II/ m/h STAT2 KI splenocytes when compared to OT-II.

OT-II and either (**A**) OT-II/IFNAR2-/- (*p < .003) Unstimulated (0 hour): OT-II, n = 3 (mean .029 +/- .003); OT-II/IFNAR2-/-, n = 3 (mean .026 +/- .0008). Five hours post-stimulation: OT-II, n = 3 (mean .999 +/- .044); OT-II/IFNAR2-/-, n = 3 (mean 1.11 +/- .039). Six hours post-stimulation: OT-II DMSO, n = 3 (mean .618 +/- .064); OT-II Act D, n = 3 (mean .672 +/- .312); OT-II/IFNAR2-/- DMSO, n = 3 (mean .581 +/- .0641); OT-II/IFNAR2-/- Act D, n = 3 (mean .352 +/- .0022). Seven hours post-stimulation: OT-II DMSO, n = 3 (mean 1.518 +/- .088); OT-II Act D, n = 3 (mean .848 +/- .061); OT-II/IFNAR2-/- DMSO, n = 3 (mean .908 +/- .0262); OT-II/ IFNAR2-/- Act D, n = 3 (mean .911 +/- .0325). Splenocytes were activated with .5 μM OVA peptide for 4 or 5 hours and either harvested or treated with actinomycin D for 2-3 hours. Every hour a sample was harvested and total RNA extracted. IL-2 mRNA was normalized to GAPDH mRNA, and referenced to the 4 or 5 hour sample. Significance determined by Student's t test, error bar represents standard error of the mean.

Figure 3.21 (continued) IL-2 mRNA degradation not increased in OT-II/IFNAR2-/- or OT-II/ m/h STAT2 KI splenocytes when compared to OT-II.

(**B**) OT-II/ m/h STAT2 KI (*p < .02) Mean of one experiment, sample size: Unstimulated (0 hour): OT-II, n = 3 (mean .027 +/- .003); OT-II/ m/h STAT2 KI, n = 3 (mean .013 +/- .003). Four hours post-stimulation: OT-II, n = 3 (mean .983 +/- .009); OT-II/ m/h STAT2 KI, n = 3 (mean .977 +/- .023). Five hours post-stimulation: OT-II DMSO, n = 3 (mean .660 +/- .035); OT-II Act D, n = 3 (mean .670 +/- .051); OT-II/ m/h STAT2 KI DMSO, n = 3 (mean .593 +/- .003); OT-II/ m/h STAT2 KI Act D, n = 3 (mean .740 +/- .023). Six hours post-stimulation: OT-II DMSO, n = 3 (mean .520 +/- .015); OT-II Act D, n = 3 (mean .510 +/- .036); OT-II m/h STAT2 KI DMSO, n = 3 (mean .597 +/- .007); OT-II/ m/h STAT2 KI Act D, n = 3 (mean .673 +/- .028). Seven hours post-stimulation: OT-II DMSO, n = 3 (mean 1.167 +/- .120); OT-II Act D, n = 3 (mean .490 +/- .021); OT-II/ m/h STAT2 KI, n = 3 (mean .570 +/- .007); OT-II/ m/h STAT2 KI Act D, n = 3 (mean .623 +/- .062). Splenocytes were activated with .5 μM OVA peptide for 4 or 5 hours and either harvested or treated with actinomycin D for 2-3 hours. Every hour a sample was harvested and total RNA extracted. IL-2 mRNA was normalized to GAPDH mRNA, and referenced to the 4 or 5 hour sample. Significance determined by Student's t test, error bar represents standard error of the mean.

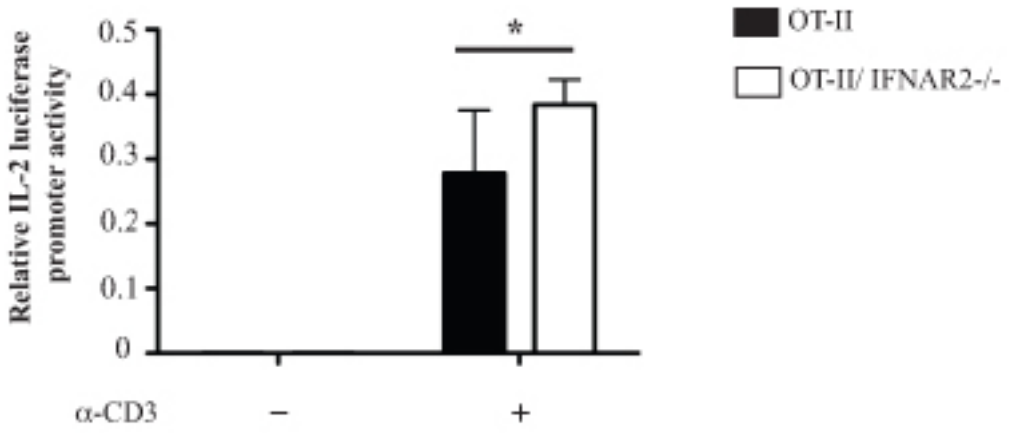

Figure 3.22 IL-2 promoter activity is augmented in OT-II/IFNAR2-/- CD4$^+$ T cells.

A 1.9kb IL-2 promoter element driving luciferase expression in OT-II and OT-II/IFNAR2-/- CD4$^+$ T cells displayed slightly enhanced promoter activity at 7 hours post-stimulation. Luciferase activity was normalized to 1 x 10^5 cells/well. Mean of two experiments, sample size: Unstimulated: OT-II, n = 4 (mean .0005 +/- .0001); OT-II/IFNAR2-/-, n = 4 (mean .0005 +/- .00009). Anti-CD3 stimulated: OT-II, n = 4 (mean .2782 +/- .097); OT-II/IFNAR2-/-, n = 4 (mean .384 +/- .0388). Significance (*p = .352) was determined by unpaired Student's t-test. Error bars represent standard error of the mean.

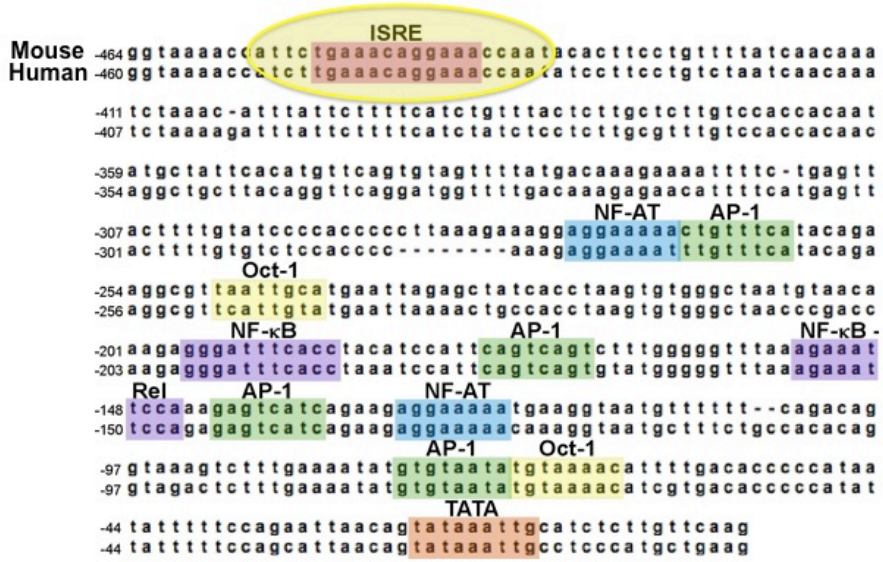

Figure 3.23 Alignment of the proximal IL-2 promoter of mouse and human.

The IL-2 promoter contains a putative interferon stimulated response element (ISRE).

Gene	Sequence	ISRE Consensus Sequence	Position
IFIT1 (h)	T**AG TTT C ACT TTC C**C	AGTTTC NN TTTCC	-125 to -93
IFI6 (h)	GG**G AAA ATG AAA C**T	GAAA NN GAAAC	-127 to -89
IFIT2 (h)	TCT **AGT TTC ACT TTC C**CT TT	AGTTTC NN TTTCC	-103 to -84
OAS1 (h)	C TCC TCC CTT CTG AG**G AAA CGA AAC** CAA CAG CAG T	GAAA N GAAAC	-113 to -74
ISG15 (h)	AAG G**GA AAC CGA AAC** TG	GAAA NN GAAAC	-111 to -94
IFITM1 (h)	AG**G AAA TAG AAA C**T	GAAA NN GAAAC	-185 to -147
IL-2 (h)	T**GA AAC AGG AAA C**	GAAA NNN GAAAC* *putative	-450 to -438

Table 3.1 Putative ISRE sequence found within IL-2 promoter, similar to ISRE consensus sequences found within ISGs.

An alignment of the putative ISRE sequence identified within the IL-2 promoter with known ISRE consensus sequences in ISGs promoter elements indicates a strong sequence similarity.

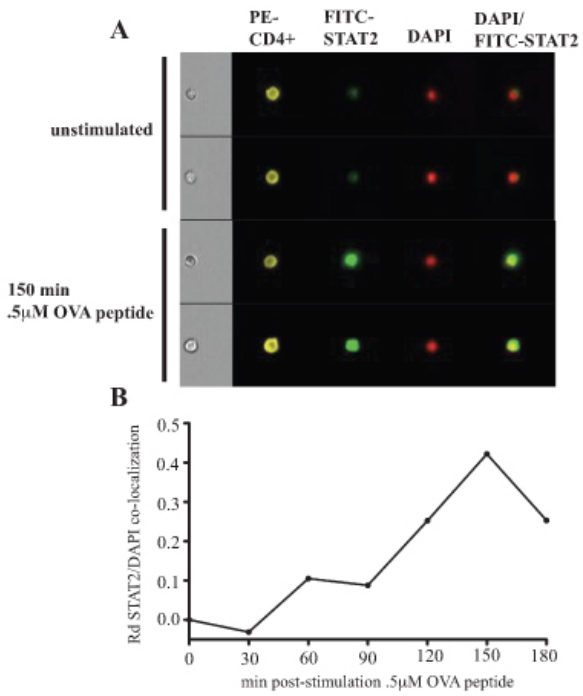

Figure 3.24 Preliminary data by ImageStream may indicate STAT2 nuclear translocation in OT-II CD4$^+$ T cells in response to TCR stimulation.

(**A**) Following 150 minutes post-stimulation with .5μM OVA peptide there is an enhancement of STAT2 nuclear localization when compared to unstimulated control. (**B**) Quantification of STAT2 nuclear co-localization with DAPI indicates a gradual increase in nuclear localization following TCR stimulation. (Performed 1 experiment, with at least 80 cells examined per time-point with .5μM OVA peptide stimulation.)

CHAPTER IV

ABSENCE OF THE INTERFERON ALPHA RECEPTOR IN MURINE CD4$^+$ T CELLS DOES NOT AUGMENT IL-2 EXPRESSION

Introduction

Type I IFN, while observed in human CD4$^+$ T cells to play to important role in differentiating IL-2 secreting central memory CD4$^+$ T cells, it is unknown if IFN plays a similar role in murine CD4$^+$ T cells. Multiple groups have observed crosstalk of the type I IFN and T cell receptor signaling pathways (54, 55, 59, 63, 73, 74). Stevens and colleagues suggest stimulating murine CD4$^+$ T cells with type I IFN and anti-CD3 slightly enhances NFAT promoter activity (55). However, activation of downstream NFAT regulated genes, such as IL-2, was not examined. Other studies have examined activation of type I IFN signaling molecules in response to T cell receptor stimulation (76, 82).

In response to T cell receptor stimulation, STAT1 is phosphorylated at serine 727 and translocates to the nucleus (82). While it is unknown how STAT1 activation occurs, it appears to be independent of type I or type II IFN signaling (82). Maldonado and colleagues also observed STAT1 accumulation at the TCR

signaling complex in response to T cell receptor stimulation (82). This indicates that in the absence of the type II IFN receptor STAT1 may be able to associate with the TCR and signal downstream in a TCR dependent manner. If in the absence of the type I IFN receptor, OT-II/IFNAR2-/- $CD4^+$ T cells optimally express IL-2, it would not exclude the possibility that downstream signaling molecules could be involved in sustaining IL-2 expression.

There are multiple signaling molecules within the type I IFN signaling pathway that could potentially be directly activated in response to TCR stimulation. Type I IFN binds the high affinity IFN receptor (IFNAR2) that leads to recruitment of IFNAR1, to form the type I IFN signaling complex. The signaling complex activates and transphosphorylates Tyk2 and JAK1, which phosphorylates the cytoplasmic tail of IFNAR1. STAT2 binds IFNAR1, providing a docking site for STAT1. STAT1 and STAT2 heterodimerize and bind IRF9 to form the ISGF3 complex, then translocate to the nucleus to bind interferon stimulated response elements (ISRE) within gene promoters (10). It may be essential to determine if the molecules normally associated with IFN signaling play a role in regulating IL-2 expression even in the absence of the type I IFN receptor.

Type I IFN signaling could also play a direct role in regulating IL-2 expression through the induction of proteins that destabilize IL-2 mRNA. Type 1 IFN signaling and TCR stimulation have been suggested to upregulate TTP

expression. TTP is a molecule involved in destabilizing IL-2 mRNA transcripts (94, 119). If TTP expression were elevated in OT-II $CD4^+$ T cells in response to TCR stimulation when compared to OT-II/IFNAR2-/- $CD4^+$ T cells, then OT-II/IFNAR2-/- $CD4^+$ T cells would be expected to express elevated levels of IL-2.

It has not been demonstrated if receptor crosstalk affects IL-2 signaling in murine $CD4^+$ T cells. The current study was designed to investigate the role of the type I IFN receptor in IL-2 expression in naïve murine $CD4^+$ T cells.

Results

IL-2 expression is not differentially expressed in OT-II/IFNAR2-/- CD4$^+$ T cells stimulated with anti-CD3/anti-CD28

Purified CD4$^+$ T cells from OT-II and OT-II/IFNAR2-/- mice were stimulated with plate bound anti-CD3/anti-CD28 or PMA/Ionomycin for 72 hours and supernatants collected for analysis by ELISA. While OT-II/IFNAR2-/- CD4$^+$ displayed slightly elevated levels of IL-2 expression, there was not a significant difference ($p = .0886$) in IL-2 secretion between OT-II and OT-II/IFNAR2-/- CD4$^+$ T cells stimulated with plate bound anti-CD3/anti-CD28 (**Figure 4.1**).

Similar intracellular IL-2 protein expression in OT-II and OT-II/IFNAR2-/- CD4$^+$ T cells in response to OVA peptide stimulation

Whole splenocyte cultures from OT-II and OT-II/IFNAR2-/- mice were stimulated with OVA peptide for 48 hours. Cells were stained for cell surface marker CD4 and intracellular IL-2 protein expression and analyzed by flow cytometry. While the sample size is relatively small ($n = 2$), there was no significant difference ($p = .0910$) in intracellular IL-2 protein expression between OT-II and OT-II/IFNAR2-/- CD4$^+$ T cells (**Figure 4.3**). Including a larger sample

size could provide insight into whether this observation is representative of OT-II/IFNAR2-/- $CD4^+$ T cells.

Discussion

The current study did not demonstrate a significant difference in IL-2 protein expression between naïve murine OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells. Interestingly OT-II/IFNAR2-/- displayed an insignificant increase in IL-2 secretion when compared to OT-II. Further studies may help determine if the insignificant increase in IL-2 observed in the current study is reflective of a natural phenomenon. If OT-II $CD4^+$ T cells are secreting significantly less IL-2 than OT-II/IFNAR2-/- $CD4^+$ T cells, then the type I IFN receptor may be involved in inducing expression of a molecule involved in destabilizing *il-2* mRNA transcripts.

Previous studies have observed upregulation of TTP in response to anti-CD3/anti-CD28 stimulation as well as type I IFN signaling (94, 119). In T cells from TTP-/- mice, IL-2 expression is increased due to a lack of negative regulation of IL-2 mRNA transcripts (94). However, previous findings from Davis and colleagues did not identify type I IFN expression in human $CD4^+$ T cells (108). Therefore, either murine $CD4^+$ T cells are secreting a low level of type I IFN that is able to further induce TTP expression or OT-II $CD4^+$ T cells are unable to shut off TTP expression following TCR stimulation. This study did not confirm previous findings that increased TTP expression correlates with decreased IL-2 expression in OT-II $CD4^+$ T cells. It will be necessary to

determine if TTP expression is elevated in TCR stimulated OT-II $CD4^+$ T cells when compared to OT-II/IFNAR2-/- $CD4^+$ T cells.

While there does not appear to be a role for the type I IFN receptor in regulating IL-2 expression in naïve murine $CD4^+$ T cells, there may be a role for components of the type I IFN signaling pathway in regulating IL-2 expression in naïve murine $CD4^+$ T cells. Receptor crosstalk of signaling molecules within the type I IFN signaling pathway and the T cell signaling pathway has previously been observed (76, 82).

Maldonado and colleagues observed STAT1 phosphorylation and nuclear translocation in response to T cell receptor signaling in murine $CD4^+$ T cells (82). While molecules within the type I IFN signaling pathway may crosstalk with the TCR signaling pathway, the presence of the type I IFN receptor may not be necessary for these downstream signaling event to occur. The current study did not evaluate activation or nuclear translocation of type I IFN downstream signaling molecules in OT-II or OT-II/IFNAR2-/- $CD4^+$ T cells. Further studies may need to evaluate activation of downstream type I IFN signaling molecules to determine if the TCR utilizes these molecules. This could occur in the absence of the type I IFN receptor if signaling molecules are interacting directly with the TCR signaling complex.

In the current study the insignificant difference observed between OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells could be representative of the role the type I

IFN receptor plays in a naïve $CD4^+$ T cell population. It is currently unknown if there is a role for the type I IFN receptor in naïve human $CD4^+$ T cells, thus this study is unable to compare naïve $CD4^+$ T cells between the two species. However, if naïve human $CD4^+$ T cells display a requirement for the type I IFN receptor while murine $CD4^+$ T cells do not, this could be due to species differences.

A previous study by Davis and colleagues observed a role for exogenous type I IFN in human $CD4^+$ T cells in the differentiation of central memory $CD4^+$ T cells (33). It may be necessary to examine the role exogenous type I IFN has on OT-II $CD4^+$ T cells, to determine if type I IFN signaling regulates memory development similarly to that observed in human $CD4^+$ T cells. A species difference in the type I IFN signaling pathway could explain differences if type I IFN does not appear to play a role in enhancing IL-2 secreting central memory $CD4^+$ T cells.

The species difference in the type I IFN signaling pathway may explain why there is not an observed difference in IL-2 expression. Farrar and colleagues demonstrated that a minisatellite insertion in the c-terminal portion of murine STAT2, a type I IFN downstream signaling molecule, could disrupt the downstream activation of STAT4 (83). In humans, STAT2 phosphorylates STAT4 in response to type I IFN, however, this is not observed in murine $CD4^+$ T cells (56, 83). The ability of human STAT2 to differentially phosphorylate

STAT4 may play a role in the type I IFN signaling pathway's ability to regulate IL-2 expression in human $CD4^+$ T cells. This hypothesis needs to be investigated further by examining downstream molecules in the type I IFN signaling pathway.

The current study does not support the hypothesis that the type I IFN receptor is essential is regulating IL-2 expression. Additional experiments should be performed to confirm these findings as well as determine if the TTP expression is elevated in OT-II $CD4^+$ T cells

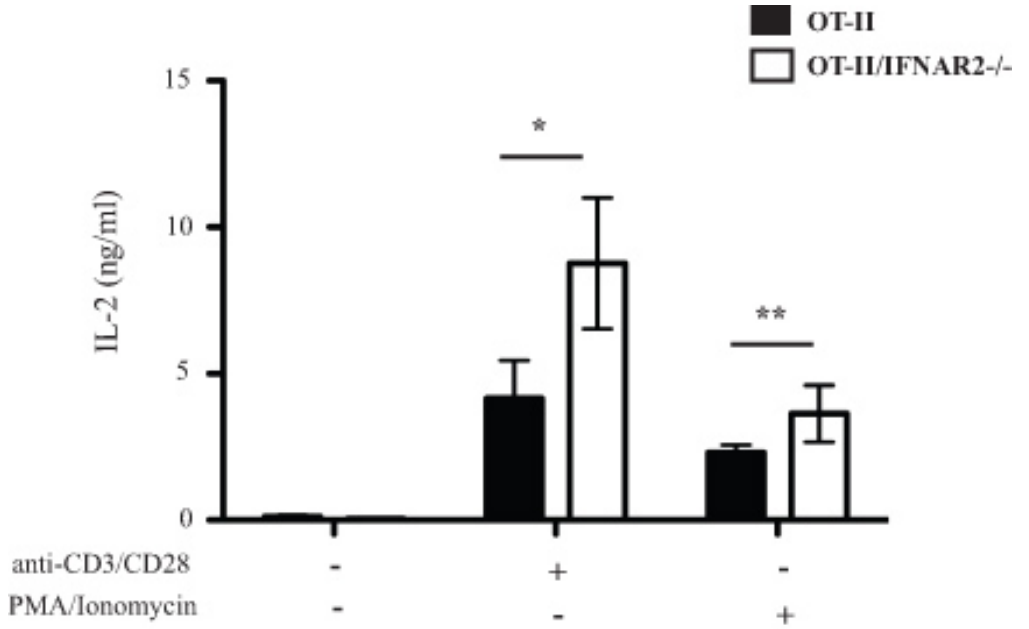

Figure 4.1 OT-II/IFNAR2-/- CD4+ T cells do not display augmented IL-2 secretion when compared to OT-II CD4$^+$ T cells at 72 hours post-stimulation.

Purified naïve CD4$^+$ T cells from OT-II and OT-II/IFNAR2-/- are stimulated with plate bound anti-CD3/anti-CD28 or PMA/Ionomycin for 72 hours and supernatants analyzed for secreted IL-2 protein by ELISA. Mean of four independent experiments, sample size unstimulated: OT-II, n = 12 (mean 117.42 +/- 60.12); OT-II/IFNAR2-/-, n = 12 (mean 53.06 +/- 21.53). Stimulated with anti-CD3/anti-CD28: OT-II, n = 12 (mean 4154.83 +/- 1297.04); OT-II/IFNAR2-/-, n = 12 (mean 8766.50 +/- 2240.37). Stimulated with PMA/Ionomycin: OT-II, n = 9 (mean 2304.56 +/- 262.72); OT-II/IFNAR2-/-, n = 9, (mean 3641.11 +/- 971.10). Error bars represent standard error of the mean. * p = .0886 and ** p = .2024 as determined by unpaired Student's t-test.

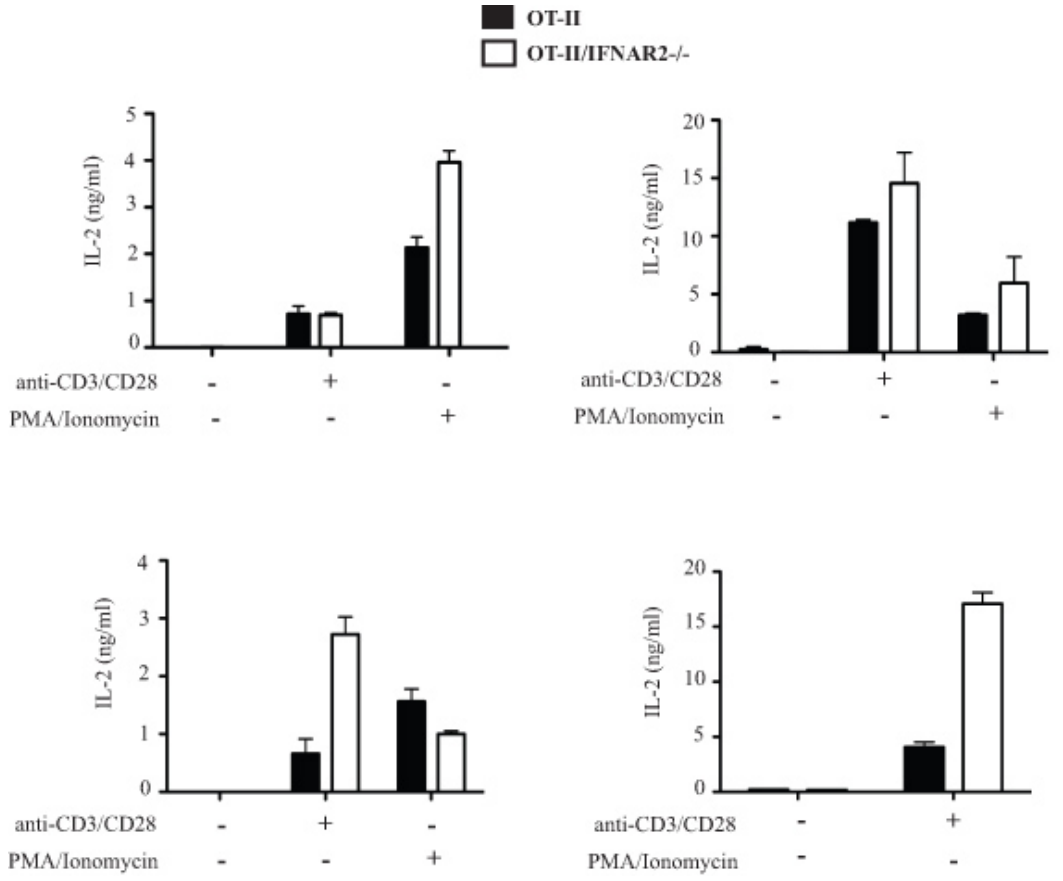

Figure 4.2 Representative experiments of data presented in Figure 4.1

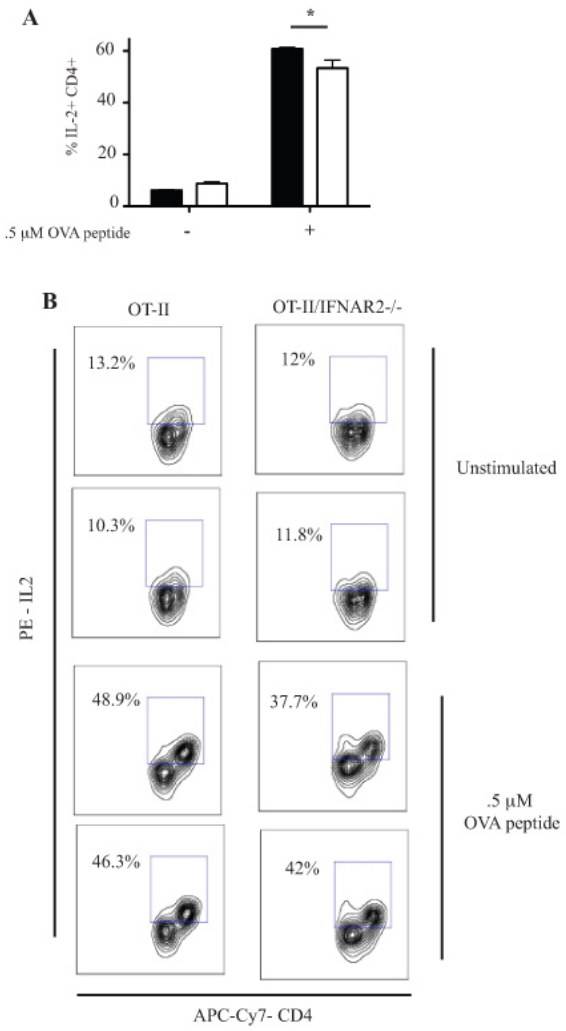

Figure 4.3 Similar intracellular protein expression in OT-II/IFNAR2-/- CD4$^+$ T cells when compared to OT-II CD4$^+$ T cells.

(**A**) Percent of CD4$^+$ T cells that stain positive for intracellular IL-2. Mean of one independent experiments sample size unstimulated: OT-II, n = 2 (mean 6.185 +/- .0235); OT-II/IFNAR2-/-, n = 2 (mean 8.80 +/- .60). OVA peptide stimulated: OT-II, n = 2 (mean 60.75 +/- .65); OT-II/IFNAR2-/- n = 2 (mean 53.4 +/- 3.0). Error bar represents the standard error of the mean. * p = .0910 as determined by unpaired Student's t-test. (**B**) Representative FACS plots of intracellular IL-2 protein expression in OT-II and OT-II/IFNAR2-/- CD4$^+$ T cells

CHAPTER V

DISCUSSION

Overview

Type I IFN activates ISGs that assist the immune system in clearing pathogen infections. The role of type I IFN in the adaptive immune response was recently shown to differentially regulate IL-2 expression and central memory development in human T_H1 $CD4^+$ T cells (33). However the mechanism behind IFN-α has not been elucidated.

The current study was designed to examine signaling molecules downstream of ligand binding that may contribute to enhanced IL-2 secretion in $CD4^+$ T cells. To perform these studies a mouse model was utilized that allowed antigen specific $CD4^+$ T cells to be examined. This study did not provide concrete evidence that the type I IFN signaling pathway definitively plays a role in IL-2 expression in naïve murine $CD4^+$ T cells. However, because there was some supporting data that indicates the type I IFN signaling pathway may play a role in IL-2 expression, the null hypothesis was note ruled out.

In mice lacking a component of the type I IFN signaling complex, OT-II/ IFNAR2-/-, conflicting observations were made. This may indicate that in most culture conditions, the type I IFN receptor is necessary for optimal IL-2 secretion in $CD4^+$ T cells. However, further studies will need to be performed in order to determine in what specific condition the type I IFN receptor is required for IL-2 secretion. Interesting, the observation that the type I IFN receptor plays a role in IL-2 secretion was recapitulated in OT-II $CD4^+$ T cells treated with an antibody directed to IFNAR2. These results may point to a role for the type I IFN signaling pathway in regulating IL-2 expression in naïve cells. Understanding how IL-2 expression is regulated in $CD4^+$ T cells will allow for manipulation of *il2* gene expression in human health conditions that display altered expression.

However, it is unknown if for optimal IL-2 expression in murine $CD4^+$ T cells requires IFN-α signaling. Treatment of naïve $CD4^+$ T cells with IFN-α appears to enhance IL-2 expression, but it is unknown if optimal expression requires a low level of type I IFN signaling. Further tests will need to be performed to determine if murine $CD4^+$ T cells both express and secrete type I IFN that feed back on the type I IFN receptor. Interesting, murine $CD4^+$ T cells that express a mutant STAT2 molecule can express the T_H1 cytokine IFN-γ and ISGs, but fail to express IL-2. This result may suggest a specific role for STAT2 in regulating IL-2 expression.

Our attempts at identifying a defect in IL-2 promoter activity have not provided conclusive results. Preliminary data indicates OT-II/ IFNAR2-/- display an insignificant enhancement of IL-2 promoter activity. However, the current system to measure promoter activity contains caveats and it may be necessary to answer this question by studying the endogenous promoter.

Preliminary data points at a putative ISRE sequence within the IL-2 promoter. While *in situ* analysis results must be confirmed it does suggest STAT2, STAT1 and IRF9 may directly bind the IL-2 promoter. Additional preliminary data obtained from a collaboration with Amnis Corporation in Seattle, WA suggests STAT2 may translocate to the nucleus in response to TCR stimulation in $CD4^+$ T cells. Further tests will need to be performed to determine if the kinetics of STAT2 translocation are similar to STAT1. This along with future experiments will determine if TCR stimulation activates ISGF3.

These observations are only the start of understanding how the type I IFN pathway can modulate IL-2 expression in naïve $CD4^+$ T cells. Based on the initial data it is possible to speculate on a potential mechanisms that ultimately must be tested. It will be necessary to perform biochemical experiments to dive into the molecular mechanism behind STAT2 regulation of IL-2 expression.

Possible role for type I IFN receptor complex in TCR induced cytokine expression.

The type I IFN receptor has long been suggested to cross talk with the TCR to elicit specific signaling events in response to IFN-α signaling. Previous studies suggest IFN-α signaling requires ZAP-70 and SLP76 to induce anti-proliferative effects (54, 55, 63). However, it has not been determined if the TCR requires specific components of the type I IFN receptor complex to regulate gene expression upon stimulation.

This study describes a possible role for the type I IFN receptor in regulating IL-2 expression. While IFNAR2 appears to play a role in optimal IL-2 secretion the majority of the time, there were multiple observations when $CD4^+$ T cells from OT-II/IFNAR2-/- mice did not display a defect in IL-2 expression. There may be experimental conditions that $CD4^+$ T cells are responding to that could affect their ability to secrete optimal levels of IL-2 in response to TCR stimulation. However, the decreased IL-2 secretion observed in OT-II/IFNAR2-/- $CD4^+$ T cells does not appear to be due to a genetic variation in IFNAR2-/- mice. As the result is duplicated in WT cells treated with a blocking antibody to IFNAR2. It is unknown if the blocking antibody is disrupting IFN signaling or preventing the movement of the type I IFN receptor in the cell membrane. While the type I IFN and TCR signaling pathways have been implicated to crosstalk, it

is unknown if the receptors co-localize in response to TCR stimulation (54, 55, 63). Co-localization of the two signaling complexes may allow for more efficient activation of shared downstream signaling molecules.

It has not been determined if IFN signaling is required for optimal IL-2 expression. Previous attempts by Davis and colleagues suggested human $CD4^+$ T cells do not express type I IFN mRNA (108). This current study observed that exogenous IFN-α might be able to enhance IL-2 expression in naïve murine $CD4^+$ T cells. It will be necessary to investigate whether murine $CD4^+$ T cells secrete a low level of type I IFN that allows for optimal IL-2 expression. Treating $CD4^+$ T cells with a blocking antibody to type I IFNs may help to address this question. However, there are 14 type I IFN subtypes, so a negative result would not conclude type I IFN is not produced in response to TCR stimulation (120). A negative result could suggest that an IFN subtype, not blocked by the blocking antibody, is eliciting effects that lead to optimal IL-2 expression. To help conclude whether or not type I IFNs are produced, it may be necessary to examine type I IFN mRNA transcripts from TCR stimulated murine $CD4^+$ T cells. Investigating downstream signaling molecules in the type I IFN receptor pathway can also help determine if IFN-α signaling is required for IL-2 expression.

Taking advantage of a mouse with a C-terminal substitution in STAT2, may suggest $CD4^+$ T cells from this strain display a similar phenotype when compared to IFNAR2-/- $CD4^+$ T cells. This may suggest the type 1 IFN pathway

is involved in regulating IL-2 expression. Having identified a possible transcription factor, STAT2, involved in IL-2 expression provides a molecule to investigate whether it is directly or indirectly involved in IL-2 expression. Transcription factors are activated in the cytoplasmic portion of the cell and translocate to the nucleus where they bind specific promoter elements. STAT1, STAT2 and IRF-9 interact to form ISGF3 and bind ISRE consensus sequences in gene promoter regions. Taking advantage of a STAT1-/- and IRF-9-/- mouse, it would be possible to provide evidence that STAT1 and IRF-9 may be involved in regulating IL-2 expression.

C-terminal domain of STAT2 may play role in regulating IL-2 expression in murine CD4$^+$ T cells.

Mice with a unique substitution in STAT2 appear to display a specific defect in IL-2 expression. The C-terminal portion of STAT2 contains a transactivation domain (TAD) that has been suggested to engage in species-specific interactions (85). The substitution made in murine STAT2 may prevent interactions necessary to sustain IL-2 expression. A few hypotheses can be made on the mode of action of the C-terminus of STAT2, 1) binding to 'protein x' and this complex is involved in directly binding the IL-2 promoter or 2) STAT2 regulates expression of 'protein x' that is involved in stabilizing IL-2 mRNA or

regulating *il-2 gene* expression or 3) STAT2 is directly activated in response to TCR stimulation and requires the C-terminus to stabilize a transcription factor complex at the *il-2 gene* promoter.

Analysis of IL-2 mRNA stability in IFNAR2-/- and m/h STAT2 KI may suggest STAT2 is not involved in stabilizing mRNA. Previous findings from Ogilvie and colleagues suggest TCR signaling destabilizes IL-2 mRNA through the induction of TTP (94). TTP, a protein involved in destabilizing IL-2 mRNA, is expressed in response to anti-CD3 and anti-CD28 stimulation (94). Ogilvie and colleagues stimulated purified $CD4^+$ T cells with immobilized anti-CD3 and anti-CD28, and observed a peak expression of at 3 hours post-stimulation (94). Interestingly, type I IFN has also been demonstrated to induce expression of TTP (119). The data presented in Chapter 4 would support this finding, as there is decreased IL-2 secretion in OT-II $CD4^+$ T cells when compared to OT-II/IFNAR2-/- $CD4^+$ T cells. However, the data presented in Chapter 3 of this study is contradictory to the hypothesis that TTP is induced in response to type I IFN signaling. There could be experimental conditions that are variable between experiments that could contribute to this difference.

An avenue to investigate is the role of STAT2 in de novo protein synthesis that may feed back on the *il-2 gene* promoter. The initial induction of IL-2 expression requires rapid activation of NFAT, NF-κB, Oct1 and AP-1. However, to sustain IL-2 expression de novo protein synthesis is required. McKarns and

colleagues observe that in the absence of TNFR, sustained IL-2 expression in $CD4^+$ T cells is defective (37). They attribute this to decreased induction of c-rel expression that is thought to enhance chromatin remodeling at the *il-2 gene* promoter (37). In the absence of c-rel, IL-2 expression and proliferation is severely defective (121).

Neither STAT2 nor IFN signaling has been implicated in regulating protein expression that is required for sustained IL-2 expression. However, since there is a defect in the secondary burst of IL-2 expression, STAT2 could play a role in regulating de novo protein synthesis that feeds back on the IL-2 promoter. One method to examine genes that are differentially regulated in WT, IFNAR2-/- and m/h STAT2 KI $CD4^+$ T cells is to perform a gene chip examining mRNA from select strains at different times points post stimulation. Amongst the three strains there would theoretically be many shared genes, but those genes that are exclusively up regulated in WT $CD4^+$ T cells, would be hits to more closely examine. It will be necessary to examine the promoter regions of the gene list to determine if one contains an ISRE sequence.

STAT2 could also be acting directly on the IL-2 promoter. The IL-2 promoter has been extensively studied and the -300bp region, known as the enhancesome. This is where NF-κB, NFAT, Oct1 and AP-1 bind with histone acetyltransferases and RNA polymerase to drive gene expression. The murine IL-2 promoter is described to be as large as -10kb, providing ample room for ISGF3

to bind. This study id identified a putative ISRE sequence at -450bp. To determine if ISGF3 binds to this region, an IL-2 ChIP could be performed to place STAT2 on the promoter.

It is also of interest how m/h STAT2 may be defective in sustaining IL-2 expression. The C-terminus, as mentioned, can direct species-specific interactions. To determine what specific interactions WT STAT2 participates in compared to m/h STAT2 KI, it is be possible to immunoprecipitate STAT2 from TCR stimulated cells and perform liquid chromatography mass spectroscopy. This will allow interacting proteins to be identified and compared between WT and m/h STAT2 KI to determine what is not capable of binding the mutant murine STAT2 molecule.

Asforementioned, STAT1 is phosphorylated and translocates to the nucleus in response to TCR stimulation (76, 82). Preliminary results suggest STAT2 translocates to the nucleus by 2.5 hours post TCR stimulation. Although, due to experimental differences it is difficult to determine if STAT1 and STAT2 share similar kinetics. Examining both proteins in response to the same stimulus will allow us to conclusively determine if the kinetics are similar.

This study purposes three potential models for the role of the type I IFN receptor, and STAT2 in regulating IL-2 expression. The first model suggests activation of STAT2 is independent of signaling through the type I IFN receptor, but is activated potentially through cross talk with TCR stimulation (**Figure 6.1**).

This activation results in nuclear translocation of ISGF3 to the IL-2 promoter where it binds the putative ISRE sequence. The C-terminus of STAT2 is responsible for forming an essential interaction that is required for recruiting a protein that is then required for stabilizing or enhancing *iL-2 gene* expression. The second model suggests TCR stimulation drives expression of type I IFN or an IFN like gene that then feeds back on the IFN receptor (**Figure 6.2**). Signaling through the type I IFN receptor activates ISGF3, resulting in nuclear translocation and binding to the putative ISRE sequence within the IL-2 promoter. Alternatively, STAT2 could be directly activated in response to T cell receptor stimulation (**Figure 6.3**). Activation of STAT2 would not require the presence of the type I IFN receptor, due to accumulation of STAT2 at the TCR signaling complex. STAT2 could then be involved in stabilizing a transcriptional complex at the *il-2 gene* promoter. The common theme within the working models is that the C-terminus of STAT2 may be involved in stabilizing a protein complex that is responsible for enhancing *IL-2 gene* expression.

This study presented conflicting data that questions the involvement of the type I IFN receptor in regulating IL-2 expression. However, subsequent data presented helps support a possible role for the type I IFN signaling pathway in regulating IL-2 expression. Further studies should be performed to help solidify the findings in the current study as well as potentially understand the variation in experimental results. After further understanding the current findings, this project

could initiate a number of new avenues of research ranging from understanding the activation of STAT2 in response to TCR signaling, to what role the C-terminus of STAT2 plays in interacting with potential proteins, and how and if STAT2 localizes to the IL-2 promoter.

Experimental variation

Results presented in this study suggest there is inconsistency when repeating the initial observation obtained with anti-CD3 and anti-CD28 stimulation of naïve murine $CD4^+$ T cells. Possible explanations for the variability observed are strength of stimulation, culture conditions, purity of cells and percent of naïve cells in cell culture. The initial findings were performed using anti-CD3 and anti-CD28 as described in materials and methods, while most recent experiments were performed using commercially purchased reagents. In a pilot experiment comparing anti-CD28 from each the two sources, results may suggest stronger TCR stimulation may overcome the defect initially observed in OT-II/IFNAR2-/- (results not shown). Purified $CD4^+$ T cells stimulated with commercially purchased anti-CD28 and anti-CD3 displayed significantly increased IL-2 secretion from both OT-II and OT-II/IFNAR2-/- at 72 hours post-stimulation. However, when cells were stimulated with the initial source of anti-CD28 and commercially purchased anti-CD3, the total concentration of secreted

IL-2 was significantly less from OT-II $CD4^+$ T cells. Additionally the initial observation of defective IL-2 secretion in OT-II/IFNAR2-/- $CD4^+$ T cells was observed. This pilot experiment would suggest strength of stimulation might override the defect initially observed in OT-II/IFNAR2-/- $CD4^+$ T cells. Further testing, by titrating anti-CD3 and anti-CD28 or OVA peptide may provide understanding how strength of stimulation affects IL-2 expression.

There may also be difference in the preparation time that allows cells to rest prior to stimulation. During euthanasia mice are exposed to a lethal dose of carbon dioxide, creating hypoxic tissue conditions that have been observed to affect $CD4^+$ T cells. Roman and colleagues demonstrate purified $CD4^+$ T cells secrete increased IL-2 in hypoxic conditions when compared to normoxic conditions (122). This finding supported previously published findings that hypoxic conditions elicited increased cytokine expression in $CD4^+$ T cells (122, 123). However, Caldwell and colleagues suggest mixed lymphocyte cultures activated in the presence of hypoxic conditions secrete a decreased concentration of IL-2 (124). In the presence of normoxic conditions, mixed lymphocyte cultures were capable of secreting 2.5 fold more IL-2 than cells in hypoxic conditions (124). It is suggested IL-2 secretion is decreased in hypoxic stimulated cells due to fewer cells capable of secreting IL-2 in the culture (124). The role of hypoxic verses normoxic conditions needs to be further studied to determine how this experimental condition affects $CD4^+$ IL-2 secretion.

Culture conditions for the current study only treat samples to transient rather than constant hypoxic conditions. However, the preparation time from CO_2 asphyxiation to cell stimulation has decreased, leaving less time for tissues to return to normoxic conditions. This cell culture condition may have some influence on IL-2 expression in OT-II/IFNAR2-/- $CD4^+$ T cells. In a pilot experiment, testing 'resting' vs 'no rest' for splenocytes activated with OVA peptide, there was an observed difference in the two stimulatory conditions. In the 'no rest' condition both OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells displayed very similar intracellular IL-2 staining. However, allowing a rest there was an observed two fold difference in intracellular IL-2 staining when comparing OT-II and OT-II/IFNAR2-/- $CD4^+$ T cells (results not shown). The rest may provide cells the opportunity to return to normoxic conditions as well as recover from the physical stress encountered during isolation.

It has been suggested that in response to cellular stress IFN-β expression is increased (125, 126). The role and mechanism of IFN-β during a stress response is currently unknown. However, if OT-II $CD4^+$ T cells are responding to exogenous type I IFN in this study that may explain the increased production of IL-2 in OT-II cells. The present study demonstrated that exogenous IFN-α could enhance IL-2 expression in cells expressing the type I IFN receptor. From experiment to experiment the relative level of cellular stress may vary leading to a variation of IL-2 production. 'Resting' cells an equivalent amount of time prior to

stimulation may allow more consistent stimulatory conditions from one experiment to another.

An additional condition that could explain the variability is the percent of naïve $CD4^+$ T cells obtained during isolation and used subsequently in downstream applications. Contamination of APCs could result in the presence of exogenous cytokines in a controlled experimental condition. Macrophages can secret TGF-β and IL-10 two cytokines that have been demonstrated to inhibit IL-2 production (127, 128). IL-2 production is the experimental readout in the current study thus it may be necessary for future studies to contain minimal contamination of IL-10 and TGF-β secreting cells. By setting a standard for the percent of naïve $CD4^+$ T cells that are applied to downstream applications, we can feel confident that the defect in IL-2 production is an intrinsic $CD4^+$ T cell defect.

While the variability of IL-2 expression in OT-II/IFNAR-/- $CD4^+$ T cells is potentially troubling, it may also help the scientific community better understand how IL-2 expression is regulated. The cytokines, strength of signal, and environment in which cells are activated may dictate the need for specific signals in order to elicit a similar response (124, 129, 130). Future studies will provide insight into understanding how the microenvironment influences activation of naïve $CD4^+$ T cells.

The primary observation in IFNAR-/- $CD4^+$ T cells is the presence of a defect in *in vivo* expansion in response to an acute viral infection (65). A similar

observation has been repeated in this study while utilizing a different acute viral pathogen. However, in response to an acute bacterial infection, *Listeria monocytogenes,* IFNAR2-/- $CD4^+$ T cells are capable of expansion (65). While this observation was not confirmed in the present study, the initial findings from Havenar-Daughton and colleagues suggest each pathogen can illicit a unique immune response (65).

Viral and bacterial infections can induce expression of unique subsets of innate cytokines that can differentially regulate responses from the adaptive immune system. Havenar-Daughton and colleagues speculate IL-12, expressed at high levels in response to a *Listeria monocytogenes* infection, promotes expansion and survival of murine $CD4^+$ T cells (65). While Havenar-Daughton and colleagues did not further investigate this speculation in $CD4^+$ T cells, it was suggested IL-12 could promote survival in $CD8^+$ T cells in response to antigen specific stimulation (131). Type I IFN has been demonstrated to inhibit proliferation of murine $CD4^+$ T cells, thus we would not speculate the high expression of type I IFN in response to a viral infection promotes expansion *in vivo* (65). However, it is clear pathogen infections can differentially regulate cytokine expression. Future investigations may provide a deeper insight into in the differences between viral and bacterial infections that could differentially result in proliferation in the latter in the absence of the type I IFN receptor.

Relevance of the presented research to human health and disease

The pinnacle of biomedical research is to enhance treatment and our understanding of diseases that afflict humans. A human subject with an inherit defect in IL-2 secretion has not been identified, although multiple studies have suggested cancer patients show a significant decrease in IL-2 secretion.

Patients diagnosed with small cell lung cancer (SCLC), gastrointestinal cancer and various other cancers show a statistically significant decrease in IL-2 secretion from peripheral blood cells when compared to healthy controls (132-134). Fischer and colleagues suggest TGF-β secreted from SCLC cells plays a role in decreased cytokine secretion, including IL-2, although this observation has only been made in SCLC cell lines (135). Recent studies suggest blood from healthy patients shows a similar decrease in cytokine secretion in response to TGF-β, although future tests will need to be performed to determine if primary SCLC cells behave in a similar manner (132). Although this is an interesting observation seen in cancer patients, this is not a specific decrease in IL-2 expression.

Interestingly, exogenously delivered IL-2 has been used in cancer treatment. While not all cancers respond to IL-2, there are subsets that regress in human subjects (136). IL-2 in the past has been used as a systemic treatment but due to severe side effects alternative methods of delivery have been investigated.

Localized delivery of IL-2 appears to be an effective method that is associated with fewer side effects (136). IL-2 treatment of lymphocytes in conjunction with antigen stimulation promotes proliferation and effector function. Lymphocytes in cancer patients display defective IL-2 expression, thus it may be possible that delivery of exogenous cytokine can recover and enhance proliferation. Exogenous delivery of IL-2 has been shown to enhance proliferation of $CD8^+$ T cells in vivo, while in $CD4^+$ T cells enhance differentiation of $CD4^+$ T_{reg} cells (114, 137).

In this study, IL-2 mRNA expression is enhanced in naïve $CD4^+$ T cells after treatment with IFN-α. Davis and colleague also observe enhanced IL-2 secretion from human T_H1 $CD4^+$ T cells treated with IFN-α (33). However, *in vivo* tests have not been performed that can substantiate the findings observed *in vitro*. Murine studies have shown IFN-α treatment reduces tumor size and enhances infiltration of $CD4^+$ and $CD8^+$ T cells (138). IFN-α is a common cancer treatment that is used in combination with chemotherapy and/or radiation (139, 140). However, it is still poorly understood how IFN-α enhances $CD4^+$ T cell function in cancer patients. Studies have suggested IFN-α plays an important role in driving differentiation and maturation of DCs (140). It would be of interest to determine if IFN-α treatment in patients correlates to enhanced IL-2 secretion in $CD4^+$ T cells as this *in vitro* study described.

The present study provides initial observations of the type I IFN signaling pathways' involvement in enhancing and sustaining IL-2 expression in $CD4^+$ T cells. The uses of exogenous IL-2 and IFN-α are common treatments for cancer patients, but our understanding of the molecular mechanism behind these molecules remains unclear. Investigating the role of IL-2 and IFN-α in T cell biology, may give us greater insight behind the mode of action. As the pathways are elucidated, this may provide additional targets for future cancer immunotherapy treatments. Future generation immunotherapy may develop specific agonist or antagonists that target molecules farther downstream in the IL-2 and IFN-α signaling pathways in $CD4^+$ T cells.

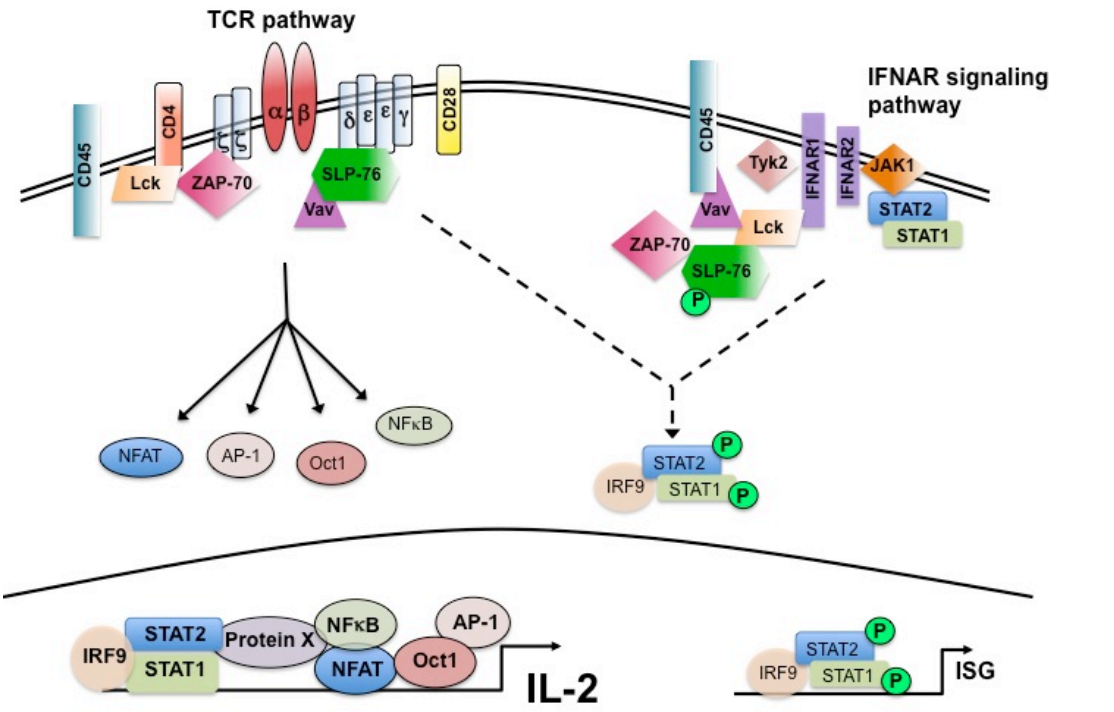

Figure 5.1 Model 1: Type I IFN signaling independent mechanism of sustained IL-2 expression.

Activation of STAT2 is dependent on crosstalk with T cell receptor stimulation and the type I IFN signaling complex. STAT2 may form the ISGF3 signaling complex, and translocate to the nucleus in response to activation. ISGF3 binds to the putative ISRE sequence within the IL-2 promoter, and is directly involved in regulating IL-2 expression. The C-terminus of murine STAT2 is necessary for maintaining an interaction with Protein X and this is essential for sustained IL-2 gene expression.

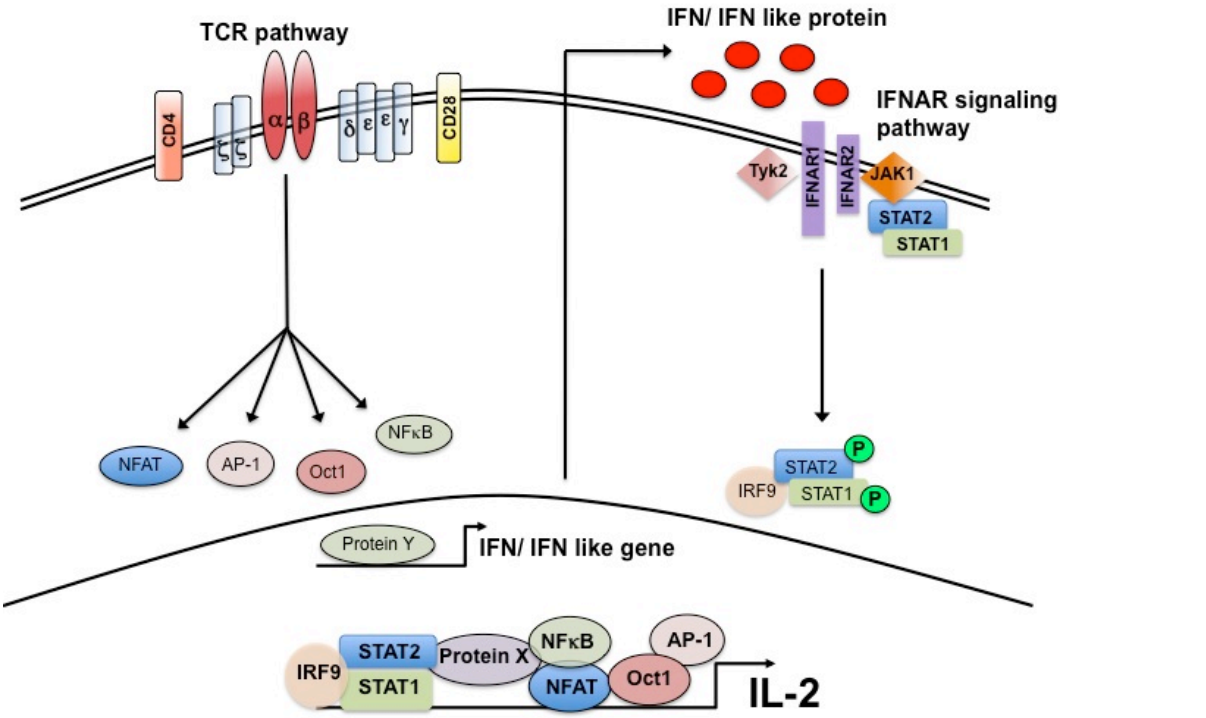

Figure 5.2 Model 2: Type I IFN dependent mechanism of sustained IL-2 expression.

TCR signaling results in activation of transcription factors, "Protein Y" involved in IL-2 expression and upregulation of IFN or an IFN like protein. IFN or an IFN like protein binds to the IFN signaling complex to activate downstream STAT1 and STAT2. ISGF3 binds to the IL-2 promoter at the putative ISRE sequence to act in sustaining IL-2 expression in $CD4^+$ T cells. The C-terminus of STAT2 is hypothesized to play a role in species specific interactions, which could help maintain the interaction of proteins in a large signaling complex located at the IL-2 promoter.

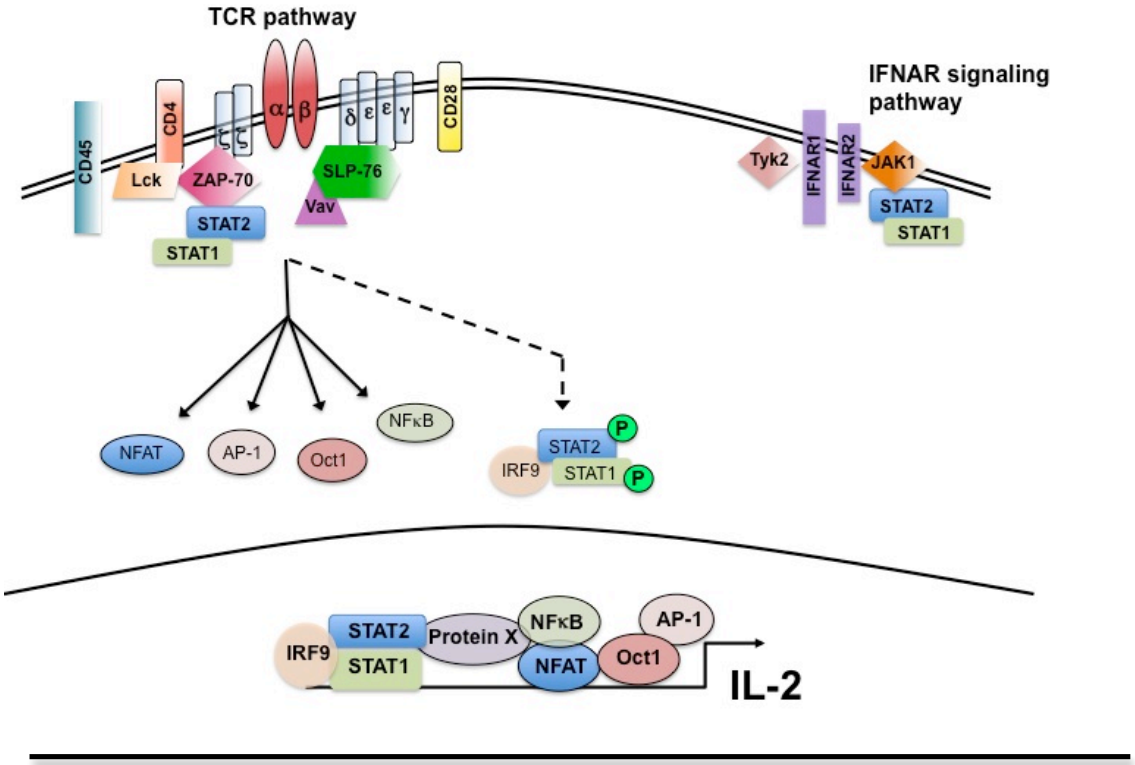

Figure 5.3 Model 3: T cell stimulation directly activates STAT2.

T cell receptor stimulation could directly activate STAT2 independent of the type I IFN receptor. Stimulation may result in the accumulation of STAT2 and STAT1 at the TCR immunological synapse. Following stimulation, nuclear translocation of STAT2, STAT1 and IRF9 results in ISGF3 binding to IL-2 promoter elements. The C-terminal domain of STAT2 could be involved in stabilizing an interaction at the IL-2 promoter and this interaction may be necessary for optimal IL-2 expression.

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