TYPE I INTERFERON MEDIATES TH2 REPROGRAMMING AND ACUTE SUPPRESSION OF EFFECTOR FUNCTIONS

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DEDICATION

The work presented in this thesis is dedicated to the advancement of human understanding, one small step at a time, in hopes of advancing our capacity to help those that suffer from disease.

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TYPE I INTERFERON MEDIATES TH2 REPROGRAMMING AND ACUTE SUPPRESSION OF EFFECTOR FUNCTIONS

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TYPE I INTERFERON MEDIATES TH2 REPROGRAMMING AND ACUTE SUPPRESSION OF EFFECTOR FUNCTIONS

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The type I interferon (IFN-α/β) family is a pleiotropic set of cytokines that play a role in regulating many biological functions, including suppressing viral replication and modulating adaptive immune functions. Although IFN-α/β has been extensively studied regarding the activation of interferon sensitive genes, much less is known regarding its role as a negative regulator. Here, I demonstrate a role for IFN-α/β in the regulation of Th2 development as well as memory Th2 cell function. The Th2 master transcription factor GATA3, promotes its own expression through a positive regulatory loop, uncoupling the cell from the requirement of IL-4

signaling. IFN-α/β inhibits this process by inducing epigenetic modifications within the GATA3 locus that prevent this positive regulatory loop from being established. Reduced DNase hypersensitivity and enhanced H3K27me3 correlates with a reduction in GATA3 gene expression by targeting the IL-4-sensitive alternative transcript exon 1a for suppression. These results demonstrate that IFN-α/β interferes with IL-4-mediated programming and induction of GATA3 through the enhancement of gene-silencing histone modifications within the GATA locus.

In addition to mediating chromatin modifications required for the long-term suppression of genes, IFN-α/β signaling also acutely suppresses gene expression in pre-committed Th2 cells. Here, I demonstrate the cellular and molecular pathways involved in suppressing the TCR-mediated expression of the human *IL5* gene. IFN-α treatment potently suppressed *IL5* and *IL13* gene expression by reducing the rate of nascent transcription, independent of *de novo* expression of ISGs. Further, I show that IFN-α-mediated STAT4 activation is required to suppress gene expression. Furthermore, IFN-α/β-mediated acute suppression occurs in a species-specific manner, since murine Th2 cells are not regulated by IFN-α/β signaling in contrast to human Th2 cells. This robust suppression of acute *IL5* and *IL13* expression, paired with the suppression of Th2 development, provide further evidence that IFN-α/β is a candidate for the treatment of allergic disease.

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J.P. Huber*, **S.R. Gonzales-van Horn*** K.T. Roybal, M.A. Gill, J.D. Farrar. (2014) IFN-α suppresses GATA3 transcription from a distal exon and promotes H3K27 tri-methylation of the CNS-1 enhancer in human Th2 cells. *Journal of Immunology* 192(12): 5687-5694.

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Ab – antibody

AP-1 – activation protein-1

APC – allophycocyanin

APC – antigen-presenting cell

AHR – airway hyperresponsiveness

BCR – B cell receptor

BSA – bovine serum albumin

Bt – biotin

cDNA – complementary DNA

CD – cluster of differentiation

CFSE – carboxyfluorescein succinimidyl ester

ChIP – chromatin immunoprecipitation assay

cIMDM – complete Iscove's modified Dulbecco's medium

CIRE – conserved intronic regulator element

CNS – conserved non-coding sequence

CRTH2 – chemoattractant receptor expressed on Th2 cells

DAMPs – danger-associated microbial patterns

DC – dendritic cell

DNase – deoxyribonuclease I

DTT – dithiothreitol

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetracetic acid

ELISA – enzyme-linked immunosorbent assay

EMSA – electrophoretic mobility shift assay

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

GAS – γ-IFN activation site

GFP – green fluorescent protein

h – human

H – histone

HRP – horseradish peroxidase

IFN-α/β – Type I interferon

IFN-γ – Type II interferon

IFN-λ – Type III interferon

IFNAR – interferon-α/β receptor

Ig – immunoglobulin

IL – interleukin

ILC2 – innate lymphoid type 2 cell

IP – immunoprecipitate

IRF – interferon regulatory factor

IRS – insulin receptor substrate

ISG – interferon-stimulated gene

ISGF3 – interferon stimulated gene factor 3

ISRE – interferon sensitive response element

JAK – Janus kinase

LCR – locus control region

mRNA – messenger ribonucleic acid

MFI – mean fluorescence intensity

MHC – major histocompatibility complex

NF-κB – nuclear factor κB

NFAT – nuclear factor in activated T-cells

NK – natural killer cell

NKT – natural killer T cell

PAMPs – pathogen-associated microbial patterns

PBMC – peripheral blood mononuclear cells

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

pDC – plasmacytoid dendritic cell

PE – phycoerythrin

PGD2 – prostaglandin D2

PI3K – phosphatidylinositol 3-kinase

PMA – phorbol 12-myristate 13-acetate

PRR – pattern recognition receptor

qPCR – quantitative real-time PCR

rh – recombinant human

RAG – recombination activating gene

RNA – ribonucleic acid

SA – streptavidin

SH2 – Src homology 2 domain

SOCS – suppressor of cytokine signaling

STAT – signal transducer and activator of transcription

T-bet – T-box expressed in T cells

TCR – T cell receptor

Th1 – T helper type 1

Th2 – T helper type 2

Th9 – T helper type 9

Th17 – T helper type 17

TLR – Toll-like receptor

TSLP – thymic stromal lymphopoietin

U – units

UTP – uradine triphosphate

WT – wild type

Y – tyrosine

YAC – yeast artificial construct

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Evolution of the adaptive immune response to 'non-self'

The ability to distinguish self from non-self in an immunological sense has proven to be a great evolutionary advantage for multicellular organisms. Many of the basic pathways that recognize self from non-self are conserved in plants, worms, fruit flies, mice, and humans. The Toll pathway was first described to have a role in innate immunity in *Drosophila melanogaster* by Hoffmann and colleagues (Lemaitre et al., 1996). Similar pathways have since been identified in many eukaryotic organisms (Belvin and Anderson, 1996; Medzhitov et al., 1998). In vertebrates, this pathway is stimulated by cell surface, intracellular and endosomal proteins binding to pattern- and danger- associated molecular patterns (PAMPs, DAMPs), which leads to the activation of NF-κB, a transcription factor critical to the activation of the immune response, regulating the expression of hundreds of genes (Janeway et al., 2001).

A further adaptation in combating non-self is the adaptive immune system, which confers immunological memory in jawed vertebrates. Development of immunological memory to non-self requires the presence of immense diversity to respond specifically to a particular invading organism. Clonal diversity is thought to have evolved from the invasion of a transposable element into a common jawed ancestor (Janeway et al., 2001). This led to the ability of the antigen-receptor and immunoglobulin genes to rearrange different gene segments into approximately 10^{14} and 10^{18} possible combinations, respectively (Janeway et al., 2001). The

immense power of this system was an obvious advantage to our common jawed ancestor, and the advantage of the adaptive immune response is exploited through vaccine utilization, driving memory formation without the host having ever been exposed to the pathogen. Although the ability to detect and respond to non-self by the immune system prevents invasion from the majority of pathogens, errors can arise that lead to autoimmune diseases and atopy, the immune response to self and non-innocuous environmental signals, respectively. It is imperative that the adaptive immune response be regulated by external cues from the immune system. This body of work demonstrates how one arm of the adaptive immune system is regulated in the context of atopy, and has implications for the treatment of allergic patients with a dysregulated Th2 immune response.

Activation of the CD4 T cell response to varied stimuli

$CD4^+$ T helper cells are able to differentiate into a variety of different lineages depending on the cytokine milieu and co-stimulation present during T cell receptor (TCR) activation by mature antigen presenting cells (APCs). These cytokine cues lead to downstream transcription factor activation and epigenetic programming, allowing a distinct set of genes to become activated and poised for quick and robust expression in response to TCR-mediated restimulation. APCs undergo a series of events in order to mature and present peptides to $CD4^+$ T cells. APCs express germline pattern recognition receptors (PRRs) on their cell surface, within endosomes, and within the cytoplasm. These receptors bind unique pattern associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) (Janeway and Medzhitov, 2002; Takeda et al., 2003), which include LPS, dsRNA, and extracellular ATP. The diverse receptor

signals lead to cellular maturation through the activation of NF-κB, upregulation of chemokine and co-stimulatory receptors, and the production of cytokines specific to the insulting PAMP (Janeway and Medzhitov, 2002). For example, in response to LPS and peptidoglycan, which bind TLR4 and TLR2 respectively, dendritic cells become activated and produce IL-12, which drives a Th1-dominant response (Kadowaki et al., 2001). Further, chemokine expression is altered in response to stimulation. The chemokine receptor, CCR7 mediates the recruitment of APCs to lymph node, where they interact with naïve $CD4^+$ T cells that express the TCR specific to the priming antigen (Sallusto and Lanzavecchia, 2000). Antigen stimulation in the context of MHC II, co-stimulatory proteins, and cytokines expressed by the APC, or other sources, define which lineage a $CD4^+$ T cell will commit (Figure 1.1). These three signals are necessary for driving the primary $CD4^+$ T cell response.

$CD4^+$ T cell lineages can be defined by the master transcription factor expressed in response to cytokine programming, as well as the cytokines produced upon restimulation. Some common $CD4^+$ T cell subtypes and their respective master transcription factors are shown in Figure 1.2. Th1 and Th2 cells were the first $CD4^+$ T cell lineages to be described (Mosmann et al., 1986). Murine Th1 clones were shown to secrete IL-2, IFN-γ, GM-CSF, and IL-3, while the Th2 clones produced IL-3, IL-4, IL-5, IL-10 and IL-13. Each of the $CD4^+$ T cell lineages is driven by a specific set of cytokines (Figure 1.2). Specifically, Th1 lineage programming occurs in response to IL-12 signaling. Binding of this cytokine to the IL-12R induces a series of downstream events, activating a specific set of IL-12-sensitive genes. The IL-12R consists of two subunits, IL12R α and IL12R β chains (Chua et al., 1994; Presky et al., 1996; Yao et al., 1999). IL-12R ligation induces the phosphorylation and activation of the Jak kinases Tyk2 and Jak2, which are necessary to recruit and phosphorylate STAT4 (Bacon et al., 1995a; 1995b).

Phosphorylation of STAT4 induces homodimer formation of STAT4, which translocates to the nucleus, where it binds specific STAT4 enhancers within the promoters of specific genes that define the Th1 phenotype (Bacon et al., 1995b; Jacobson et al., 1995). Further, co-stimulation through CD28 enhances IL-12R expression, further reinforcing Th1 lineage commitment (Igarashi et al., 1998). In response to IL-12 signaling, STAT4 drives T-bet expression, the master transcription factor of Th1 cells (Szabo et al., 2000; 2002; Thieu et al., 2008). Further, STAT4 signaling induces IFN-γ expression, which, in a positive feedback loop, activates T-bet and IL-12R expression and suppresses IL-4R β chain expression (Bach et al., 1995). However, IFN-γ is not sufficient to drive Th1 development, as it does not induce STAT4 activation (Robinson et al., 1997). The master transcription factor T-bet, in turn, enhances the expression of many Th1-specific genes, including IFN-γ (Szabo et al., 2002). STAT4 is necessary in driving Th1 commitment, as STAT4 knockout mice fail to mount a Th1 immune response to *Listeria monocytogenes* infection, but rather, mount a Th2 response, characterized by IL-4, IL-5 and IL-10 expression (Kaplan et al., 1996b). This IL-12-induced process is unique in driving Th1 development, and parallel signaling mechanisms exist for each $CD4^+$ T cell lineage in response to a unique cytokine milieu during TCR engagement.

Th2 cell functions

In contrast to Th1 cells, Th2 cells orchestrate a distinct inflammatory response against helminth infection and allergens. Committed Th2 cells circulate through the blood and peripheral tissues, and travel through the lymph nodes and spleen, waiting to be activated in response to cognate antigen by mature antigen presenting cells. Although the helminth-mediated stimulation

drives Th2 lineage commitment and activation, allergens also elicit a Th2-mediated response. *In silico* analysis has revealed that there are a number of conserved domains between helminth proteins and allergens (Bielory et al., 2013). For example, *Schistosoma mansoni* albumin protein is highly conserved with that of Fel d 2 (Bielory et al., 2013), a minor cat allergen. Further, both cat allergens and *Schistosoma mansoni* infection induce a Th2-dominant response in human and mouse (Boros, 1989; Kaplan et al., 1998b; Ragheb and Boros, 1989).

Memory Th2 cells are predominantly characterized by the expression of the classical Th2 cytokines IL-4, IL-5 and IL-13. Each of the Th2 cytokines has unique functions that drive immune activation and inflammation. IL-4, previously named B-cell stimulatory factor-1 (Howard et al., 1982; Hu-Li et al., 1987; Isakson et al., 1982), belongs to the common γ chain family of cytokines. IL-4 induces B cell activation and isotype switching to produce IgE antibodies, which are critical for inducing hypersensitivity through IgE-mediated granulocyte-induced activation (Isakson et al., 1982). IL-4 activates alternatively activated macrophages, which play a role in tissue remodeling (Pechkovsky et al., 2010; Wang and Joyce, 2010). Furthermore, IL-4 is necessary but not sufficient in driving Th2 development (see next section). However, this cytokine is dispensable in driving the pathology of an allergic response, whereas IL-5 and IL-13 play a more central role in this process (Cohn et al., 1998).

IL-5 is a member of the common β chain family of cytokines, and signals through the IL-5R, which consists of a unique α chain and the common β chain, which is shared by IL-3 and GM-CSF (Plaetinck et al., 1990; Tavernier et al., 1991). This cytokine plays a major role in inducing eosinophilia through eosinophil activation and survival (Jabara et al., 1988; Yamaguchi et al., 1988). Further, IL-5 has been shown to enhance the production of leukotriene C4 and histamine release from basophils in the presence of the complement component C5a (Bischoff et

al., 1990). Mouse models using IL-5 deficient mice demonstrate abolished eosinophilia, airway hyperreactivity, and tissue pathology in an ovalbumin-induced asthma model (Foster et al., 1996). Further, an *in vivo* model using *Strongyloides stercoralis* infection demonstrate that IL-5-deficient animals fail to clear the larval parasite compared to wild type controls, and eosinophil reconstitution in these mice rescued the lack of a functional immune response to the parasite (Herbert et al., 2000).

Finally, IL-13 signaling contributes to tissue architecture rearrangement and inflammation in the lung in chronic allergic diseases. Il-13 induces effector function by signaling through the high affinity IL-13 receptor, composed of IL-13Rα1 chain and the IL-4Rα chain (Smerz-Bertling and Duschl, 1995). Alternatively, a decoy receptor exists that is composed of the IL-13Rα2 chain and the IL-4Rα chain. Binding of IL-13 to this receptor is thought to be important for reducing the severity of response to IL-13, as mice lacking the IL-13Rα2 chain have enhanced allergic disease (Wood et al., 2003). Effector functions of IL-13 are widespread; the high affinity IL-13 receptor is expressed on both lymphoid and non-lymphoid cells. IL-13 recruits and activates eosinophils and mast cells to the site of inflammation (Kaur et al., 2006; Pope et al., 2001), induces goblet cell hyperplasia and mucus secretion (Whittaker et al., 2002; Zhu et al., 1999), and promotes tissue remodeling and airway hyperresponsiveness (Kim et al., 2002; Zhen et al., 2007; Zhu et al., 1999). Each of these cytokines plays a critical role in driving the prolonged Th2 response seen in chronic allergic diseases.

Together, these cytokines induce global changes that have wide spread effects on different tissues in response to helminth infection or allergic stimulation. Other cytokines reportedly expressed by Th2 cells include IL-6, IL-9 and IL-10 (Fiorentino, 1989; Larché et al., 2003; Zubiaga et al., 1990). However, some of these observations were made in T cell lines, and

each of these cytokines have been shown to be produced by other distinct T cell lineages (Ahyi et al., 2009; Chang et al., 2010; 2007b; Dardalhon et al., 2008; Kishimoto, 2010; Lotz et al., 1988; Veldhoen et al., 2008). The classical Th2 cytokines IL-4, IL-5 and IL-13 are the predominant ones expressed by committed Th2 cells that drive Th2-mediated inflammation in response to allergic stimulus and helminth infection.

In addition to the production of cytokines in response to restimulation, activated Th2 cells express specific chemokine receptors enable them to migration to the site of insult. CCR4 is expressed on a subset of committed Th2 cells and binds CCL17 and CCL22, which are expressed by $CD11c^+$ dendritic cells, which attracts Th2 cells to the lung (Perros et al., 2009). Blocking CCR4 in mice inhibits airway eosinophilia, goblet cell hyperplasia, and the recruitment of $CD4^+$ and $CD8^+$ T cells to the lung, demonstrating that this chemokine/ligand interaction is able to drive lung inflammation (Perros et al., 2009). Other tissue-tropic chemokines are important for the recruitment of memory Th2 cells to the site of inflammation. In mouse and man, CCR8 expression recruits IL-5-producting Th2 cells to the skin by interacting with the ligand, CCL8 (Islam et al., 2011). CCR8- and CCL8-deficient mice have significantly less Th2 cells recruited to the skin in a mouse model of chronic atopic dermatitis (Islam et al., 2011). Finally, in mouse and man, memory Th2 cells express CXCR3, and can be divided into four separate populations based on their expression of CXCR3 and CD62L (Endo et al., 2011). Although CXCR3 is predominantly expressed on activated $CD8^+$ and $CD4^+$ Th1 cells, all CXCR3-expressing Th2 populations can make IL-4 and IL-13, while only the $CXCR3^{lo}/CD62L^{lo}$ population produces IL-5 (Endo et al., 2011).

In addition to the classical chemokines, other receptors play a role in Th2 migration. The prostaglandin D_2 receptor, CRTH2, is expressed on eosinophils, basophils, innate lymphoid type

2 cells (ILC2s), and Th2 cells (Cosmi et al., 2000; Nagata et al., 1999a). Human CRTH2 is a specific marker for the identification of Th2 cells, and individuals with allergic diseases, such as atopic dermatitis, have increased peripheral blood $CRTH2^{+}CD4^{+}$ T cells (Cosmi et al., 2000). Prostaglandin D_2 is a lipid mediator produced by mast cells in response to IgE crosslinking (Lewis et al., 1982). In addition to chemotaxis, CRTH2 binding to prostaglandin D2 enhances cytokine production by Th2 cells (Tanaka et al., 2004; Xue et al., 2005), and induces the contraction of bronchial smooth muscle (Coleman and Sheldrick, 1989).

Th2 lineage commitment

In order for naïve $CD4^{+}$ T cells to differentiate into functional Th2 cells, a series of well-defined events occur in response to TCR stimulation, costimulatory signals, and the cytokine IL-4. The initial source of this cytokine remains unclear. Many cell types are able to produce IL-4 upon activation and thus could contribute to Th2 development, including dendritic cells (d'Ostiani et al., 2000; Maroof et al., 2006), eosinophils (Moqbel et al., 1995), basophils (Sokol et al., 2007), mast cells (Bradding et al., 1992), NKT cells (Yoshimoto and Paul, 1994), as well as naïve T cells (Noben-Trauth et al., 2000; 2002). Despite these observations, many of these cell types are not necessary to drive Th2 commitment (Brown et al., 1996; Kim et al., 2010; Zhang et al., 1996). Several reports have demonstrated that naïve T cells may be the initial source of IL-4 that drives Th2 development. Paul and colleagues demonstrated that IL-4R deficient $CD4^{+}$ T cells produce similar levels of IL-4 compared to $CD4^{+}$ T cells expressing the IL-4R (Noben-Trauth et al., 2000). This IL-4 production is sufficient to drive Th2 polarization, particularly in the absence of IL-12 or IFN-γ. Further, IL-4 deficient mice reconstituted with IL-4-expressing

$CD4^+$ splenic T cells rescued the Th2 response, and IgE production in response to ovalbumin challenge was restored (Schmitz et al., 1994). Taken together, these findings demonstrate that the production of IL-4 by naïve $CD4^+$ T cells is sufficient to induce Th2 programming and drive downstream effector functions, including IgE isotype switching, even in the absence of other sources of IL-4.

There are two unique receptors in which IL-4 is able to bind (Nelms et al., 1999), however only one is expressed on $CD4^+$ T cells. This receptor is comprised of the IL-4R α chain and the common γ chain. The α chain is required for binding IL-4, while the common γ chain is necessary for cytoplasmic signaling (Kammer et al., 1996; Russell et al., 1993). Bound to the cytoplasmic components of the IL-4 receptor α and common γ chains are the Jak1 and Jak3 kinases, respectively (Figure 1.3) (Chen et al., 1997; Miyazaki et al., 1994). IL-4 binding to the receptor results in the tyrosine phosphorylation of these kinases, leading to their activation (Chen et al., 1997). This leads to the phosphorylation of the cytoplasmic portion of the IL-4R α chain at five tyrosine residues, including Y575, Y603, and Y631 (Reichel et al., 1997; Smerz-Bertling and Duschl, 1995). It is here where STAT6 is recruited to the cytoplasmic IL-4R to become activated (Figure 1.3). Deletion and/or mutation of these tyrosine residues reduces STAT6 phosphorylation and subsequent DNA binding (Ryan et al., 1996). Once STAT6 is recruited to the three phosphorylated tyrosine residues via its SH2 domain, it becomes phosphorylated at C-terminus residue Y641 (Darnell, 1997; Mikita et al., 1996). Y641 is critical to drive STAT6 homo-dimerization and DNA binding. Upon dimerization, STAT6 translocates to the nucleus and binds the STAT6-specific motif TTC-$(N)_4$-GAA within enhancers and promoters of IL-4-sensitive genes (Ihle, 1996; Mikita et al., 1996; Schindler et al., 1995). STAT6 is necessary to drive Th2 development, as naïve $CD4^+$ T cells lacking STAT6 fail to develop into Th2 cells in

the presence of IL-4, nor do they proliferate in response to this cytokine (Kaplan et al., 1996a). Furthermore, mice lacking STAT6 infected with the nematode *Nippostrongylus brasiliensis* failed to generate a Th2 response and produce less IgG_1 and IgE (Shimoda et al., 1996; Takeda et al., 1996). the lack of a Th2 response is seen with other infection models; for example, STAT6 deficient mice infected with *Trypanosoma cruzi* develop persistent infection compared to wild-type mice due to reduced Th2 responses (Tarleton et al., 2000). Taken together, these studies demonstrate that STAT6 plays a critical role in driving Th2 responses to pathogens. In addition to STAT6, IL-4R signaling activates the insulin receptor substrate (IRS)-1/2 signaling pathway. IRS-1/2-deficient cells are unable to proliferate in response to IL-4 signaling, demonstrating that the IRS signaling plays a role in driving cell cycle progression (Sun et al., 1995; Wang et al., 1993). IRS-1/2 becomes phosphorylated in response to IL-4R activation in T cells, and subsequently is able to interact with phosphoinositide-3-kinase (PI3K) and the signaling intermediate Grb-2, both of which play a role in cell survival and proliferation (Sun et al., 1995; 1993).

A critical gene induced by STAT6 is the Th2 master transcription factor GATA3 (Figure 1.3). STAT6 dimers bind to the murine GATA3 promoter at several sites (Onodera et al., 2010; Scheinman and Avni, 2009). Nakayama and colleagues demonstrated by ChIP and EMSA that these sites were specific for STAT6, with mutation of these site abrogating STAT6 binding (Onodera et al., 2010). GATA3 is a member of the GATA family of zing-finger transcription factors. All six family members contain two N-terminal transactivation domains and two zinc finger domains that are responsible for DNA binding. Several groups have demonstrated the importance of GATA3 in Th2 development, and that it is both necessary and sufficient to drive Th2 lineage commitment (Ouyang et al., 1998; Zhang et al., 1997; Zheng and Flavell, 1997).

Using GATA3 conditional knockout mice, this transcription factor was shown to be necessary for the production of Th2 cytokines, both *in vitro* and *in vivo* (Pai et al., 2004). *In vivo* models using a dominant negative GATA3 protein demonstrated that a lack of GATA3 activity attenuates Th2 function *in vivo* in response to ovalbumin stimulation (Zhang et al., 1999). Furthermore, individuals lacking one functional allele of GATA3 have reduced circulating Th2 cells, and reduced serum IgE, IgG_4, and are biased towards a Th1 phenotype (Skapenko, 2004). Thus, GATA3 is unequivocally necessary and sufficient to drive Th2 development and downstream *in vivo* Th2 responses (Figure 1.3).

Once GATA3 becomes activated by IL-4-induced STAT6 activation, the requirement for these exogenous signaling cues no longer exists. Ectopic expression of GATA3 in STAT6 deficient cells overrides the need for STAT6 signaling, and restores the production of Th2 cytokines (Ouyang et al., 2000). It has been demonstrated that GATA3 binds to its own promoter as well as at specific conserved non-coding sequences (CNS), which could explain how the auto-activation loop occurs in the absence of STAT6 signaling (Figure 1.3) (Fang et al., 2007; Scheinman and Avni, 2009). Notably, the DNA binding domains of the GATA family members are remarkably similar, and Murphy and colleagues demonstrated that GATA1, GATA2, and GATA4 were also able to induce GATA3 expression in murine Th2 cells (Ranganath and Murphy, 2001).

In addition to STAT6, other transcription factors are necessary, but not sufficient, to induce GATA3 expression. NFAT1 binds to the GATA3 promoter, with GATA3 expression suppressed in the presence of cyclosporin A, an NFAT inhibitor (Scheinman and Avni, 2009). Epigenetic modifications undoubtedly play a role in T cell development that lead to heritable programming in daughter cells (Kanno et al., 2012). The polycomb group (PcG) protein complex

plays a role in driving epigenetic modifications in different cell types. One of the PcG proteins, MEL18, induces GATA3 expression in mice. MEL18-deficient cells showed reduced GATA3 expression compared to wild type cells, suggesting its role in enhancing GATA3 during Th2 development (Kimura et al., 2001). Further, Notch signaling selectively enhances GATA3 expression through the use of the exon 1a splice variant (Amsen et al., 2007; Fang et al., 2007). Other transcription factors, co-stimulatory molecules, and cytokines reinforce Th2 development, but do so through the regulation of the Th2 cytokine locus without directly acting on GATA3. In addition to activating GATA3, STAT6 also binds to the Il4, Il5, Il4ra, Batf, and Maf genes (Betz et al., 2010; Elo et al., 2010; Wei et al., 2010). The role of STAT6 in activating these genes and its correlation with changes in histone modifications in STAT6 sufficient and deficient cells has been studied (Wei et al., 2010). The repressive histone modification, H3 lysine 27 trimethylation (H3K27me3), is enhanced in STAT6 knockout cells, suggesting that STAT6 enhances accessibility of the surrounding genes, leading to a distinct transcription profile during Th2 cell development (Wei et al., 2010). Furthermore, STAT6 plays a role in driving T cell proliferation. STAT6 suppresses the expression of the cdk inhibitor, p27-KIP1, through the upregulation of growth factor independent-1 (GFI-1), a transcriptional repressor (Zhu et al., 2002). STAT6 knockout cells have enhanced expression of p27-KIP1, which correlates with a reduction in cells that transition from G_1 to S phase of the cell cycle (Kaplan et al., 1998a).

In addition to STAT6-induced gene expression, Th2 cell development requires other secondary signals as well. IL-2 signaling activates STAT5a and STAT5b, which is required for maintaining IL-4 gene accessibility in differentiating Th2 cells (Cote-Sierra et al., 2004). IL-2 plays a supportive role in Th2 development, as it is not sufficient to induce Th2 commitment in the absence of IL-4 signaling (Cote-Sierra et al., 2004; Swain et al., 1990). Further,

costimulatory receptor activation plays a role in the initial T cell activation. Lack of CD28 signaling strongly suppresses T cell development by reducing global cell proliferation by at least 60% (Green et al., 1994), thus, this co-stimulatory molecule is indirectly necessary in driving T cell differentiation through the induction of cell proliferation. While T cells from CD28 knockout mice respond to antigen and upregulate activation markers such as CD69 and CD25, they fail to sustain an optimal TCR-induced response (Lucas et al., 1995). CD28 signaling induces IL-2 production, and when exogenous IL-2 is added to the CD28 knockout T cell cultures, proliferation and cell survival is almost completely restored (Green et al., 1994). Thus, although CD28 signaling does not play a direct role in driving Th2 cell development per se, it is necessary for the proliferation and survival of T cells. Additionally, OX40/OX40 ligand interactions play a role in Th2 development, which are expressed on T cells and mature DCs, respectively (Jenkins et al., 2007; Rogers and Croft, 2000). OX40 ligand-deficient DCs fail to induce the survival and/or proliferation of $CD4^+$ Th2 cells, but did not prevent Th2 polarization (Jenkins et al., 2007). This study observed that the kinetics of IL-4, IL-5 and IL-13 production by T cells activated by OX40 ligand-deficient dendritic cells or wild-type dendritic cells looked similar. However, the overall expansion of the cells, and perhaps the quality of priming, is reduced (Jenkins et al., 2007). Further, the use of OX40 blocking antibodies reduces the number of eosinophils in bronchial lavage fluid from house dust mite-challenged mice (Burrows et al., 2014). This reduction in cells within the lung correlates with reduced IL-13 production by murine Th2 cells isolated from the anti-OX40 antibody-treated mice (Burrows et al., 2014). A similar observation was seen in human Th2 cells; OX40 neutralization inhibites IL-5 and IL-13 production from PBMCs isolated from house dust mite-allergic individuals (Burrows et al., 2014). Taken together, these studies demonstrate a role for OX40/OX40 ligand interactions in

inducing a robust Th2 response. Finally, Notch signaling regulates Th2 differentiation, and as previously described, induces GATA3 expression through the distal alternative exon 1a to initiate transcription (Amsen et al., 2007; Fang et al., 2007). Different Notch ligands expressed on APCs play a role in driving one specific T cell lineage versus another. Amsen *et al* demonstrated that Delta induces a Th1 phenotype, while Jagged induces a Th2 phenotype as deteremined by IL-4, IL-5 and IFN-γ expression (Amsen et al., 2004). Furthermore, specific regions within the Th2 cytokine locus are responsive to Notch-mediated signaling. Using a truncated version of an IL-4 transgene containing a luciferase reporter, Amsen *et al* demonstrated that lack of a specific hypersensitive site downstream of the IL4 gene reduces the amount of luciferase expressed, and further, mutating the DNA binding sites for Notch signaling intermediates ablated reporter expression, suggesting that direct Notch signaling enhances IL-4 production (Amsen et al., 2004).

Other roles of GATA3

In addition to Th2 development, GATA family members are expressed in a variety of different cell types and play critical roles in other aspects of development. GATA3 knockout mice are embryonic lethal at approximately day 11 to 12, and display brain and spinal cord deformities, incomplete fetal liver hematopoiesis, growth retardation, and internal hemorrhaging (Pandolfi et al., 1995). GATA3 can be detected in many adult tissues, including mammary and adrenal glands, kidneys, skin, and the central nervous system, to name a few. In addition to Th2 development, GATA3 is critical in shaping overall T cell development at multiple levels. When GATA3 is absent, CD4/CD8 double negative T cells fail to develop (Hendriks et al., 1999).

Furthermore, GATA3 is necessary during single-positive $CD4^+$ T cell thymocyte selection, and conditional knockout cells deficient in GATA3 during the DN3 stage lack the TCR β chain (Pai et al., 2003). This transcription factor plays multiple roles in a variety of tissues, thus it is tightly regulated. Such regulation likely occurs through a variety of mechanisms, however there is evidence for two mechanisms in particular. The first involves the presence of transcriptional cis-regulators surrounding the GATA3 gene that are bound by tissue-specific proteins (Hosoya-Ohmura et al., 2011; Lakshmanan et al., 1999). Karis and colleagues demonstrated that, in a mouse model using a 650 kb yeast artificial chromosome containing a transgenic GATA3 gene, organ-specific expression of GATA3 was only partially restored in transgenic embryos, demonstrating that very distal sites are necessary to regulate GATA3 expression in a tissue-specific manner (Lakshmanan et al., 1999). The second method of regulation revolves around the presence of two unique non-coding first exons approximately 10 kb apart (exon 1a and exon 1b). Both of these alternative exons are spliced onto the same exon 2, which contains the translation start codon, resulting in the same protein being expressed (Asnagli et al., 2002). Both of the alternative first exons contains a unique promoter, sensitive to unique signals that mediate the expression of GATA3 in different contexts. Asnagli *et al* demonstrated that each of these exons induced GATA3 transcript expression in a tissue-specific manner, whereby exon 1a-induced transcripts are found in the brain, while exon 1b-induced transcripts are expressed in the thymus (Asnagli et al., 2002). Furthermore, evidence for exclusive regulation of each of these non-coding first exons is evident in the observation that murine Th2 cells have different patterns of Gata3 transcript expression when exon 1a and 1b-induced transcripts are compared (Asnagli et al., 2002). Although both are present at day 3 post-Th2 polarization, exon 1b transcripts are almost completely gone while exon 1a transcript expression continues to increase over time

(Asnagli et al., 2002). Other groups have demonstrated that these transcripts are sensitive to different transcription factors. Notch signaling has been shown to selectively enhance GATA3 expression through the use of the exon 1a splice variant, and plays a role in driving Th2 lineage commitment (Amsen et al., 2007; Fang et al., 2007). Finally, Scheinman and Anvi showed that the distal exon 1a, but not exon 1b, is an IL-4 sensitive promoter that is activated in response to STAT6 signaling (Scheinman and Avni, 2009). Taken together, these studies demonstrate the complexity of GATA3 expression, and validate the importance of its cell-specific regulation.

Epigenetic modifications within the Th2 cytokine locus

After a $CD4^+$ T cell has received the three signals necessary to differentiate into a Th2 cell (TCR stimulation in the presence of cognate antigen, co-stimulation, and IL-4-mediated programming), the cell will divide several times. Cell division is a critical step in the lineage commitment process, and cells that have divided more times have been shown to produce greater amounts of cytokines (Bird et al., 1998). Daughter cells that arise from the differentiated Th2 cell will have inherited a Th2-specific epigenetic program, characterized by accessible genomic regions, such as the Th2 cytokine locus and GATA3, to be poised and easily accessible to transcription factors that activate the committed Th2 cell, such as TCR signaling. Other genes that are not activated in Th2 cells, including Tbx21 and Ifng, will be less accessible to transcriptional activation. This closed chromatin, or heterochromatin, has been shown to be methylated on histones and DNA (CpG islands), reinforcing the suppression of these genes (Weng et al., 2012). Alternatively, hyperacetylated histones and demethylation of histones and DNA will be more open and accessible to transcriptional enhancers. Other histone modifications,

including phosphorylation and ubiquitination, have also been shown to play a role in regulating gene expression (Baarends et al., 1999; Cheung et al., 2000; El-Osta and Wolffe, 2000; Strahl and Allis, 2000; Turner, 2000; Wei et al., 1999). H3 acetylation on lysine residues 9 and 14 (H3K9Ac and H3K14Ac) and methylation on lysine 4 (H3K4me) correlate with transcriptional activation, and these modifications are often found within the promoters and enhancers of transcriptionally active genes. Alternatively, H3 trimethylation of lysine residues 3 and 27 (H3K3me3 and H3K27me3) are associated with genes that are transcriptionally inactive, especially di- and tri-methylation of these residues (Lee et al., 2006; Weng et al., 2012). The histone modification patterns that associate with transcriptionally active or repressed genes are not black-and-white, but the presence of these marks generally correlates with a particular state of gene expression. Histone modifications are induced by a variety of proteins, including histone acetylases and deacetylases (HATs, HDACs), histone methyltransferase (HMTs), and DNA methyltransferases (DNMTs). Each of these categories of enzymes are made up of a number of family members that are regulated by different proteins in a tissue-specific manner (Weng et al., 2012). The importance of these epigenetic modifications has been demonstrated through the use of inhibitors during T cell development. Reiner and colleagues demonstrated that naïve T cells treated with the DNMT inhibitor 5-azacytidine or the HDAC inhibitor sodium butyrate during Th2 polarization express enhanced IL-4 as a function of cell division (Bird et al., 1998).

The Th2 cytokine locus is one of the most well characterized loci in the genome, and many studies have demonstrated how TCR and STAT6 induction drives histone modifications during Th2 commitment (Agarwal and Rao, 1998; Ansel et al., 2003; Fields et al., 2002; 2004; Kim et al., 2007; Lee et al., 2006). The Th2 cytokine locus is found on human chromosome 5q31 and mouse chromosome 11. Approximately 140 kb long in both species, this locus contains four

genes (Figure 1.4). The first is *IL5*, which is transcribed in the opposite direction compared to the other genes. *IL5* is separated from *IL4* and *IL13* by the *RAD50* gene, approximately 50 kb in length (Figure 1.4). *IL4* and *IL13* are on the 3' site of *RAD50*, and are transcribed in the same direction and are approximately 10 kb apart (Figure 1.4). Despite the presence of *RAD50*, a constitutively expressed DNA damage gene, *IL4*, *IL5* and *IL13* are often activated as a group, especially in response to TCR stimulation in committed Th2 cells. DNA regions that are important for driving transcriptional expression have been characterized using two specific observations. If a region is transcriptionally active, it will often be hypersensitive to degradation by DNase. These sites are termed 'hypersensitive sites' (HSS). Further, when a particular region of DNA is highly conserved in multiple species, it is potentially important for regulating gene expression across species. These sites are termed 'conserved non-coding sequences' (CNS). Together, these definitions have allowed us to distinguish the cis regulatory regions within the Th2 cytokine locus and other gene loci. A detailed description of all of the sites within the Th2 cytokine locus is beyond the scope of this thesis, but sites that have critical roles in driving Th2 cytokine expression will be described here; specifically, sites that have been shown to have functional significance in Th2 cytokine production. Characterization of other sites that play a role in Th2 cytokine gene regulation have been studied in detail, and have been thoroughly reviewed (Ansel et al., 2006; Lee et al., 2006).

There are several regions shown to act as cis enhancers in regulating gene expression of the Th2 cytokines. The largest CNS region, CNS-1, lies within the intergenic region between the Il4 and Il13 genes (Figure 1.4) (Takemoto et al., 1998). This region contains three HSS sites (HSS-1, HSS-2, HSS-3), 1 and 2 being hypersensitive to DNase treatment only in Th2 cells, and not in naïve or Th1 cells (Takemoto et al., 1998). Transgenic mice containing a 450 kb yeast

artificial chromosome composed of the extended human Th2 locus were utilized to demonstrate that the CNS-1 region is important during Th2 development (Loots et al., 2000). When polarized under Th2 conditions, naïve T cells from CNS-1-deficient transgenic mice produce approximately half as many IL-4-producing cells and less than one-third of the IL-13 producing cells than wild-type controls (Loots et al., 2000). Other groups have determined the significance of this region using alternative models. Locksley and colleagues demonstrated that ablation of this region reduced the amount of IL-4- and IL-13-producing T cells when CNS-1-knockout naïve T cells were polarized under Th2 conditions (Mohrs et al., 2001). Furthermore, knockout mice fail to mount a strong Th2 response in a *Nippostrongylus brasiliensis* infection model (Mohrs et al., 2001). Finally, another study showed this site is bound by GATA3 in Th2 cells (Takemoto et al., 2000), demonstrating that this cis-regulatory region is important in driving IL-4 and IL-13 production in Th2 cells. Because of the sheer size of the Th2 cytokine locus, and the fact that the Th2 cytokine genes are often concordantly expressed in TCR-stimulated Th2 cells, several groups looked for a locus control region (LCR) that could be responsible for regulating all three genes, unlike CNS-1, which regulated IL-4 and IL-13, but modestly suppressed IL-5 expression in CNS-1 knockout cells (Mohrs et al., 2001). Simultaneous discoveries by two groups revealed the presence of a LCR localized within the 3' end of the Rad50 gene containing four unique DNase hypersensitivity sites, RHS4, RHS5/RAD50-O, RHS6/RAD50-(A+B), and RHS7/RAD50-C (Figure 1.4) (Fields et al., 2004; Lee and Rao, 2004). RHS4, 5 and 7 are hypersensitive sites specific to Th2 cells, but not in naïve or Th1 cells, and RHS7 knockout mice challenged with alum-precipitated keyhole limpet hemocyanin (KLH) failed to produce IL-4, IL-5 and IL-13 upon *in vitro* splenocyte restimulation (Lee et al., 2004).

The Th2 cytokine locus is under extensive regulation, and the aforementioned examples of enhancer regions demonstrate the complexity of this regulation. The presence of HSS and CNS regions correlate with histone and DNA epigenetic modifications that alter the accessibility of these regions in the presence or absence of different cellular signals. For example, the region surrounding RHS4 through RHS7 within the LCR of the Th2 cytokine locus is rapidly demethylated and hyperacetylated in committed Th2 cells, compared to the same region in naïve T cells (Fields et al., 2004). RHS7 is rapidly demethylated in response to Th2 polarization, which correlates with demethylation of the Il4 and Il13 promoters (Fields et al., 2004). Furthermore, these patterns of epigenetic modifications correlate with the presence of STAT proteins and/or binding to specific cis-regulatory elements, which are thought to drive lineage-specific epigenetic programming (Avni et al., 2002; Fields et al., 2002; Lee et al., 2002; Wei et al., 2010). $CD4^+$ T cells have been shown to become hyperacetylated on H4 within the Il4 promoter and enhancer elements in response to Th2 development (Avni et al., 2002). However, in STAT6 deficient cells, H4 acetylation is abolished at these sites. More recent studies have used next generation sequencing to look at STAT binding and histone modifications in different knockout mice, and correlate trends with the expression or silencing of a particular gene (Wei et al., 2010). For example, in STAT6 deficient Th2 cells, H3K4me3 and H3K36me3 were relatively unchanged at the Gata3 gene, however, other genes like Il24 and Il4 had reduced H3K4me3 and H3K36me3 in STAT6 knockout cells (Wei et al., 2010). The presence of these two epigenetic modifications correlated with transcriptional expression of these genes (Wei et al., 2010). Additionally, the presence of STAT6 inhibited the repressive mark H3K27me3 at the Th2 cell genes, Il4, Gata3, Il4ra, and Il24, whereas the STAT6 knockout mice had an enhancement of H3K27me3 at these genes, correlating with reduced gene expression (Wei et al., 2010). In

addition to STAT binding correlating with enhanced gene expression, this binding was also shown to correlate with gene silencing in a lineage-specific manner. The Th1 gene locus Il18r1-Il18rap was bound by STAT6 in Th2 cells, and STAT6 knockout cells failed to induce the repressive mark, H3K27me3 at this gene (Wei et al., 2010). This observation correlates with the data from Nakayama and colleagues, showing that binding of the histone methytransferase Ezh2 (Enhancer of Zeste Homolog 2) within specific gene loci correlates with enhanced H3K27me3 and suppression of these genes. For example, Tbx21 is bound by Ezh2 in Th2 cells, which correlates with its suppression in this cell population (Tumes et al., 2013). These examples demonstrate how STAT proteins can regulate histone modifications and subsequent lineage commitment, although the exact mechanism for STAT-mediated recruitment of histone modifying proteins remains undefined.

In addition to GATA3 and STAT6, other proteins work in concert to promote Th2 development through the induction of the Th2 cytokine genes. The AP-1 family member c-Maf, has been shown to be important for the induction of IL-4 expression in both T and NK cells (Ho et al., 1998; Kim et al., 1999). c-Maf-deficient cells failed to produce IL-4 expression *in vitro* and *in vivo*, while IL-5 and IL-13 expression is unaffected (Kim et al., 1999). The proto-oncogene c-Myb has also been shown to play a role in upregulating Th2 cytokine production in Th2 cells (Kozuka et al., 2011). siRNA-mediated knockdown of c-myb in naïve T cells reduced levels of IL-4, IL-5 and IL-13 expression at multiple time points post-Th2 differentiation (Kozuka et al., 2011). In concert with GATA3, c-Myb bound to specific enhancer elements within the murine Il13 promoter, which correlates with enhanced gene expression. Mutation of these sites prevented the expression of a reporter construct driven by the Il13 promoter. Furthermore, c-Myb and GATA3 associate with each other at the Il13 promoter (Kozuka et al.,

2011). The pathways described here are examples of the many different transcription factors that become activated during Th2 lineage commitment; however, the only gene that is both necessary and sufficient to drive Th2 development is GATA3. In concert with other proteins, including STAT6, GATA3 is able to promote Th2 stability and effector function through the activation of the Th2 cytokine genes. Each of the Th2 cytokine genes contain GATA3 consensus binding motifs within their promoters (Kaminuma et al., 2005; Lavenu-Bombled et al., 2002; Tanaka et al., 2011; Zheng and Flavell, 1997). GATA3, in the presence of the TCR-induced transcription factors NF-κB, AP-1 and NFAT, binds to the promoters of the Th2 cytokine genes and enhance their expression (Ferber et al., 1999; Horiuchi et al., 2011; Kaminuma et al., 2005; Lavenu-Bombled et al., 2002; Ranganath et al., 1998; Zheng and Flavell, 1997). For example, Horiuchi *et al* demonstrated that in STAT6 deficient cells, overexpression of GATA3 by retroviral transduction enhances IL-4, IL-5 and IL-13 expression (Horiuchi et al., 2011). Furthermore, Kaminuma *et al* observed that GATA3 overexpression induces IL-5 expression, and mutating the GATA3 binding sites within the Il5 locus suppresses IL-5 production (Kaminuma et al., 2005). Other studies have demonstrated GATA3 binding sites in the Il4 and Il13 loci as well (Lavenu-Bombled et al., 2002; Tanaka et al., 2011; Zheng and Flavell, 1997). It is through these STAT6-dependent, GATA3-dependent mechanisms that the Th2 cytokine locus is unwound, and accessible to TCR-induced transcription factors that will induce a recall response of these committed Th2 cells.

The interferon signaling networks

To date, three unique families of interferon have been characterized. Type I (IFN-α/β) and Type III (IFN-λ) interferons are produced in response to viral infections and regulate the expression of hundreds of Interferon Sensitive Genes (ISGs). In contrast, Type II Interferon (IFN-γ) plays a more dominant role in bacterial infections, but is also expressed by cytotoxic $CD8^+$ T cells in response to viral infections. The term 'interferon' was first utilized in 1957 to describe the antiviral effects of this cytokine by Isaacs and Lindenmann (Isaacs and Lindemann, 1957). This study observed that heat-inactivated influenza virus induced the expression of a secreted factor that led to a reduction in virus growth, as measured by hemagglutination assay (Isaacs and Lindemann, 1957). To date, the classical signaling mechanisms by which these cytokines alter the cellular gene profile have been well characterized.

The human IFN-α/β family consists of 16 unique IFN-α genes, along with individual genes encoding the IFN-β, IFN-ε, IFN-κ, and IFN-ω proteins (Stark et al., 1998). Each of these ligands binds to the Interferon Alpha Receptor (IFNAR), which is ubiquitously expressed on all nucleated cells. The IFNAR is composed of the IFNAR1 and IFNAR2 chains (Figure 1.5), both of which are necessary for ligand binding and IFN-α/β signaling, as the absence of either of these chains results in abolished IFN-α/β signaling (Cohen et al., 1995; Russell-Harde et al., 1995). Prior to IFNAR ligation, Jak1 and Tyk2 are pre-associated with the IFNAR2 and IFNAR1 chains, respectively (Colamonici et al., 1994a; Li et al., 1997). Upon IFNAR ligation by IFN-α/β, a series of well-defined events occurs within the cell that leads to the activation of Jak kinases and STAT transcription factors. The signaling cascade begins with the cross phosphorylation of the Jak kinase members, Jak1 and Tyk2. Tyk2 is pre-associated with IFNAR1 before receptor

ligation (Colamonici et al., 1994a; 1994b; Richter et al., 1998; Yan et al., 1996b; 1998), and becomes phosphorylated by Jak1, which is associated with the IFNAR2 chain (Figure 1.5) (Gauzzi et al., 1996). The phosphorylation of Tyk2 leads to the subsequent cross phosphorylation of Jak1 by Tyk2. Cross phosphorylation of these kinases leads to the phosphorylation of Y466 of the IFNAR1 chain, creating a docking site for STAT2 (Figure 1.5) (Krishnan et al., 1996). The Src Homology 2 (SH2) domain of STAT2 binds to phosphorylated Y466 on the IFNAR1 chain (Yan et al., 1996a), where it becomes phosphorylated on Y690 residue (Improta et al., 1994). STAT2 Y690 phosphorylation acts as a docking site for STAT1 via its SH2 domain, and the absence of STAT2 prevents STAT1 activation from occurring (Improta et al., 1994; Leung et al., 1995). Once STAT1 is bound to phosphorylated STAT2 Y690, STAT1 Y701 is phosphorylated, and STAT1 and STAT2 proteins heterodimerize and disassociate from the IFNAR1 chain (Gupta et al., 1996; Improta et al., 1994; Shuai et al., 1993). The STAT1/STAT2 complex associates with the DNA binding protein Interferon Regulatory Factor 9 (IRF9), creating the Interferon-Stimulated Gene Factor 3 (ISGF3) (Figure 1.5) (Kessler et al., 1990; Schindler et al., 1992). This interaction occurs in the cytoplasm, as enucleated cells are able to create the ISGF3 complex in response to IFN-α/β treatment (Dale et al., 1989a). STAT1 seems to play a dominant role in the direct interaction with IRF9, as STAT1 mutants that still bind STAT2 fail to induce ISGF3 formation in response to IFN-α/β signaling (Horvath et al., 1996). Once the ISGF3 complex is in the nucleus, it binds the cis DNA regulatory element, Interferon Stimulated Response Element (ISRE) with high affinity (Figure 1.5) (Dale et al., 1989b; Kessler et al., 1988; Levy et al., 1988). The ISRE motif is characterized by the canonical cis element, AGTTT-$(N)_3$-TTTCC, and is found within the promoters of ISGs (Friedman and Stark, 1985; Israel et al., 1986; Levy et al., 1988; Porter et al., 1988; Reich et al., 1987; Sugita et al., 1987; Wathelet et al., 1987).

In addition to the classical STAT1/STAT2 activation pathway that leads to the nuclear localization of ISGF3, other STAT dimers become activated in response to IFN-α/β signaling. IFN-α/β signaling has been shown to activate all the STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6) (Farrar et al., 2000b; Fasler-Kan et al., 1998; Ghislain and Fish, 1996; Kessler et al., 1990; Matikainen et al., 1999; Tanabe et al., 2005; Uddin et al., 2003). dimers formed that exclude IRF3 bind to a cis DNA regulatory element, called the Gamma Activated Sequence (GAS) (Schindler and Darnell, 1995). Although the GAS element, TTC-$(N)_3$-GAA, is bound by a number of STAT dimers, it is thought that binding specificity is provided by cell-specific transcription factors and other signals that provide context for GAS-induced gene expression (Kang et al., 2013). In particular, STAT3 and STAT4 activation have been described in detail in response to IFN-α/β treatment. STAT3 interacts with IFNAR1 chain at residues Y527 and Y538 in response to IFN-α/β signaling in a Tyk2-dependent manner (Pfeffer et al., 1997; Rani et al., 1999; Yang et al., 1996), and can form homodimers and heterodimers with STAT3 and STAT1, respectively. IFN-α/β signaling recruits and activates STAT4 in a species-specific manner (Berenson et al., 2004; 2006; Cho et al., 1996; Farrar et al., 2000b; Rogge et al., 1998). Human IFN-α/β, but not mouse, is able to induce STAT4 phosphorylation in a STAT2-dependent manner (Farrar et al., 2000a; 2000b). Species specificity of STAT4 activation is due to a minisatellite insertion within the murine STAT2 C-terminus that is absent in the human STAT2 protein (Farrar et al., 2000a). This minisatellite sequence consists of 12 copies of a 24-bp-long sequence that encodes the consensus protein sequence PAPQVLLE. In human, STAT4 is recruited to the IFNAR signaling complex by interactions with the distal carboxy (C) terminus of STAT2 via the STAT4 SH2 domain (Farrar et al., 2000a). Further, a murine-human chimeric STAT2 protein that consists of a murine STAT2 protein containing the

human C-terminus was fully functional in inducing human STAT4 phosphorylation in STAT2 knockout cells, while the wild-type murine STAT2 protein was not (Farrar et al., 2000a). These findings demonstrate how a species-specific difference between the human and mouse systems have biological effects in driving STAT activation in response to IFN-α/β signaling.

Like IFN-α/β, Type III (IFN-λ) interferons play a role in combating viral infections, and share many of the same signaling intermediates induced by IFN-α/β. To date, there are three ligands included in the IFN-λ family; IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). Each of these cytokines bind the IFN-λR (IL-28R) that induces signaling within the cell (Kotenko et al., 2003; Sheppard et al., 2002). The receptor consists of the unique IFN-λR1 chain and the IL-10R2 chain, which is common among the IL-10 family member receptors (Donnelly et al., 2004; Kotenko et al., 2003). IFN-λ binds to the IFN-λR1 chain, which induces the complex to bind to the IL-10R2 chain, leading to the activation of the Jak/STAT pathway. IFN-λ-induced intracellular signaling occurs in a very similar manner to that of IFN-α/β; both Jak1 and Tyk2 have been shown to be involved in IFN-λ-induced signaling (Dumoutier et al., 2004; Kotenko et al., 2003). As described above, the STAT1/STAT2 heterodimer binds IRF-9, and the ISGF3 complex translocates to the nucleus, where it will bind ISGs containing the canonical cis regulatory ISRE. Further, IFN-λ signaling has also been shown to activate STAT3, STAT4 and STAT5 in specific cell types (Dumoutier et al., 2004), which will bind accessible GAS elements within the genome. The IFN-λ-induced ISGF3 complex has been shown to activate similar sets of IFN-α/β-induced genes, including *OAS1*, *MX1* and *IRF7*, and microarray analysis has demonstrated that the ISG profile activated by IFN-λ and IFN-α/β are nearly identical, however, the kinetics and intensity of ISG-enhancement is slower and more modest in response to IFN-λ signaling in human hepatocytes and PBMCs (Doyle et al., 2006; Freeman et al., 2014; Marcello

et al., 2006). Despite the similarities between IFN-λ and IFN-α/β ISG expression, the most striking difference is the lack of IFN-λR expression on many cell types, including certain hematopoietic lineages. DCs, macrophages and non-hematopoietic cells, including epithelial cells and hepatocytes, express a functional IFN-λR (Doyle et al., 2006; Hermant et al., 2014). Studies are unclear as to which T cell subsets express the IFN-λR. Many reports have demonstrated a lack of response to IFN-λ signaling by human PBMCs compared to hepatocytes, regardless of cytokine concentration (Freeman et al., 2014; Sommereyns et al., 2008; Witte et al., 2009). However, one group has demonstrated that IFN-λ signaling can alter $CD4^+$ T cell commitment. Polarization of human naïve $CD4^+$ T cells with MHC-mismatched mDCs in the presence of IFN-λ reduced IL-13 production (Jordan et al., 2007). Their follow-up study corroborated their initial findings, and they observed suppressed GATA3 in naive T cells cultured under Th2 conditions in the presence of IFN-λ (Dai et al., 2009). Taken together, these observations demonstrate the tissue-specific differences in IFN-λ versus IFN-α/β responsiveness, regardless of almost identical signaling pathways and enhancement of ISG expression.

In contrast to IFN-α/β and IFN-λ, IFN-γ induces a more unique gene signature that is independent of STAT2 activation, although there is some overlap. Type II interferon (IFN-γ) consists of a single ligand in both mouse and human (Derynck et al., 1982). IFN-γ binds the Interferon Gamma Receptor (IFNGR), which consists of the IFNGR α and β chains (Farrar and Schreiber, 1993). Similar to the IFNAR, IFNGR is ubiquitously expressed by most nucleated cells. Prior to ligand-induced signaling, Jak1 and Jak2 are associated with IFNGR α and β chains, respectively (Igarashi et al., 1994). Activation of the Jak kinases leads to the phosphorylation of IFNGR α chain at the Y440 residue, which acts as docking site for STAT1, where it will binds via its SH2 domain (Greenlund et al., 1994; Heim et al., 1995). STAT1

becomes phosphorylated at Y701, which leads to the formation of STAT1 homodimers, and the dissociation from the IFNGR α chain (Greenlund et al., 1995). STAT1 homodimers translocate to the nucleus and bind GAS elements (TTC-$(N)_3$-GAA) within the promoters and enhancers of specific genes.

The nature of STAT-mediated ISRE/GAS binding is highly promiscuous, thus, it has been suggested that other signaling events dictate the specific set of genes that respond to STAT signaling in a cell-specific manner. Different homo- and hetero-dimerized STAT complexes, including STAT1/STAT2 that lacks the IRF-9 protein, have been shown to bind the canonical GAS element (Brierley and Fish, 2002; 2005; Ghislain et al., 2001). Furthermore, due to the promiscuity of STAT activation, it has been observed that ISRE elements can become activated in response to IFN-γ treatment, and IRF-9 is able to interact with STAT1 homodimers, potentially enhancing its affinity for ISRE (Bluyssen et al., 1995; Gao et al., 1993). In addition to the classical Jak/STAT activation, a number of other signaling intermediates have been shown to be responsive to interferon signaling, and those that occur in response to IFN-α/β signaling have been described in detail. The PI3K pathway has been shown to be activated in response to IFN-α/β treatment (Uddin et al., 1997), as well as activation of the GTPase Rap1 through CrkL activation (Lekmine et al., 2002). It is thought that these alternative signaling pathways play a role in the anti-proliferative effects seen in response to IFN-α/β treatment, and are relatively separate from GAS/ISRE-induced gene regulation (Platanias, 2005).

Immune modulation by type I interferon

The cellular responses that occur in response to IFN-α/β are vast, and the antiviral effects in T cells and other hematopoietic lineages are relatively well characterized (Crouse et al., 2015). Although IFN-α/β is critical in controlling viral infections, as well as some bacterial infections, it can also be detrimental to the host in response to chronic infection, including HIV and *M. tuberculosis* (Katze et al., 2002; McNab et al., 2015). Homeostatic production of IFN-β in specific pathogen-free mice has also been observed by several groups, and has been somewhat characterized (Gough et al., 2012; Gresser, 1990; Tovey et al., 1987; Viti et al., 1985; Yaar et al., 1986). It has been hypothesized that this low-level production of IFN-β is due to ongoing exposure to microbes at mucosal surfaces, as well as tissue remodeling (Bocci, 1980), but this has not been tested in a germ-free setting. Interestingly, deletion of IRF3 or IRF9, two proteins involved in viral-induced IFN-β production, do not inhibit constitutive IFN-β production (Hata et al., 2001). However, AP-1 and NF-κB are important in driving homeostatic production of IFN-β by directly interacting with the IFN-β promoter in the absence of viral infection (Basagoudanavar et al., 2011; Gough et al., 2010; Wang et al., 2010). Tonic IFN-β signaling has been shown to be involved in hematopoietic stem cell maintenance and mobilization (Essers et al., 2009; Sato et al., 2009), immune cell homeostasis specifically in NK cells, B cells, and myeloid lineages (Hamilton et al., 1996; Honda et al., 2003; Hwang et al., 1995; Swann et al., 2007; Teige et al., 2003), as well as osteoclast formation (Takayanagi et al., 2002), all of which have been determined through the use of IFNAR- or IFN-β-deficient mouse models.

The induction of ISGs has been characterized in different cell types in response to either direct IFN-α/β treatment or viral infection (de Veer et al., 2001; Hartman et al., 2005; Honda et

al., 2006; Liu et al., 2012; Samarajiwa et al., 2009; Schneider et al., 2014; Testoni et al., 2011b). IFN-α/β signaling induces the expression of hundreds of antiviral genes, including well studied examples like 2'5'-oligoadenylate synthetase (OAS), myxoma resistance protein 1 and 2 (MX1, MX2), and latent ribonuclease L (RNase L), all of which directly act to suppress viral replication (Schneider et al., 2014). Hartman *et al* demonstrated that in addition to antiviral genes, a set of ISGs were suppressed in response to IFN-α/β treatment that correlate with STAT2 binding to these gene loci, and some overlapped with IFN-γ-sensitive genes (Hartman et al., 2005). In addition to other genes, interleukin-17 receptor and prostaglandin E synthase were selectively repressed in response to IFN-α treatment. Other studies have observed that IFN-α/β signaling suppresses gene expression as well (Hartman et al., 2005). IFN-α treatment has been shown to negatively regulate cyclin D3 and cdc25A genes, which correlates with the observation that in some cell types, IFN-α/β suppresses proliferation (Tiefenbrun et al., 1996). Th17 lineage commitment, and the expression of IL-17 mRNA and protein, have been shown to be suppressed in response to IFN-α/β signaling in both mouse and human (Moschen et al., 2008; Ramgolam et al., 2009). Further, *in vitro* treatment of human eosinophils with IFN-α/β showed inhibited release of granule proteins, including neurotoxin and eosinophil cationic protein, and suppressed IL-5 expression (Aldebert et al., 1996). Basophils have also been shown to be responsive to IFN-α/β signaling. Human basophils treated with IFN-α prevented IL-3 mediated priming, and reducing IL-4 and IL-13 expression (Chen et al., 2003). Finally, IFN-α has been shown to regulate IL-4-mediated B cell isotype switching to IgE in both mouse and human, however, this observation can also be induced by treatment with IFN-γ (Finkelman et al., 1991; Pène et al., 1988). The examples described here demonstrate the vast effects IFN-α/β has on different compartments of the immune system.

Due to the activation of STAT4 in response to IFN-α/β in human, it was thought that this signaling cascade could lead to Th1 development in human (Parronchi et al., 1992). Early studies observed that IFN-α/β enhanced IFN-γ-production from human $CD4^+$ T cells (Brinkmann et al., 1993). Several studies have demonstrated, however, that IFN-α/β does not induce Th1 development in human, despite STAT4 activation. Hilkens and colleagues compared STAT4 activation in response to IL-12 and IFN-α/β, and found that IFN-α/β induced modest STAT4 activity compared to IL-12 (Athie-Morales et al., 2004). zrecent studies have observed the same phenomenon and also showed that IFN-α/β-mediated STAT4 activation fails to sustain T-bet expression compared to IL-12 signaling (Ramos et al., 2007). Thus, IFN-α/β-induced STAT4 activation is insufficient at driving Th1 development in both mouse and human (Berenson et al., 2004; 2006; Davis et al., 2008; Persky et al., 2005; Ramos et al., 2007). The majority of cellular consequences of IFN-α/β-mediated STAT4 activation have yet to be described.

Initiation of the Th2-mediated allergic response

Atopic diseases are initiated as an antigen-specific response to otherwise innocuous substances, including ragweed, house dust mite, and cat dander. Allergic asthma affects over 300 million individuals worldwide, with 250,000 deaths occurring annually (World Health Organization, 2013). Although asthma is a heterogeneous disease, 70% of individuals suffering have an allergic-mediated disease in which symptoms are triggered by specific allergens (Novak and Bieber, 2003). Allergic diseases are characterized by increased serum IgE antibodies, and enhanced allergen-specific Th2 response at the site of insult. Research over the past three decades has described in detail how the immune system responds to allergen, however, the role

of epithelial-derived cytokines has changed our understanding of the initial response to allergen, and how downstream events are triggered. Upper airway epithelial cells are the first to directly contact the insulting allergen, and in response, produce thymic stromal lymphopoietin (TSLP) (Allakhverdi et al., 2007; Lee and Ziegler, 2007; Liu et al., 2007), IL-33 (Byers et al., 2013; Gregory et al., 2012), and IL-25 (Beale et al., 2014; Cheng et al., 2014). TSLP suppresses IL-12 production by dendritic cells (Ito et al., 2005), and enhances IL-8 and eotaxin-2, which recruit neutrophils and eosinophils to the site of inflammation (Isaksen et al., 2002; Su et al., 2013). Further, TSLP enhances OX40L expression by dendritic cells, enhancing their ability to drive Th2 differentiation (Ito et al., 2005), and also directly acts on antigen-specific $CD4^+$ T cells to enhance cytokine production and survival (He et al., 2008; Wang et al., 2015). IL-33 enhances IL-5 production by Th2 cells (Kurowska-Stolarska et al., 2008), and is necessary for the secretion of IL-13 by mast cells and basophils (Junttila et al., 2013). IL-33 also enhances OX40L expression by dendritic cells (Chu et al., 2013), and enhances airway remodeling (Saglani et al., 2013). IL-25 directly induces the production of collagen from fibroblasts and recruits endothelial progenitor cells to the lung, resulting in airway remodeling (Gregory et al., 2012), and also enhances IL-9 production by Th9 cells (Angkasekwinai et al., 2010). Although these events occur in both healthy and atopic individuals, the levels of these cytokine are higher in allergic individuals (Beale et al., 2014; Cheng et al., 2014; Saglani et al., 2013).

Furthermore, TSLP, IL-33 and IL-25, activate the lineage negative innate lymphoid type 2 cells (ILC2s), which are critical in driving the initial development of a Th2-dominant environment by producing IL-5 and IL-13 in an antigen-independent manner (Halim et al., 2014; Huang et al., 2015; Klein Wolterink et al., 2012). The importance of this cell population in Th2-mediated responses has been demonstrated in mice lacking a functional adaptive immune

system. In a papain-induced asthma model, Rag1-deficient mice still developed inflammation in the lungs, characterized by IL-5 and IL-13 production, infiltration of eosinophils, and enhanced mucus production (Halim et al., 2012). Other studies have shown that these cells play a critical role in clearing helminth infection. ILC2s were shown to expand *in vivo* during *Nippostrongylus brasiliensis* infection in Rag2-deficient mice, but not Rag 2-common γ chain-deficient mice, which lack both adaptive immunity and lineage negative cells (Price et al., 2010). Although these cells do not produce IL-4, they play a central role in driving eosinophil recruitment and structural changes at the site of insult. This, paired with IL-25-, IL-33-, and TSLP-induced DC maturation, indirectly drives Th2 cell lineage commitment.

Exactly how allergen activates epithelial cells to produce the innate cytokines is somewhat unclear. Epithelial damage occurs in response to allergen, and it is thought that damage-induced signaling mediates innate cytokine production (Lambrecht and Hammad, 2012). In some cases, damage-mediated signaling pathways can lead to epithelial cell apoptosis, which enhances inflammation at the site of challenge (White, 2011). Allergens seem to have protease activity, which could directly damage epithelial cells. House dust mite allergens Der p 1, 2, 3 and 4 have been characterized as cysteine protease, lysozyme, trypsin and amylase proteins, respectively (Chua et al., 1988; Lake et al., 1991; Stewart et al., 1992a; 1992b). Additionally, early reports demonstrate that the purified house dust mite allergen, Der p 1, enhances permeability of epithelial cell layers to albumin *in vitro* (Herbert et al., 1995). Further, addition of proteinase inhibitors, especially cysteine proteinase inhibitors, prevented house dust mite allergen-mediated epithelial cell damage *in vitro* (Winton et al., 1998). In addition to direct damage, it has been demonstrated that secreted proteins from activated eosinophils also disrupt epithelial cell adhesion, including gelatinase, a matrix metalloproteinase that degrades collagen

and other basement membrane proteins (Herbert et al., 1996; 1993). Finally, TLR4, expressed on epithelial and stromal cells bind components of allergens like LPS, which is found in total house dust mite and contributes to the allergic response in a murine system (Hammad et al., 2009; Lambrecht and Hammad, 2012; Tan et al., 2010). Taken together, these danger signals induced by direct and indirect allergen interaction with the epithelial cell likely play a role in inducing the expression of the innate cytokines IL-25, IL-33 and TSLP.

In addition to activating epithelial cells, allergen also directly activates cells by binding allergen-specific IgE antibodies bound to the Fc epsilon receptor (FcεR). Allergen-specific IgE is produced by B cells in response to BCR-mediated signals and enhanced through IL-4 and IL-13 signaling (Del Prete et al., 1988; Geha, 1992; Pene et al., 1988; Punnonen et al., 1993). Further, B cell CD40 interacts with CD40L expressed on T cells, which enhances the production of IgE in isotype-switched B cells (Shapira et al., 1992). The high affinity FcεR (FcεRI) is expressed as a tetramer ($\alpha\beta\gamma 2$) on mast cells, basophils and or at lower levels as a trimer ($\alpha\gamma 2$) on various APCs, including dendritic cells. The low-affinity receptor, FcεRII (CD23) on B cells, platelets, and APCs (Novak et al., 2001). Depending on the cell type, allergen-induced FcεR crosslinking can lead to cytokine production, degranulation of preformed molecules, and the production of inflammatory lipid mediators, including prostaglandins and leukotrienes (Gilfillan and Tkaczyk, 2006). In granulocytes, FcεRI crosslinking leads to immediate degranulation of preformed effector molecules which causes increased vascular permeability, mucus secretion, and induces the contraction of smooth muscle in the airways (Fahy and Dickey, 2010). Taken together, these processes, as well as the priming and activation of Th2 cells, induce a long-lasting Th2-dominant phenotype in atopic individuals that are acutely reactivated when the insulting allergen is present.

The duality of allergic diseases and viral infections

PAMPs and DAMPs drive the activation of the innate immune system, leading to the secretion of cytokines that mobilize the adaptive immune response. Epithelial cells respond to pathogens by producing many different antimicrobial peptides, including defensins, reactive oxygen species, and cytokines like TSLP, all of which directly impact the clearance of the insulting pathogen (Bals, 2000). Asthmatic individuals seem to have a dysfunctional response to viral infections, including rhinoviruses (Gavala et al., 2011; Gern and Busse, 1999; Rowe and Gill, 2015). Bronchial epithelial cells isolated from asthmatics stimulated with dsRNA produced more TSLP and less IFN-β compared bronchial epithelial cells from healthy controls given the same stimulation (Uller et al., 2010). This suggests that asthmatic epithelial cells are predisposed to the allergic response, and this pathway appears to override the antiviral response. Furthermore, rhinovirus enhances TSLP production in non-allergic cells as well, and co-treatment with IL-4 or IL-13 further enhances TSLP expression (Kato et al., 2007). Thus, the Th2-dominant environment that exists when an asthmatic individual becomes infected with a respiratory viral infection appears to alter the nature of the epithelial cell antiviral response. Although asthmatic individuals clear respiratory viral infections at a rate similar to non-asthmatic controls, respiratory infections are the leading cause of asthma exacerbations in both children and adults (Gern and Busse, 2002). 80% of asthma exacerbations are associated with a viral respiratory infection, and human rhinovirus and respiratory syncytial virus are the most commonly associated viral infections found to induce asthma exacerbations (Gern and Busse, 2002).

It is well documented that TLR signaling in plasmacytoid DCs (pDCs) and certain conventional DCs induces the expression of IFN-α/β and IFN-λ, and that these cells make the

majority of interferons in response to viral infection (Yin et al., 2012). This mechanism also occurs in epithelial cells, which have been shown to produce IFN-λ (Khaitov et al., 2009). Reports have revealed that FcεR crosslinking dampens this antiviral response. pDCs from non-atopic adults cultured with an anti-IgE antibody that crosslinks and activates the FcεR produced less IFN-α in response to human influenza infection compared to isotype-treated cells (Gill et al., 2010). Further, pDCs from asthmatic adults produced less IFN-α in response to *in vitro* human influenza infection compared to pDCs isolated from non-atopic adults (Gill et al., 2010). Similar effects are seen in pediatric asthma as well; PBMCs from asthmatic children expression higher FcεR than healthy controls, and FcεR crosslinking reduces the production of IFN-α and IFN-λ in response to *in vitro* human rhinovirus infection compared to PBMCs from non-asthmatic children (Durrani et al., 2012). These reports demonstrate that IgE receptor activation on pDCs is dominant over the production of IFN-α/β and IFN-λ in response to viral challenge. Studies have demonstrated that bronchial lavage cells from asthmatic individuals produce less IFN-λ and IFN-β compared to non-asthmatic cells when infected with rhinovirus *in vitro* (Sykes et al., 2012; Wark et al., 2005). Further, primary human bronchial epithelial cells from asthmatics produced less IFN-λ compared to healthy controls in response to experimental rhinovirus challenge (Contoli et al., 2006). Reduced IFN-λ production correlated with an increase in virus-induced asthma exacerbations. However, there are other studies demonstrating that bronchial epithelial cells from well-controlled asthmatics produce equivalent amounts of *IL28A* and *IFNB1* mRNA compared to cells isolated from healthy controls (Bochkov et al., 2009). Lopez-Souza *et al* have also seen this observation in both nasal and bronchial epithelial cells; asthmatic BAL cells produced similar IFN-β1 in response to rhinovirus infection (Lopez-Souza et al., 2009). Furthermore, Sykes *et al* observed that there was no difference in the production of rhinovirus-

induced IFN-β, IFN-α, and IFN-α2 by PBMCs from asthmatic individuals compared to healthy controls, despite differences in bronchial lavage cells using the same challenge (Sykes et al., 2012). Based on the donor information provided in these studies, there is similar expression of interferon when the atopic disease is mild or well-controlled. These findings suggest that there is a subset of individuals whose' ability to appropriately activate the interferon pathways are suppressed, which correlates with the ability to control their atopic disease.

"How [Th2] cells respond to interferon"

The immune response to IFN-α/β signaling in the context of viral infection has been well characterized, but much less is understood about its role in Th2 commitment and effector function. A number of reports have observed that IFN-α/β, and potentially IFN-λ, suppress Th2 development and function, but very little is understood about how this process occurs. The depletion of pDCs from non-atopic human PBMC cultures enhanced *in vitro* Th2 cytokine production in response to human rhinovirus infection (Pritchard et al., 2012a). Further, IFN-α treatment of human $CD4^+$ T cells suppresses IL-5 and enhances IL-10 production (Schandené et al., 1996). Hirohata and colleagues observed similar results in that IFN-α suppressed IL-5 and IL-13 production, as well as enhanced IL-4 production, by $CD4^+$ T cells (Shibuya and Hirohata, 2005). These studies, as well as others by a number of groups, demonstrate the clear observation that IFN-α/β suppress Th2 function in human $CD4^+$ T cells (Kaser et al., 1998; McRae et al., 1997; Nakajima et al., 1994; Pritchard et al., 2012b; Ramgolam et al., 2009; Tiefenbrun et al., 1996; Zhang et al., 2009). There are two reports demonstrating that IFN-λ may also play a role in negatively regulating human Th2 cells. Treatment of human $CD4^+$ T cells with IFN-λ1

suppressed IL-13 production in the presence of mitogen or DC-induced stimulation, and a follow-up study showed that IFN-λ1 treatment can suppress human naïve $CD4^+$ T cell production of GATA3 and IL-13 expression (Dai et al., 2009; Jordan et al., 2007). Work from our lab, conducted by Dr. Jonathan Huber, demonstrated that human naïve T cells treated with IFN-α prevents IL-4-mediated Th2 lineage commitment by limiting the expression of GATA3 mRNA and protein (Huber et al., 2010). Further, pre-committed human Th2 cells are also amenable to IFN-α-mediated repression, demonstrating that IFN-α/β signaling overrides TCR- and GATA3-induced Th2 lineage commitment (Huber et al., 2010). In contrast to this finding, murine Th2 cells are not negatively regulated in response to IFN-α/β, and early studies demonstrate that IFN-α treatment alone does not alter IL-4 production by murine $CD4^+$ T cells (Wenner et al., 1996). Indeed, studies conducted by Dr. Huber indicate that IFN-α/β cannot override IL-4-mediated Th2 development in murine $CD4^+$ T cells (Huber et al., 2010). Thus, there is a species-specific response to IFN-α/β by $CD4^+$ T cells, whereby human Th2 development and cytokine production are arrested by IFN-α/β signaling, but murine Th2 cell lineage commitment is largely unaffected by this signaling pathway.

The literature described in this section set the stage for the work I am presenting in this thesis. IFN-α/β signaling suppresses human Th2 development and effector function, but the mechanism by which this occurs is largely unknown. A handful of case reports have demonstrated that IFN-α/β therapy reduces the number and severity of asthma attacks in adult steroid-resistant asthma (Gratzl et al., 2000; Kroegel et al., 2006; Simon et al., 2003). To date, there is one published clinical trial testing the effects of IFN-β in allergic asthma patients. Asthmatic adults inhaled IFN-β or placebo at the onset of cold symptoms, and the endpoint of viral infection was measured, among other parameters (Djukanović et al., 2014). Although IFN-β

did not alter the endpoint of the study, treatment with inhaled IFN-β in difficult-to-treat asthmatics reduced the number of virus-induced moderate asthma attacks, and reduced the number of times these individuals used their rescue inhalers on days 5 and 6 post-symptom onset (Djukanović et al., 2014). Taken together these findings demonstrate the potential of this cytokine in treating atopic diseases, especially those in which current therapies are unsuccessful at controlling disease symptoms. In order to understand why IFN-α/β is an effective therapeutic, the following experiments were conducted. This work aimed to determine how human naïve $CD4^+$ T cells and committed Th2 cells respond to IFN-α/β signaling, and how these findings can be applied to our understanding of the dysregulated Th2 response in atopic diseases. These questions were tackled by designing experiments that address the following research aims:

Aim 1: Determine how IFN-α/β signaling alters IL-4-induced Th2 commitment, thus inhibiting Th2 development in human naïve $CD4^+$ T cells.

Aim 2: Determine the molecular mechanism by which IFN-α/β signaling suppresses TCR-mediated Th2 cytokine production by human memory Th2 cells.

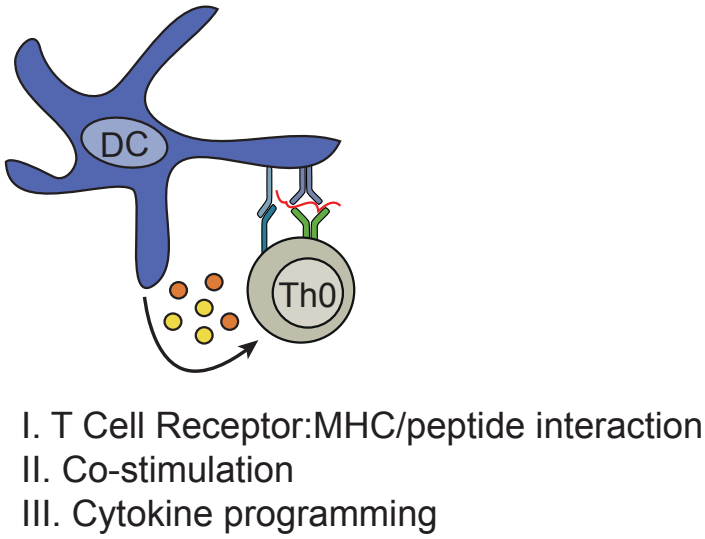

Figure 1.1: CD4$^+$ T cell activation and programming requires three external signals. Naïve CD4$^+$ T cells require I) TCR activation through the interaction with MHC class II presenting processed peptide that is specific for the TCR expressed, II) Co-stimulation through CD28, and other proteins expressed on the surface of the T cell, which interact with surface proteins upregulated by a mature antigen presenting cell, and III) Cytokine programming to mediate the upregulation of a specific master transcription factor necessary to direct the cell to a specific lineage, as well as help to mediate epigenetic programming that allows subsequent daughter cells to inherit lineage-specific programming. Once the cell has received these three signals, it can respond to restimulation more quickly and robustly, and the need for co-stimulation and cytokine programming no longer exists.

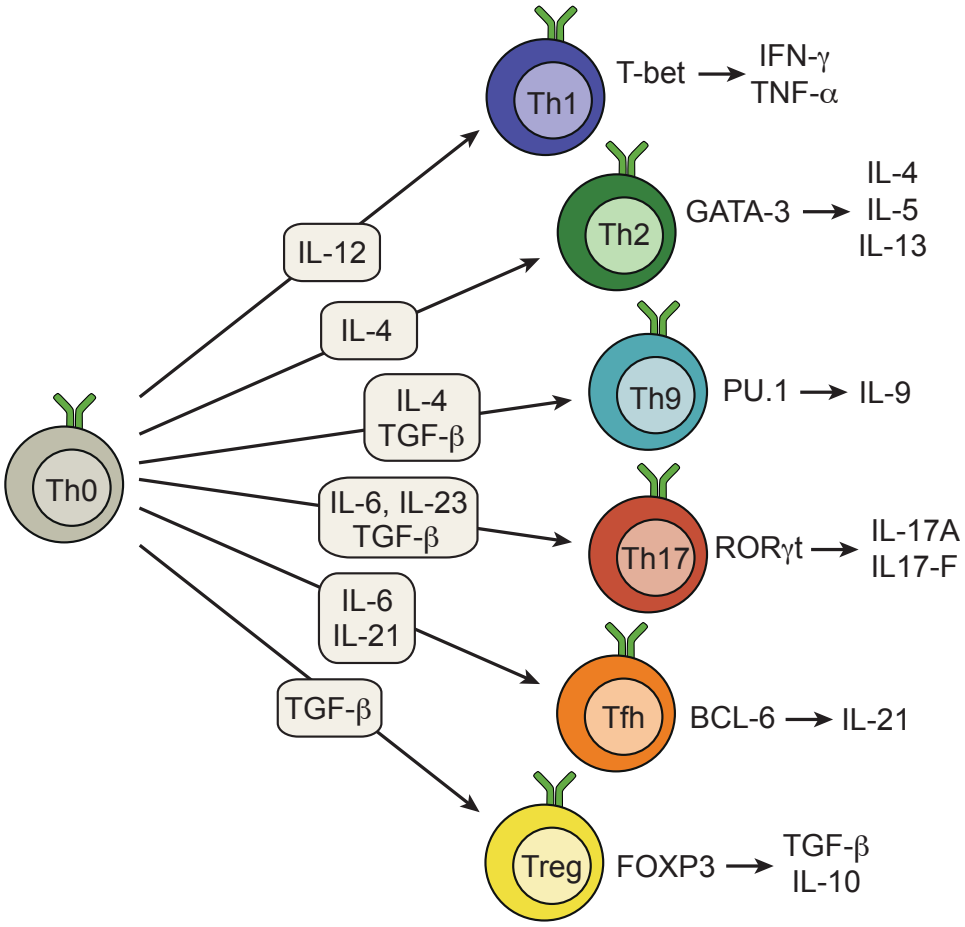

Figure 1.2: CD4$^+$ T cell lineages and the relevant master transcription factors and cytokines. Depending on the antigen presented and the cytokine cues received from the environment, a naïve T cell can differentiate into a wide array of cell lineages, depending on the insult. The boxed cytokines are the signals received by the naïve CD4$^+$ T cell to differentiate into a specific lineage. Master transcription factors that are necessary for the respective lineage, as well as cytokines the activated cells make, are listed on the right.

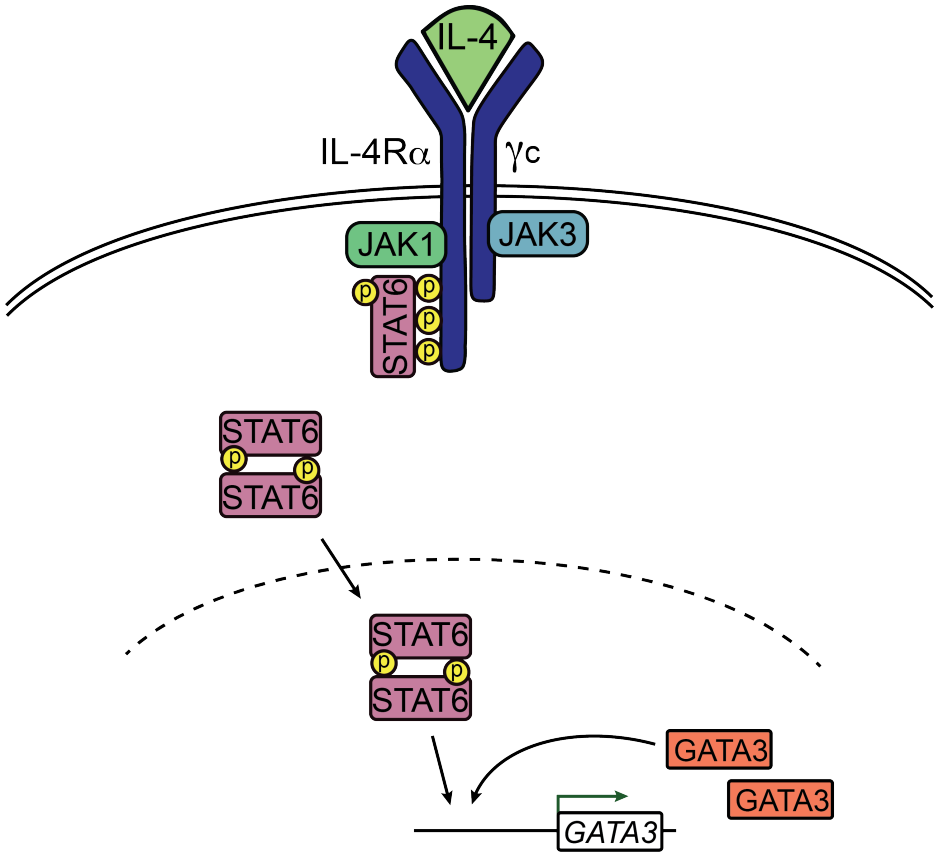

Figure 1.3: IL-4-mediated signaling drives Th2 lineage commitment. IL-4 signaling through the IL-4Rα and common γ chain induces phosphorylation and activation of the JAK kinases, JAK1 and JAK3. Subsequent phosphorylation of the IL-4Rα chain leads to the recruitment of STAT6, which becomes phosphorylated at the C-terminus, and forms a homodimer with another phosphorylated STAT6 protein via the SH2 domains. This dimer translocates to the nucleus, where it will bind to promoters and enhancers of IL-4-sensitive genes, including *GATA3*. Once *GATA3* has been activated by STAT6, it is able to maintain its own expression in a feed-forward auto-regulatory loop, making STAT6 dispensable after initial GATA3 upregulation.

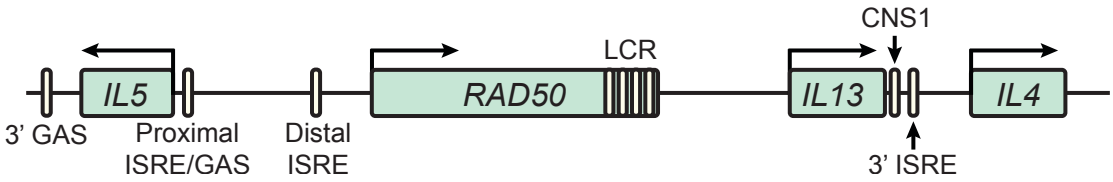

Figure 1.4: Organization of the Th2 cytokine locus. The Th2 cytokine locus is found on human chromosome 5q31 and murine chromosome 11. In both species, the region is approximately 140 kb long, and contains four genes. The *IL5* gene is located on the 5' end of the locus, and is transcribed in the opposite direction compared to the other genes. *IL4* and *IL13* genes are approximately 10 kb apparent and are present at the 3' end of the region. *IL4* and *IL13* genes are transcribed in the same direction and opposite to IL5. Finally, *IL5* is separated from *IL4* and *IL13* by the DNA repair gene *RAD50*, whose expression is unaffected by signals that regulate the *IL5*, *IL4* and *IL13* genes. LCR: Locus control region, CNS: Conserved noncoding sequence, GAS: Gamma activated sequence, ISRE: Interferon stimulated response element.

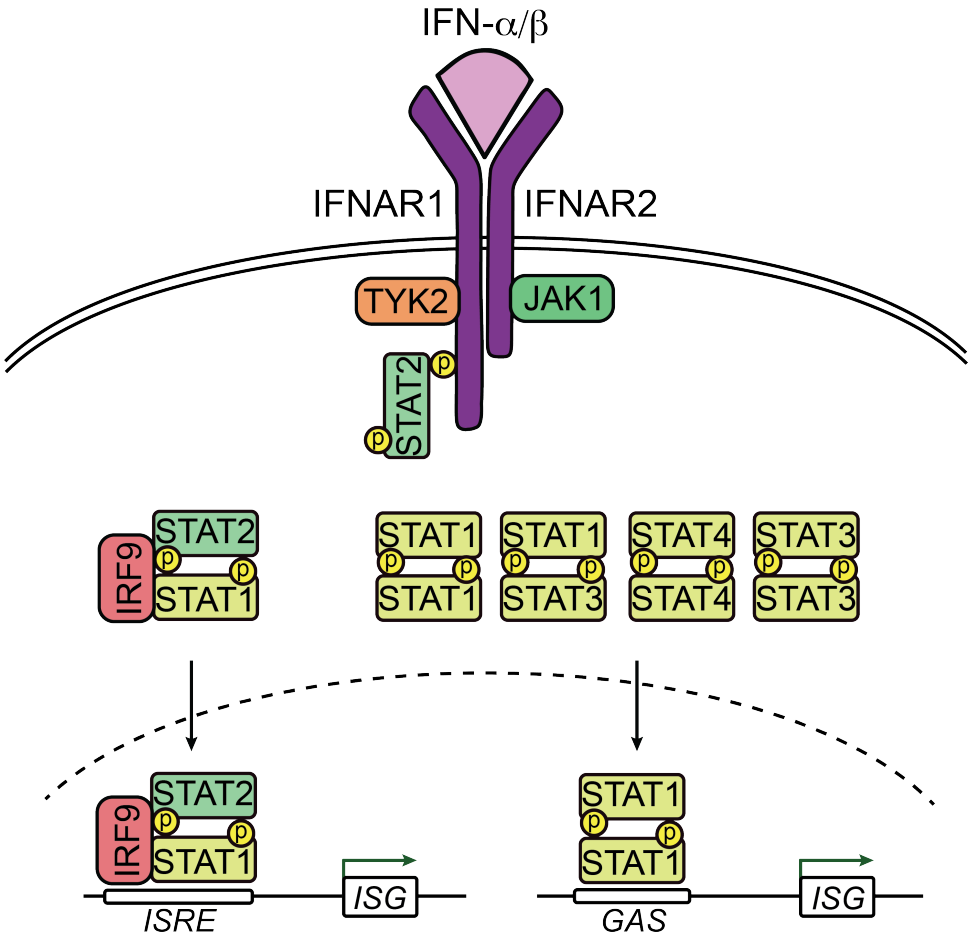

Figure 1.5: IFN-α/β signaling drives Jak/STAT activation and ISG expression. Classical IFN-α/β signaling induces Tyk2 and Jak1 cross-phosphorylation and subsequent IFNAR1 tyrosine phosphorylation. STAT2 is recruited to the IFNAR1 chain, where it becomes phosphorylated and interacts with STAT1. The STAT2/STAT1 heterodimer disassociates from IFNAR1, and interacts with IRF9, creating the ISGF3 complex. ISGF3 translocates to the nucleus and binds the cis regulatory ISRE, found in the promoters of ISGs. Additionally, other STAT hetero- and homodimers will form in response to Jak/STAT activation, including, but not limited to STAT1/STAT1, STAT1/STAT3, STAT3/STAT3, STAT1/STAT4, and STAT4/STAT4 (some not shown above). These dimers lacking IRF9 will bind to the canonical GAS elements within ISGs, although genes that contain ISREs may not necessarily contain GAS elements.

CHAPTER II

MATERIALS AND METHODS

Human Donors

Peripheral blood (120 - 180 ml) was collected from healthy adults by venipuncture. Informed consent was obtained from each donor in accordance with the guidelines set by the Internal Review Board (University of Texas Southwestern Medical Center). Healthy donors were selected based on a defined set of criteria for participation prior to the initiation of the study. These include no past/present drug use; acute or chronic infection or test positive for HIV, pneumonia, influenza, any sexually transmitted diseases, or tuberculosis; autoimmune disorders including rheumatoid arthritis, lupus, juvenile diabetes, multiple sclerosis, inflammatory bowel disease, or myasthenia gravis; asthma or severe allergies; past or present diagnosis of cancer, including Hodgkin's Disease; organ transplant recipient; or fever and/or malaise. Blood was collected in 60 ml syringes prepared with 3ml heparin at 5,000 U/ml (Sigma-Aldrich, Inc. St. Louis, MO).

Mice

All mice were housed in a specific pathogen-free facility in accordance with the guidelines established by the Institutional Animal Care and Use Committee at UT Southwestern Medical

Center. All mice used for experiments were euthanized by CO_2 asphyxiation and subsequent cervical dislocation.

Cytokines and Reagents for Tissue Culture and Assays

Recombinant human IL-4 (rhIL-4), rhIL-12, rhIFN-γ, and anti-human IL-4 (anti-hIL-4) were purchased from R&D (Minneapolis, MN). rhIFN-α(A), rhIFN-ω, and anti-hIFN-α/β receptor (IFNAR2) antibody were purchased from PBL Laboratories (Piscataway, NJ). rhIL-2 was obtained from the NIAID Resources for Researchers (Bethesda, MD). rhIFN-β1 was a gift from M. Racke (University of Ohio). rhIFN-λ1 (rhIL-29) was purchased from Peprotech (Rocky Hill, NJ). Anti-hCD3 (OKT3) was purified from ascites fluid. Mouse anti-hCD3 (M305.2) was a gift from N. van Oers (University of Texas Southwestern Medical Center) and anti-hIL-12 (20C2) and anti-hIFN-γ (4S.B3) antibodies were purified from hybridomas. Anti-hCD28 was purchased from BioLegend (San Diego, CA). Phorbol-12-myristate-13-aetate (PMA) and cycloheximide were purchased from AG Scientific (San Diego, CA). Ionomycin, 5-azacytidine, and actinomycin D were all purchased from Sigma-Aldrich, Inc. (St. Louis, MO), while Monensin was purchased from eBioscience (San Diego, CA). Trichostatin A was a gift from G. Yu (University of Texas Southwestern Medical Center), and the γ-secretase inhibitor dibenzazepine (DBZ) was a gift from T. Eagar (University of Texas Southwestern Medical Center). Deoxyribonuclease I used in DNase hypersensitivity assays was purchased from Worthington Biochemical Corp. (Lakewood, NJ), and biotin-UTP used in the nuclear run-on assays was purchased from Roche Applied Science (Indianapolis, IN).

Chromatin Immunoprecipitation Antibodies and Reagents

The protein A/G Agarose and Magnetic beads were purchased from Thermo-Pierce (Rockford, IL) and Millipore (Billerica, MA), respectively. Rabbit polyclonal antisera against STAT1, (H-119), STAT2 (C-20 and L-20), and STAT4 (C-20 and E-23) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse IgG2a against EZH2 was purchased from Active Motif (Carlsbad, CA), and whole rabbit IgG was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Mouse IgG2a was purchased from Biolegend (San Diego, CA) and rabbit polyclonal antiserum against tri-methyl-H3K27 and acetyl-H4 were purchased from EMD Millipore (Billerica, MA). Rabbit polyclonal antiserum against H3 and tri-methyl-H3K4 were purchased from Abcam (Cambridge, MA). Murine IgG_1 and rabbit polyclonal antisera against STAT3 was purchased from Cell Signaling Technology (Danvers, MA).

Flow Cytometry Antibodies

Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Sigma-Aldrich (St. Louis, MO). The fluorescein isothiocyanate (FITC)-conjugated anti-hIFN-γ, phycoerythrin (PE)-Cy7-conjugated anti-hIFN-γ, FITC-conjugated anti-hIL-4, PE-conjugated anti-hIL-4, peridinin-chlorophyll (PerCP)-Cy5.5-conjugated anti-hIL-4, PerCP-Cy5.5-conjugated anti-hIL-13, and allophycocyanin (APC)-conjugated anti-hIL-13 antibodies were purchased from BD Biosciences (San Jose, CA). The FITC-conjugated anti-hCD4, PE-conjugated anti-hCD4, and APC-conjugated anti-hCD4 antibodies were purchased from Invitrogen (Carlsbad, CA). The FITC-conjugated anti-hCD4, Pacific Blue-conjugated anti-hCD4, Pacific Orange-conjugated anti-

hCD45RA, PE-conjugated anti-hCD45RO, Alexa 647-conjugated anti-hCD294 (CRTH2), Alexa 647-conjugated anti-hGATA3 (Poly6071), and the Alexa 647-conjugated anti-mIgG1κ isotype antibodies were purchased from BD Biosciences (San Jose, CA).

Human T cell Isolation

Peripheral human blood was diluted 1:1 with Phosphate Buffered Saline (PBS), then underlayed with Lymphocyte Separation Medium (Cellgro, Manassas, VA) and centrifuged at room temperature for 20 minutes at 2000 rpm. Buffy coats were collected and rinsed with 2% FBS in complete IMDM (cIMDM), then incubated for 45 minutes at 37°C on 150 mm x 25 mm tissue culture dishes to enrich for lymphocytes. Enriched lymphocytes were collected from the plates, and T cells were purified by either fluorescently activated cell sorting (FACS) using a MoFlo (Beckman-Coulter) or Special Order Aria (BD Biosciences), or by magnetic isolation kit (Naive $CD4^+$ T Cell Enrichment Set - DM (BD Biosciences, San Jose, CA), Memory $CD4^+$ T Cell Enrichment Set - DM (BD Biosciences, San Jose, CA), or EasySep Human Memory $CD4^+$ T Cell Enrichment Kit (StemCell Vancouver, BC). Purified $CD4^+CD45RA^+$, $CD4^+CD45RA^-$, and $CD4^+CRTH2^+$ T cells were purified to >90% purity, as verified by flow cytometry.

Murine T cell isolation

Balb/c or C56/B6 male or female mice between 6 - 8 weeks of age were euthanized by CO_2 asphyxiation and cervical dislocation. Spleens were harvested, minced, and incubated in RBC Lysis Buffer (Sigma) for 3 minutes on ice. Cells were centrifuged at room temperature for 4

minutes at 1300 rpm, and rinsed with 2% FBS in cIMDM. Splenocytes were used for experiments, or $CD4^+$ T cells were isolated by magnetic isolation kit using the Dynal Mouse $CD4^+$ Negative Isolation Kit (Life Technologies, Grand Island, NY). $CD4^+$ T cells were enriched to >90% purity. Splenocytes or purified $CD4^+$ T cells were polarized for 7 days in the presence of anti-mCD3 and anti-mCD28 with anti-mIL12, anti-mIFN-γ, rhIL-2, and rmIL-4. Cells were split 1:10 on day 3, rested overnight on day 6, then restimulated with anti-mCD3 in the presence or absence of recombinant universal IFN-α(A/D) for 24 - 36 hours. Supernatants were harvested and stored at -80°C until analysis by ELISA.

Staining for Flow Cytometry

Cells were restimulated with PMA and Ionomcycin in the presence of Monensin for 4 - 6 hours, harvested, rinsed with 1X PBS and fixed with 2.5-5.0% formaldehyde for 20 minutes. Cells were washed with 1X PBS twice, washed with 0.5% BSA/PBS once, and then with 0.1% Saponin in 0.5% BSA/PBS once. Cells were permeabilzed by incubating in 0.1% Saponin in 0.5% BSA/PBS for 10 minutes at room temperature, spun, and resuspended in 50 ul cytokine antibody panel. Cells were incubated for at least 20 minutes at room temperature, washed once with 0.1% Saponin in 0.5% BSA/PBS, then twice with 0.5% BSA/PBS. Finally, cells were resuspended in 200 ul 0.5% BSA/PBS and analyzed using a Calibur or LSR-II (BD Biosciences, San Jose, CA). If surface staining was necessary in addition to intracellular staining, the cells were stained for at least 20 minutes before fixing, rinsed twice with 1X PBS, then fixed. For transcription factor staining, the FOXP3/Transcription Factor Staining Kit was used as per the manufacturer's instructions (eBiosciences, San Diego, CA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Human or murine supernatants were stored at -80°C until analysis. Human IL-4, IL-5, and IFN-γ was measured using the ELISA MAX Standard Kits (Biolegend, San Diego, CA). Human IL-13 was analyzed using the Human IL-13 ELISA development kit (Mabtech, Cincinnati, OH). Murine IL-4 and IL-5 was analyzed using the ELISA MAX Standard Kits (Biolegend, San Diego, CA), and murine IFN-γ was analyzed using a homemade kit. All ELISAs, including the homemade murine IFN-γ kit was performed as follows: 96-well maxisorp flat bottom plates were coated with Bicarbonate Coating Buffer overnight at 4°C. Plates were washed twice with wash buffer (0.05% Tween-20 in PBS), then blocked with Assay Diluent (1% BSA/PBS) for 1 hour at room temperature with gentle shaking. Diluted supernatants from *in vitro* cell cultures or ELISA standards were incubated on the plates for at least 2 hours at room temperature with gentle shaking. Supernatants were removed and plates were washed with wash buffer at least 3 times, then were incubated with cytokine-specific detection antibody at room temperature for 1 hour. Plates were washed at least three times with wash buffer, then incubated with streptavidin-HRP for 30 minutes at room temperature. Plates were washed at least three times, then TMB Substrate solution was added to the plates, and depending on the kit, incubated at room temperature in the dark for 5 - 20 minutes. After samples were appropriately developed, 1:1 volume of 1 M H_2SO_4 was added to each well to stop the reaction. Signal was assessed at 450 nm using the iMark Microplate Absorbance Reader (BioRad, Hercules, CA). Absorbance of each experimental sample was analyzed based on a standard curve, and concentrations were calculated using Beer's Law. Data was graphed using Prism (GraphPad Software, La Jolla, CA). Statistical significance

was determined using a student's t test or one-way Anova with a bonferonni post-hoc test. $p < 0.05$.

Cell Division Analysis by CFSE

Purified $CD4^+$ T cells (naïve or memory based on CD45RA expression) were washed twice with 1X PBS and resuspended at $10e^6$ cells/ml in 1X PBS. CFSE was added to the cells at a 1:800 dilution from a 1 mM working stock, and cells immediately mixed by flicking and incubated in the dark at room temperature for 5 minutes. 20% FBS in cIMDM was added to the cells to quench the remaining CFSE by incubating at room temperature for 1 minute. Cells were washed twice with 10% FBS in cIMDM and stimulated as necessary. All CFSE experiments were analyzed at day 5 post-stimulation by flow cytometry and compared to 'CFSE-negative' and 'CFSE-positive unstimulated' controls.

Quantitative PCR

Total RNA from polarized $CD4^+CD45RA^+$ T cells or acutely stimulated $CD4^+CD45RA^-$ T cells was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) or the Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA). Each kit contained an on-column RNase-free DNase I treatment step that was used to remove contaminating genomic DNA. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Grand Island, NY) by loading 100 - 500 ng of total RNA into a 20 ul - 40 ul reaction volume. The subsequent cDNA was the template for quantitative PCR analysis using the Brilliant II SYBR Green Master Mix

(Agilent Technologies, Santa Clara, CA), the Maxima SYBR Green Master Mix (Thermo Scientific, Waltham, MA) or the TaqMan Gene Expression Mix (Applied Biosystems Inc., Grand Island, NY) using an ABI 7300 cycler (Applied Biosystems Inc., Grand Island, NY). When the SYBR technology was used, primers were designed that spanned multiple exons to exclude any signal from genomic DNA contamination and were synthesized by Integrated DNA Technologies (Coralville, IA) or Sigma Aldrich (St. Louis, MO). For the TaqMan Gene Expression approach, primers were purchased from (Applied Biosystems Inc., Grand Island, NY) that were specific to our genes of interest. All in-house designed primers can be found in Table 2.1. Primers specific for GAPDH or PPIA were used as reference genes, and relative gene expression of specific target genes were assessed using the $2^{-\Delta\Delta Ct}$ approach (Livak and Schmittgen, 2001).

Nuclear Run-On

Nuclear run-on assays were performed as described previously with some modifications (Fan et al., 2010; Mehta et al., 2010; Patrone et al., 2000). $CD4^+CD45RA^+$ T cells were polarized for 7 days in the presence of rhIL-2 (50 U/ml), rhIL-4 (20 ng/ml), anti-hIFN-γ (5 μg/ml), and anti-hIL-12 (10 ng/ml) in the presence of plate-bound anti-hCD3 (3 μg/ml) and anti-hCD28 (3 μg/ml) stimulation. On day 7, Th2 cells were restimulated for 2 hours in the presence or absence of anti-hCD3/rhIL-2 (3 μg/ml and 50 U/ml, respectively), rhIFN-α(A) (1000 U/ml) and actinomycin D (10 μg/ml). 12 - $20e^6$ cells per condition were harvested, rinsed once with cold PBS, then spun at 250 x g for 5 minutes at 4°C. The supernatant was discarded, and the cell pellet was resuspended in Cell Lysis Buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 150 mM sucrose)

containing 0.3% NP-40 (Ipegal). Cells were incubated on ice for 5 minutes, mixing by hand every minute. Cells were spun at 250 x g for 5 minutes at 4°C, and supernatants were discarded. Cell nuclei were washed once in Cell Lysis Buffer (no NP-40) and spun at 250 x g for 5 minutes at 4°C. Cell lysis was verified by staining a small aliquot of the nuclei with trypan blue. Nuclei were resuspended in Nuclei Storage Buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) then a 2X solution of Reaction Buffer (20 mM Tris-HCl, pH 8.0, 200 mM KCl, 5 mM $MgCl_2$, 200 mM sucrose, 20% glycerol supplemented with fresh 4 mM dithiothreitol) supplemented with 4 mM rATP, rCTP, rGTP, 0.4 mM Biotin-rUTPand 1.6mM rUTP was added 1:1 to each sample. Additional actinomycin D was added to the 'actinomycin D sample' at 10 µg/ml. Each treatment was incubated for 30 minutes at 29°C. The *in vitro* transcription run-on reaction was stopped by the addition of 250 mM $CaCl_2$ and DNase I with mixing, and incubated for 15 minutes at 29°C. Nuclei were spun at 6,000 x g for 5 minutes at room temperature and supernatants were discarded. Nuclei were resuspended in TRizol reagent and incubated at room temperature for 5 minutes to allow complete dissociation of nucleoproteins. 200 ul chloroform per 1 ml TRizol was added and each sample was mixed by vigorous shaking for 30 seconds, then incubated at room temperature for 3 minutes. Phases were separated by centrifugation at 12,000 x g for 15 minutes at room temperature. The aqueous phase was collected and chloroform extraction was repeated. 2.5 volumes of 100% isopropyl alcohol was added to the aqueous phase, mixed gently by hand and incubated at -80°C for 10 minutes. Precipitated RNA was centrifuged at 12,000 x g for 5 minutes at room temperature. Isopropanol was discarded, and the RNA pellet was washed once with 75% ethanol. The pellet was vortexed briefly to dislodge from the tube, then centrifuged for 5 minutes at 8,000 x g. The ethanol was discarded, then RNA pellets were air dried and resuspended in 30 ul of RNase-free H_2O.

Alternatively, the RNeasy Mini Kit (Qiagen, Valencia, CA) was utilized as per the manufacturers protocol. Samples were incubated between 55-65°C for 10 minutes then placed on ice and quantified by Nanodrop. Samples were stored at -80°C until streptavidin-capture. Biotin-labeled transcripts were enriched using Dynal M-280 Streptavidin coated magnetic beads (Invitrogen, Grand Island, NY). 20 ul beads per sample were prepared by washing for 5 minutes each as per the manufacturers instructions; twice in Solution A (DEPC-treated 0.1 M NaOH, 0.05 M NaCl), once in Solution B (DEPC-treated 0.1 M NaCl), twice in 1X Binding Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl) then resuspended in 50 ul 2X Binding Buffer. Beads were blocked with 10 µg poly(I:C) for 1 hour at room temperature with shaking on a vortexer at setting 3. Beads were washed twice in 1X Binding Buffer and resuspended in 50 ul 2X Binding Buffer. RNA samples were treated with DNase I in 1X DNase I Buffer (Roche Applied Sciences, Indianapolis, IN) for 20 minutes at 37°C, then DNase I was heat-inactivated at 75°C for 15 minutes. 3 - 4 µg of DNase-treated RNA was add to the pre-washed beads at a 1:1 volume and incubated for 20 minutes at 42°C with pipetting every 10 minutes, then for 2 hours at room temperature with vortexing. Biotin-labeled RNA captured on the streptavidin-coated magnetic beads was washed consecutively twice with 15% formamide/2X saline-sodium citrate (SSC) buffer, once with 2X SSC buffer supplemented with 0.05% NP-40 in the presence of RNase Inhibitor and DTT, and once with DEPC-treated H_2O for 5 minute each. Beads were resuspended in 30 ul DEPC-treated H_2O, and complementary DNA was made directly off the beads using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Grand Island, NY) with pipetting throughout the reaction to prevent the beads from settling. The cDNA mixture was immediately pipetted at least 10 times, spun down, and the supernatant containing the cDNA was immediately analyzed by quantitative PCR. qPCR analysis was preformed as described in the

'quantitative PCR' section using PPIA as a reference gene. See Table 2.2 for primers used for the detection of nascent labeled transcripts.

mRNA Decay Analysis

Bulk lymphocytes were isolated as described in the 'Human T cell Isolation' section. Cells were utilized after the 'panning' step. Lymphocytes were stimulated for 2 hours in the presence or absence of rhIL-2 (50 U/ml) and anti-CD3 (3 μg/ml) at 37°C. After 2 hours, actinomycin D (10 μg/ml) and/or rhIFN-α(A) (1000 U/ml) added. Cells were harvested every hour pre- and post-actinomycin D up to 5 hours post-TCR stimulation, rinsed once with 1X PBS, then total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was prepared using 300 - 500 ng total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Grand Island, NY). qPCR was performed and *IL5* and *IFNG* transcripts were assessed relative to *PPIA* for each condition: No TCR-Drift, TCR-Drift, TCR-Drift + Actinomycin D, TCR-IFN-α, TCR-IFN-α + Actinomycin D. Each treatment was analyzed relative to the 0 hour time point as described in the 'quantitative PCR' section. Primers utilized for these experiments can be found in Table 2.1.

Chromatin Immunoprecipitation (ChIP) using Protein A/G Agarose Beads

$CD4^+CD45RA^+$ T cells were polarized for 7 days in the presence of rhIL-2 (50 U/ml), rhIL-4 (20 ng/ml), anti-hIFN-γ (5 μg/ml), and anti-hIL-12 (10 ng/ml) in the presence of plate-bound anti-hCD3 (3 μg/ml) and anti-hCD28 (3 μg/ml) stimulation. On day 7, Th2 cells were restimulated

for 2 hours in the presence or absence of anti-CD3/rhIL-2 (3 µg/ml and 50 U/ml, respectively) and rhIFN-α(A) (1000 U/ml). 8 - 16e^6 cells were harvested and rinsed with cold PBS, then resuspended in PBS at a concentration of 8 - 12e^6 cells/ml. 37% formaldehyde was added to a final concentration of 1% and the cells were rotated at room temperature for 10 minutes. 2 M glycine was added to each treatment to a final concentration of 0.125 M and rotated at room temperature for 5 minutes to neutralize the formaldehyde. Cells were centrifuged for 4 minutes at 400 x g at 4°C, then washed once in cold PBS. Cells were resuspended in Cell Lysis Buffer (5 mM Pipes, 85 mM KCl, 0.5% NP-40) in the presence of proteinase and phosphatase inhibitors and incubated for 10 minutes on ice. Nuclei were pelleted at 5,000 rpm for 5 minutes at 4°C, then resuspended in Nuclear Lysis Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) in the presence of proteinase and phosphatase inhibitors (200 - 300 µl/sample) and incubated for 10 minutes on ice. Chromatin was sonicated for 28 - 31 minutes using a Bioruptor (Diagenode, Denville, NJ). Samples were sonicated on 'high' for four-7 minute intervals, 30 seconds on, 30 seconds off. Cellular debris was cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C and supernatants were quantified by Nanodrop. 14 - 25 µg of chromatin was diluted 10 fold in ChIP Dilution Buffer (16.7 mM Tris-HCl pH 8.0, 1.2 mM EDTA, 1.1% TritonX-100, 0.01% SDS, 167 mM NaCl) in the presence of proteinase and phosphatase inhibitors. Chromatin was pre-cleared with Protein A/G agarose beads for 1.5 - 2 hours at 4°C with rotation. Protein A/G agarose beads were pelleted by centrifugation at 100 x g for 1 minute and supernatants were transferred to new tubes, then 4 µg isotype or specific antibody was added. Samples were incubated at 4°C overnight with rotation, then 80 µl Protein A/G agarose beads was added to each sample, then each sample was rotated at 4°C for 1.5 - 2 hours. Samples were spun at 100 x g for 1 minute, then beads were washed consecutively for 5 minutes with 1 ml of each following

solutions: Low Salt Buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% TritonX-100, 0.1% SDS, 150 mM NaCl), High Salt Buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% TritonX-100, 0.1% SDS, 500 mM NaCl), LiCl Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Deoxycholate, 1% NP-40, 0.25 M LiCl), then twice in 1X Tris-EDTA (TE). 250 ul Elution Buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 2% SDS) was added, and each sample was vortexed at setting 3 for 10 minutes, then incubated at 37°C for 10 minutes. Beads were centrifuged at 100 x g for 1 minute, supernatant was collected, and the elution process was repeated one more time (total 500 µl). Input samples (2 - 6 µg) were diluted with 500 ul Elution Buffer, and each sample (IP and input samples) were supplemented with 25 ul of 4 N NaCl and 2 ul of Protease K (20 mg/ml), then incubated at 37°C for 30 minutes. Samples were incubated at 58 - 65°C overnight, and DNA was isolated using the QIAgen minElute PCR Kit (Qiagen, Valencia, CA) per the manufacturer protocol. Eluted DNA was stored at -80°C until used for quantitative PCR. Each immunoprecipitated sample was measured relative to its own input sample using the following formula:

$$\% \text{ ChIP Efficiency} = 2^{(\text{Input Ct} - \text{ChIP Ct})} \times \text{dilution factor} \times 100$$

Primers used to assess specific sites are listed in Table 2.4. Statistical significance was calculated using one-way Anova with a bonferonni post-hoc test ($p > 0.05$).

Chromatin Immunoprecipitation (ChIP) using Protein A/G Magnetic Beads

$CD4^+CD45RA^+$ T cells were polarized for 7 days in the presence of rhIL-2 (50 U/ml), rhIL-4 (20 ng/ml), anti-hIFN-γ (5 μg/ml), and anti-hIL-12 (10 ng/ml) in the presence of plate-bound anti-hCD3 (3 μg/ml) and anti-hCD28 (3 μg/ml) stimulation. On day 7, Th2 cells were restimulated for 2 hours in the presence or absence of anti-CD3/rhIL-2 (3 μg/ml and 50 U/ml, respectively) and rhIFN-α(A) (1000 U/ml). 2 - 12 e^6 cells were harvested and rinsed with cold PBS, then resuspended in PBS at a concentration of 8 - 12e^6 cells/ml. 37% formaldehyde was added to a final concentration of 1% and the cells were rotated at room temperature for 10 minutes. 2 M glycine was added to each treatment to a final concentration of 0.125 M and rotated at room temperature for 5 minutes to neutralize the formaldehyde. Cells were centrifuged for 4 minutes at 400 x g at 4°C, then washed once in cold PBS. Cells were resuspended in Cell Lysis Buffer (5 mM Pipes, 85 mM KCl, 0.5% NP-40) in the presence of proteinase and phosphatase inhibitors and incubated for 10 minutes on ice. Nuclei were pelleted at 5,000 rpm for 5 minutes at 4°C, then resuspended in Nuclear Lysis Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) in the presence of proteinase and phosphatase inhibitors (200 - 300 μl/sample) and incubated for 10 minutes on ice. Chromatin was sonicated for 28 - 35 minutes using a Bioruptor (Diagenode, Denville, NJ). Samples were sonicated on 'high' for four- or five-7 minute intervals, 30 seconds on, 30 seconds off. Cellular debris was cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C, and supernatants were quantified by Nanodrop. 2 - 6 μg of chromatin was diluted 10 fold in ChIP Dilution Buffer (16.7 mM Tris-HCl pH 8.0, 1.2 mM EDTA, 1.1% TritonX-100, 0.01% SDS, 167 mM NaCl) in the presence of proteinase and phosphatase inhibitors. Protein A/G magnetic beads (Protein A/G Magna Beads, EMD Millipore, Billerica, MA) were blocked with

1% BSA/PBS overnight at 4°C with rotation, and samples were incubated with specific antibody or isotype control with 14 - 25 µg chromatin overnight at 4°C with rotation. Samples were placed on a magnet for 2 minutes, then washed consecutively for 5 minutes with 1 ml of each following solutions: Low Salt Buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% TritonX-100, 0.1% SDS, 150 mM NaCl), High Salt Buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% TritonX-100, 0.1% SDS, 500 mM NaCl), LiCl Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Deoxycholate, 1% NP-40, 0.25 M LiCl), then twice in 1X Tris-EDTA (TE). 150 ul Elution Buffer (0.1 M $NaHCO_3$, 1% SDS) was added, and each sample was vortexed at setting 3 for 10 minutes, then incubated at 37°C for 10 minutes. Beads were collected by magnet for 2 minutes, supernatant was collected, and the elution process was repeated one more time (total 300 µl). Input samples (2 - 6 µg) were diluted with 300 ul Elution Buffer, and each sample (IP and input samples) were supplemented with 20 ul of 4 N NaCl and 1 ul of Protease K (20 mg/ml), then incubated at 37°C for 30 minutes. Samples were then incubated at 58 - 62°C overnight, and DNA was isolated using the QIAgen minElute PCR Kit (Qiagen, Valencia, CA) per the manufacturer's protocol. Eluted DNA was stored at -80°C until used for quantitative PCR. Each immunoprecipitated sample was measured relative to its own input sample using the formula described in the previous section. Primers and statistical significance are also listed in the previous section.

Next Generation Sequencing of Chromatin Immunoprecipitated DNA (ChIP-Seq)

Samples prepared for ChIP-Sequencing were prepared as described above in the 'Chromatin Immunoprecipitation (ChIP) using Protein A/G Agarose Beads' section, with the following

differences and additions. At least $16e^6$ cells were used for each condition, and at least 100 μg of chromatin was used for immunoprecipitation with STAT2 or STAT4. The immunoprecipitation protocol was followed as described above. DNA libraries were prepared using the NEBNext ChIP-Seq Library Prep Reagent Set for Illumina (New England Biolabs Inc., Ipswich, MA). DNA was end-repaired, dA-tailed, ligated with adaptor, size selected, and enriched by PCR using Illumina Index Primers. Purified samples were analyzed by ultra-sensitive Bioanalysis to verify size and purity. The samples were quantified by and then sequenced by the Immunology Genomics Core, using an Illumina HiSeq 1000 or 2000 for single-end sequencing, resulting in at least 21 million reads per sample. The UT Southwestern Molecular Biology-hosted cluster processing and memory resource was used to run analysis using the GALAXY graphical user interface. Samples were quality assessed by FASTQC report, mapped to the human hg19 genome by Bowtie using two different stringencies, and specific peaks were identified by MACS14 using different p values. HOMER Tools was used to create lists of statistically significant clusters of protein binding, motif analysis and general characteristics of the datasets, and the Integrative Genomics Viewer (Broad Institute) was used for visualization of the data. 'No TCR+IFN-α' and 'TCR+IFN-α' regions of protein enrichment were identified by comparing each samples to the respective 'input' sample, and then compared to each other to determine how TCR stimulation effects STAT2 binding to cis-regulatory elements of two donors, or STAT4, in one donor. Some 'hits' were validated using ChIP-qPCR using polarized Th2 cells from at least four unique donors.

Next Generation Sequencing of RNA (RNA-Seq)

$CD4^+CD45RA^+$ T cells were polarized for 7 days in the presence of rhIL-2 (50 U/ml), rhIL-4 (20 ng/ml), anti-hIFN-γ (5 μg/ml), and anti-hIL-12 (20 ng/ml) in the presence of plate-bound anti-hCD3 (3 μg/ml) and anti-hCD28 (3 μg/ml) stimulation. On day 7, Th2 cells were restimulated for 4 hours in the presence or absence of anti-CD3/rhIL-2 (3 μg/ml and 50 U/ml, respectively) and rhIFN-α(A) (1000 U/ml). Total RNA was isolated using the Quick-RNA MiniPrep Kit (ZymoResearch, Irvine CA) or the RNeasy Mini Kit (Qiagen, Valencia, CA) as per the manufacturers' protocol. Genomic DNA contamination was removed using on-column DNase I treatment as per manufacturers' protocol. Poly(A) RNA was enriched using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Inc., Ipswich, MA). mRNA libraries for sequencing were prepared using the NEBNext mRNA Library Prep Reagent Set for Illumina (New England Biolabs, Inc., Ipswich, MA). Briefly, enriched mRNA was fragmented to ~200 bp and subsequent first strand then second strand cDNA synthesis was preformed. The cDNA libraries were end-repaired and dA-tailed. Finally, unique Illumina adaptors were ligated to each sample, and PCR enrichment of each sample was performed using universal primers. Purified samples were analyzed by ultra-sensitive Bioanalysis to verify size and purity. The samples were quantified and then sequenced by the Immunology Genomics Core (University of Texas Southwestern Medical Center), using an Illumina HiSeq 1000 or 2000 for single-end sequencing, resulting in at least 23 million reads per sample. The UT Southwestern Molecular Biology-hosted cluster processing and memory resource was used to run analysis using the GALAXY graphical user interface. Samples were quality assessed by FASTQC report and mapped to the human hg19 genome by Tophat, and statistically significant differences between

the different treatments for the two donors (No TCR-Drift, No TCR-IFNα, TCR-Drift, and TCR-IFNα) were determined using CuffDiff. Data was parsed using the RNA-Seq Parsing Utility (gift, Christopher van Horn), and clustered using Gene Cluster 3.0 (de Hoon et al., 2004) and visualized using Java TreeView (Saldanha, 2004) and the UCSC Genome Browser (Rosenbloom et al., 2015).

DNase Hypersensitivity Assay (DHA)

DHA was performed as described previously (Follows et al., 2007; Lu and Richardson, 2004; Sabo et al., 2006). 8 - 12e^6 cells polarized for 7 days under the following conditions, with a 1:10 split and additional rhIL-2 added at day 3: Neutralized (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), anti-hIL-4 (2 μg/ml), and anti-hIFNAR2 (1 μg/ml)); IL-4 (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), rhIL-4 (20 ng/ml), and anti-hIFNAR2 (1 μg/ml)); IL-4 + IFN-α (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), rhIL-4 (20 ng/ml), and rhIFN-α(A) (1000 U/ml)). On day 5, cells were washed with cold 1X PBS, then washed and resuspended in Buffer A (15 mM Tris-HCl pH 8.0, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.5 mM EGTA, supplemented with fresh spermidine and spermine at 0.5 mM and 0.15 mM, respectively). NP-40 (Ipegal) was added at a final concentration of 0.3%, and the cells were gently mixed and incubated on ice for 10 minutes. Nuclei were rinsed once in Buffer A (no NP-40), and resuspended in cold DNase Buffer (Buffer A with 750 nM NaCl and 60 mM $CaCl_2$). Nuclei were added to previously prepared tubes containing the correct amount of DNase I (Worthington Biochemical, Lakewood, NJ) on ice. Nuclei containing DNase I (or no DNase I control) were incubated at 37°C for exactly 6 minutes, then Stop Buffer supplemented with RNase A (10 μg/ml, Qiagen) was added at a 1:1 dilution to

the nuclei (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% SDS, 100 mM EDTA), which were incubated at 55°C for 10 minutes. 50 μg/ml Proteinase K was added to each sample and incubated overnight at 55°C. DNA was isolated using the MinElute PCR Purification Kit (Qiagen, Valencia, CA) as described by the manufacturer, or by phenol/chloroform/isoamyl extraction and ethanol precipitation, and quantified by Nanodrop. 10 - 50 ng of DNA was added to each semi-quantitative or quantitative PCR reaction. For semi-quantitative PCR, 10 ul PCR reaction was loaded onto a 1.5% agarose gel supplemented with ethidium bromide and electrophoresed for 1.75 hours at 120 V. Gels were imaged using a Gel Doc XR+ System (Biorad, Hercules, CA) and band intensities were calculated using Image J (NIH, Bethesda, MD). The slope of decay was calculated using the band intensities and the DNase I concentration for each cell treatment. 'IL-4' and 'IL-4+IFN-α' treatments were analyzed relative to the 'Neutralized' control. Student's t-test ($p > 0.05$) was used to assess statistical significance at all the sites interrogated. Primers used to assess specific sites are listed in Table 2.5.

Electrophoretic Mobility Shift Assay (EMSA)

Two populations of cells were used to perform EMSAs. The assays shown in Chapter 3 were performed using $CD4^+CD45RA^+$ cells polarized under the following conditions for 5 days in the presence of anti-hCD3 (3 μg/ml), anti-hCD28 (3 μg/ml) and rhIL-2 (50 U/ml): Neutralized (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), anti-hIL-4 (2 μg/ml), and anti-hIFNAR2 (1 μg/ml)); IL-4 (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), rhIL-4 (20 ng/ml), and anti-hIFNAR2 (1 μg/ml)); IFN-α (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), anti-hIL-4 (2 μg/ml), and rhIFN-α(A) (1000 U/ml)); or IL-4 + IFN-α (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 μg/ml), rhIL-4 (20

ng/ml), and rhIFN-α(A) (1000 U/ml)). Cells were split at 1:10 with the addition of rhIL-2 (50 U/ml) at day 3, and on day 5, 5 - $10e^6$ polarized cells were utilized for each treatment. The assays shown in Chapter 4 were performed using PBMCs acutely stimulated with anti-hCD3 (3 µg/ml) and rhIL-2 (50 U/ml) in the presence or absence of rhIFN-α(A) (1000 U/ml) for 1-2 hour/s. In both cell populations, cells were harvested and nuclear and cytoplasmic lysates were prepared in the following manner. Harvested cells were rinsed twice in cold 1X PBS. Cells were washed once in cold HB+ Buffer (25 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 5 mM KCl with freshly added protease and phosphatase inhibitors) and centrifuged at 400 x g for 5 minutes at 4°C. Cells were resuspended in 200 ul ice cold HB+ Buffer and incubated on ice for exactly 10 minutes. 20 ul of 10% NP-40 was added to each sample, mixed by hand, then incubated for an additional 5 minutes on ice. Each sample was spun at 1,600 x g for 5 minutes at 4°C, and supernatants were transferred to new tubes (crude cytosolic fractions) and stored on ice. The remaining sample pellets were resuspended in 300 ul cold HB+ buffer and vortexed briefly and spun at 1,600 x g for 5 minutes at 4°C. Supernatant was removed and the nuclei were resuspended in 25 - 35 ul ice cold NEBHS+ Buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.1% NP-40 with freshly added protease and phosphatase inhibitors) and incubated on ice for at least 35 minutes with vortexing every 5 - 10 minutes. Crude cytosolic and nuclear fractions were spun at 1,600 x g for 5 minutes at 4°C and supernatant of cytosolic fraction was transferred to a new tube, while the DNA/RNA pellet was removed from the nuclear fraction. Protein content was quantified using the Bradford Assay (BioRad, Hercules, CA) and protein signal was assessed at 450 nm using the iMark Microplate Absorbance Reader (BioRad, Hercules, CA). Samples were stored at -80°C until use. For electrophoretic mobility shift assay (EMSA), 1 - 4 µg protein was incubated with 3'-biotin-labeled DNA probe and poly(dI-dC) in EMSA buffer (5X buffer: 50

mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM DTT, 5 mM EDTA, 25% glycerol) for at least 30 minutes at room temperature. Band specificity was determined using a cold-competitor (non-labeled) probe as well as specific antibodies against STAT1, (H-119), STAT2 (C-20 and L-20), STAT3, STAT4 (C-20 and E-23), or GATA3. Samples were run through a 4.5% non-denaturing polyacrylamide gel using 0.4X TBE at 160 V for 2.5-3 hours and transferred to a Hybond-N^+ membrane (Amersham Biosciences, Piscataway, NJ) using a semi-dry transfer at 150-155 mA for 2 - 2.25 hours. Detection of biotinylated protein-DNA complexes were performed using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific, Waltham, MA) as per the manufacturer's protocol. Biotin signal was detected and recorded using Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ). Densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD). All probes (3'-biotynolated and cold-competitors) are listed in Table 2.6. The EMSA described in Chapter 3 was performed exclusively by Dr. Jonathan P. Huber.

Assessment of GATA3 by Western blotting

Human $CD4^+CD45RA^+$ T cells were polarized under the following conditions in the presence of anti-hCD3 (3 μg/ml), anti-hCD28 (3 μg/ml) and rhIL-2 (50 U/ml) for 7 days, with a day 3 split at 1:10 with additional rhIL-2 (50 U/ml) added: Neutralized (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), anti-hIL-4 (2 μg/ml), and anti-hIFNAR2 (1 μg/ml)); IL-4 (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), rhIL-4 (20 ng/ml), and anti-hIFNAR2 (1 μg/ml)); or IL-4 + IFN-α (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 μg/ml), rhIL-4 (20 ng/ml), and rhIFN-α(A) (1000 U/ml)). On day 7, cells were lysed at 5 x 10^7 cells/ml for 1 hour at 4^oC in radioimmune precipitation assay

(RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Tween-20) plus proteinase and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM benzamidine, 1 μM pepstatin, and 1 mM sodium orthovanadate). Samples were resolved by SDS-PAGE on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA). Western blot was performed using rabbit polyclonal antisera against GATA3 (Poly6071, Biolegend, San Diego, CA) and HRP-conjugated polyclonal goat anti-rabbit Ig secondary antiserum (Jackson). Detection was performed using ECL detection reagents (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Membranes were stripped and reprobed using polyclonal rabbit antisera against β-tubulin (Cell Signaling Technology, Danvers, MA). Densitometry of GATA3 relative to β-tubulin signal was calculated using Image J software (NIH, Bethesda, MD).

Assessment of GATA3 cellular localization by immunofluorescence

Human $CD4^+CD45RA^+$ T cells were polarized under the following conditions in the presence of anti-hCD3, anti-hCD28 and rhIL-2 for 7 days, with a day 3 split at 1:10 with additional rhIL-2 added: Neutralized (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), anti-hIL-4 (2 μg/ml), and anti-hIFNAR2 (1 μg/ml)); IL-4 (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), rhIL-4 (20 ng/ml), and anti-hIFNAR2 (1 μg/ml)); IFN-α (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 ng/ml), anti-hIL-4 (2 μg/ml), and rhIFN-α(A) (1000 U/ml)); or IL-4 + IFN-α (anti-hIFN-γ (5 μg/ml), anti-hIL-12 (10 μg/ml), rhIL-4 (20 ng/ml), and rhIFN-α(A) (1000 U/ml)). On day 5, cells were acutely restimulated with anti-hCD3 (3 μg/ml) and anti-hCD28 (3 μg/ml) in the presence or

absence of rhIFN-α(A) (1000 U/ml) for 2 hours. Restimulated cells were adhered to 0.1% poly-L-lysine-coated coverslips (Sigma-Aldrich, Inc., St. Louis, MO) and fixed with 4% paraformaldehyde/PBS. Cells were stained on the coverslips by placing them facedown in 1%BSA/PBS containing anti-hCD4-PE and incubating overnight at 4°C. Cells were washed with PBS and permeabilized using the FoxP3 Staining Kit (eBioscience, San Diego, CA) as per the manufacturer's protocol to detect intracellular GATA3 using the anti-hGATA3-Alexa647 antibody or isotype control. Coverslips with fixed and stained cells were washed with PBS, drained and mounted onto slides using Prolong Gold + DAPI (Invitrogen, Carlsbad, CA). Fluorescence microscopy was performed using a Deltavision Deconvolution microscope. Images were prepared using the ImageJ software (NIH, Bethesda, MD). This assay was performed exclusively by Dr. Jonathan P. Huber.

Cloning

The human IL-5 promoter insert (1852 bp) was prepared from a human IL-5 bacterial artificial chromosome by 30 cycles of PCR using the high fidelity LA Taq DNA Polymerase (Takara, Mountain View, CA) and primers prepared by Sigma-Aldrich, Inc. (Table 2.7, Valencia, CA). The PCR product was ran on a 0.8% agarose gel containing ethidium bromide, cut from the gel and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). The human IL-5 promoter (hIL5pro) was inserted into the pCR 2.1 TOPO cloning vector and was amplified by transforming One Shot Chemically Competent E. coli (Life Technologies, Grand Island, NY). The inserted hIL5pro was sequenced using T7 and M16 primers by the McDermott Sequencing Core (University of Texas Southwestern Medical Center) to verify sequence fidelity.

The hIL5pro was digested from the cloning vector using Sac I and Xho I and ligated into a Sac I and Xho I-digested pGL3-Basic vector containing the Firefly luciferase gene (Promega, Madison, WI). Insertion was verified by restriction digest and gel electrophoresis. Sequence fidelity of the 1852 bp hIL5pro insert was verified by tiled sequencing by the McDermott Sequencing Core (University of Texas Southwestern Medical Center). Because the transcriptional start site (ATG) was still in tact in the hIL5pro, a point mutation was used to abolish the site, making the Firefly luciferase gene the only start site available. This step (ATG to CCG) was completed using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). This modified vector was used as a backbone for the human-to-mouse mutants. The Firefly luciferase gene driven by the hamster ISG54 promoter was used as a positive control for type I interferon treatment and was provided by J. Schoggins (University of Texas Southwestern Medical Center). All Firefly luciferase expression was measured relative to a constitutively expressed Renilla luciferase-containing vector, pBS-Renilla, under the control of the CMV promoter. All hIL5pro mutants were prepared using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), or using overlap PCR, with in-house designed primers (Table 2.7). Mutant 4 was made using Mutant 1 as the template, and Mutant 5 was made in a two-step process using Mutant 2 as the template.

Luciferase Reporter Assay

SV40 T Antigen-expressing Jurkat cells (SV40 T Ag) were utilized for all transfection experiments, and were generously provided by Nicolai S.C. van Oers (University of Texas

Southwestern Medical Center). The GATA3-GFPRV, STAT4-GFPRV, and STAT4-R599K-GFPRV constructs have been described previously (Farrar et al., 2000a; 2001; Ranganath et al., 1998). Cells were electroporated at 220V and 950 μF using a Gen-Pulser II (BioRad, Hercules, CA), with 10 μg Fluc-expressing hIL5pro plasmid, 7-9 μg GATA3-GFP plasmid, 7-9 μg murine STAT4-GFP plasmid or murine STAT4-R599K-GFP plasmid, and 4 μg CMV-renilla plasmid (total 28-30 μg DNA) in antibiotic-, serum- free cIMDM, then incubated on ice for 20 min. Cells were cultured for 24 hrs at 37°C in antibiotic-free cIMDM supplemented with 10% FBS. Cells were stimulated with mouse anti-hCD3 (M305.2, 1:1000) and rhIL-2 (50 U/ml) in the presence or absence of rhIFN-α(A) (1000 U/ml) for 6 hrs, then harvested and lysed. The Dual-Glo Luciferase Reporter Assay was used to quantify Luciferase activity relative to Renilla expression (Promega, Madison, WI). Signal was measured using the MicroBeta TriLux Counter (Perkin Elmer, Waltham, MA) and graphed as 'Arbitrary Units' as a ratio of Luciferase to Renilla signal.

Statistical analysis

All data are shown as mean ± SEM. Statistical analysis was performed by student's t-test, one-way, or two-way ANOVA with a bonferroni post-hoc test using GraphPad Prism software, depending on the experimental design. All p values ≤ 0.05 were considered significant.

Table 2.1: Primers utilized in qPCR to assess gene expression and mRNA decay.

Gene	Direction	Primer Sequence
hPPIA	F	5’-GCGTCTCCTTTGAGCTGTTTGC-3’
	R	5’-ATGGACTTGCCACCAGTGCC-3’
hIL5	F	5'-GCACTGCTTTCTACTCATCG-3'
	R	5'-ACTCTCCGTCTTTCTTCTCC-3'
hIL13	F	5'-CGGTCATTGCTCTCACTTGC-3'
	R	5'-GCTGTCAGGTTGATGCTCCA-3'
hIFNG	F	5'-TGCAGGTCATTCAGATGTAGC-'3
	R	5'-ATGTCTTCCTTGATGGTCTCC-3'
hMX1	F	5'-GTGGCTGAGAACAACCTGTG-3’
	R	5'-GGCATCTGGTCACGATCCC-3’
hGATA3 (total)	F	5’-AGGGACGTCCTGTGCGAACT-3’
	R	5’-GGTCTGGATGCCTTCCTTCTTCAT-3’
hGATA3 (exon 1a)	F	5’-GAACTCTGCCTGTCATTTCTGCC-3’
	R	5’-AATTCTGCGAGCCAGGCTCC-3’
hGATA3 (exon 1b)	F	5’-TCCTCCTCTCTGCTCTTCGCTAC-3’
	R	5’-TTATCTTGGAGGGGTCGTTTAGC-3’

h: human

Table 2.2: Primers utilized in nuclear run-on assays by qPCR.

Gene	Direction	Primer Sequence
hPPIA	F	5’-GCGTCTCCTTTGAGCTGTTTGC-3’
	R	5’-ATGGACTTGCCACCAGTGCC-3’
hIL5	F	5'-GCACTGCTTTCTACTCATCG-3'
	R	5'-ACTCTCCGTCTTTCTTCTCC-3'
hIL13	F	5'-CGGTCATTGCTCTCACTTGC-3'
	R	5'-GCTGTCAGGTTGATGCTCCA-3'
hIFNG	F	5'-AGTGTGGAGACCATCAAGGA-'3
	R	5'-CTTCCCTGTTTTAGCTGCTG-3'
hMX1	F	5'-GTGGCTGAGAACAACCTGTG-3’
	R	5'-GGCATCTGGTCACGATCCC-3’
	R	5’-TTATCTTGGAGGGGTCGTTTAGC-3’

h: human

Table 2.3: Primers utilized in ChIP by qPCR.

Gene	Direction	Primer Sequence
hGATA3 CNSI Site 1	F	5'-AAACATTGGGAGCTGAAAGG-3'
	R	5'-ACGACTCTTTCCTTATCTGTGC-3'
hGATA3 CNSI Site 2	F	5'-AAACACTGGTGAGCAGGC-3'
	R	5'-CAGACCTATTCCACCCAGG-3'
hGATA3 Exon1A Site 1	F	5'-GGACTCTGGCCTTTCTACCC-3'
	R	5'-CCTGCAAAACCACCTCTCTC-3'
hGATA3 Exon1A Site 2	F	5'-CTCCTTCGACCTGCTAATGG-3'
	R	5'-TGGCAGGGTTGGGATTAAC-3'
hGATA3 Exon1A Site 3	F	5'-AACCCTGCCAGAAGACAGCG-3'
	R	5'-CAGCGAGGGAATGAATTTCACC-3'
hIL-4 CIRE	F	5’-GCAGAAGGTGAGTACCTATCTGGC-3’
	R	5’-CTGCCACCAACCACCAGTTC-3’
hIL5pro 1 (proximal)	F	5’-GCCTAATAATGGCATATCGTGA-3’
	R	5’-AAATGTGGGGCAATGATGTA-3’
hIL5pro 2 (distal)	F	5’-AGCGGCTCAAGCCTGTAATC-3’
	R	5’-CCTCCTGAGTAGCTGGGACT-3’
hIL13 (3’)	F	5’-AGGTTCCTCGGGATCCTCC-3’
	R	5’-CCTGGAAGGTGTGAGGGACA-3’
hIL4 (intron 2)	F	5’-TCACTCCGCATCATTTGGGG-3’
	R	5’-GAGAGGAGAGCAGCACATGG-3’
hMXB (promoter)	F	5’-GGAGTTTGGGGACCACTCTG-3’
	R	5’-TGCACGTTCAGGATGGAGAAA-3’
hCD25 (intronic)	F	5’-AAAGACTGTGGGTGGGAGTC-3’
	R	5’-CCACAAGCTCACCACAGAAT-3’
hGAPDH (promoter)	F	5’-TACTAGCGGTTTTACGGGCG-3’
	R	5’-TCGAACAGGAGGAGCAGAGAGCGA-3’
hMYT1 (promoter)	F	5’-ACAAAGGCAGATACCCAACG-3’
	R	5’-GCAGTTTCAAAAAGCCATCC-3’
mIL5pro (promoter)	F	5’-TCCGCCATATATGCACAACT-3’
	R	5’-AAACTCAGGGTTTCCAGTGC-3’
mCD25 (promoter)	F	5’-GTTGAGCAACTTCCTGATATGTGA-3’
	R	5’-TTCAAACCTGCTGGAAATAGG-3’
mOas1b (promoter)	F	5’-GAAGCCCTAACGCCATTGG-3’
	R	5’-AGGGCGCGGATATGCA-3’

h: human, m: mouse

Table 2.4: Primers utilized for DHA analysis in semi-quantitative PCR and qPCR in human cells.

Gene	Direction	Primer Sequence
GATA3 Locus Site 1	F	5'-AGTAAAGCCTGTTGCGTTGA-3'
	R	5'-ACGCTGCTCTTTAATTAGTGTGTC-3'
GATA3 Locus Site 2	F	5'-GCTGGGGAGAAACTAAGCAC-3'
	R	5'-CCCGTTTGCAAATTCCTATC-3'
GATA3 Locus Site 2	F	5'-CACGTCCACGTGCTTTATTT-3'
	R	5'-TCTTCCTAAGAACGTGTGGC-3'
GATA3 Locus Site 2	F	5'-TGGTTCTGCAGCTCTACACAG-3'
	R	5'-CTTTGCTCCGAGGTTTTAGG-3'
GATA3 Locus Site 2	F	5'-GTGCGAGGAAGAGGTGACCA-3'
	R	5'-CAATTTAATGATCAGACAGGGGC-3'
GATA3 Locus Site 2	F	5'-CAAGACAGCAAGAAATGTCCA-3'
	R	5'-CTGGGAAGAGAAGTGTCGCT-3'
GATA3 Locus Site 2	F	5'-GAATGCCAGCGACACTTCT-3'
	R	5'-AACCGGTTGGGTAGGAAGA-3'
GATA3 Locus Site 2	F	5'-GCCCCATGAAAATAGCAACT-3'
	R	5'-TCCCTCTCCCTTCCGACTAT-3'
GATA3 Locus Site 2	F	5'-TTGGAGGCATCAGCATAGAC-3'
	R	5'-AGGGAGTGCGGAGTTAGAGA-3'
GATA3 Locus Site 2	F	5'-GAGCTGCCCAGGTCAGTGAATA-3'
	R	5'-TCCCGGGAGTTAAGTCCAAAT-3'
GATA3 CNSI Site 1 (qPCR)	F	5'-AAACATTGGGAGCTGAAAGG-3'
	R	5'-ACGACTCTTTCCTTATCTGTGC-3'

Table 2.5: Primers utilized in EMSA analysis.

Gene	Direction	Primer Sequence
hIL-4 CIRE probe	F	5’-TGAGTACCTATCTGGCACCATCTCTCCA-3'BioTEG
	R	5’-TGGAGAGATGGTGCCAGATAGGTACTCA-3'BioTEG
hGATA3cons competitor	F	5’-CACTTGATAACAGAAAGTGATAACTCT-3’
	R	5’-AGAGTTATCACTTTCTGTTATCAAGTG-3’
hIL5pro ISRE/GAS probe	F	5’-TAAATTTACTTTTATCTTTTAATAATAAAA-3'BioTEG
	R	5’-TTTTATTATTAAAAGATAAAAGTAAATTTA-3'BioTEG
mIL5pro ISRE/GAS probe	F	5’-TGCATTTTTTTTTTTTTGCTCTTTAATAAT-3'BioTEG
	R	5’-ATTATTAAAGAGCAAAAAAAAAAAAATGCA-3'BioTEG
hIL5pro ISRE/GAS competitor	F	5’-TAAATTTACTTTTATCTTTTAATAATAAAA-3’
	R	5’-TTTTATTATTAAAAGATAAAAGTAAATTTA-3’
mIL5pro ISRE/GAS competitor	F	5’-TGCATTTTTTTTTTTTTGCTCTTTAATAAT-3’
	R	5’-ATTATTAAAGAGCAAAAAAAAAAAAATGCA-3’
EaY probe	F	5’-AAATATTTTTCTGATTGGCCAAAGAGTAAT-3'BioTEG
	R	5’-ATTACTCCTTGGCCAATCAGAAAAATATTT-3'BioTEG
EaY competitor	F	5’-AAATATTTTTCTGATTGGCCAAAGAGTAAT-3’
	R	5’-AATACTCTTTGGCCAATCAGAAAAATATTT-3’

h: human, m: mouse

Table 2.6: Primers utilized in the production of the hIL5pro-Fluc and IL5pro mutants. Mutations within the primers sets are lowercase in red. * Indicates site-directed mutagenesis, while ^ indicates overlap PCR.

Gene	Dir.	Primer Sequence
hIL5pro from BAC (1852bp)	F	5'-AAGCACTCAGAACTTTGTGTATT-3'
	R	5'-CTTTGGCAAAGAAAGTGCAT-3'
*IL5pro ATG to CCG	F	5'-GGACGCAGTCTTGTACTccGCACTTTCTTTGCC-3'
	R	5'-GGCAAAGAAAGTGCggAGTACAAGACTGCGTCC-3'
*hIL5pro Mutant 1	F	5'-GGGAAACAGGGATTTTaacTATTATTAAAgagcAaATAA AAGTAAATTTA-3'
	R	5'-TAAATTTACTTTTATtTgctcTTTAATAATAgttAAAATCC CTGTTTCCC-3'
^hIL5pro Mutant 2 forw. fragment	F	5'- CACCTCCATCATATGTGTTCAGGCC-3'
	R	5'- AAATgcATTTttTTTTtTtTgctcTTTAATAAT AAAAATCCC-3'
^hIL5pro Mutant 2 rev. fragment	F	5'-TTATTAAAgagcAaAaAAAAaaAAATgcATTTTTTAAGAT ATAAGGC-3'
	R	5'- CTTAGATCGCAGATCTCGAGCGGCCG-3'
^hIL5pro Mutant 2 overlap amplification	F	5'- CACCTCCATCATATGTGTTCAGGCC-3'
	R	5'- CTTAGATCGCAGATCTCGAGCGGCCG-3'
*hIL5pro Mutant 3	F	5'- GATAAAAGTAAATTTATTTTgTttgAAGAcccAgGGCAT TGGAAACAT-3'
	R	5'- ATGTTTCCAATGCCcTgggTCTTcaaAcAAAATAAATTT ACTTTTATC-3'
*hIL5pro Mutant 4	F	5'-GTAAATTTATTTTgTttgAAGAcccAgGGCATTGG AAACAT-3'
	R	5'-ATGTTTCCAATGCCcTgggTCTTcaaAcAAAATAA ATTTAC-3'
*hIL5pro Mutant 1 Step 1	F	5'-GAAACAGGGATTTTaacTATTATTAAAgagcAAAA-3'
	R	5'- TTTTgctcTTTAATAATAgttAAAATCCCTGTTTC-3'
*hIL5pro Mutant 5 Step 2	F	5'-AAATgcATTTTgTttgAAGAcccAgGGCATTG GAAACAT-3'
	R	5'-ATGTTTCCAATGCCcTgggTCTTcaaAcAAAATgcATTT-3'

h: human

CHAPTER III

IFN-α SUPPRESSES GATA3 TRANSCRIPTION FROM A DISTAL EXON AND PROMOTES H3K27 TRIMETHYLATION OF THE CNS-1 ENHANCER IN HUMAN TH2 CELLS

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Introduction

A critical transcriptional regulator, GATA3, is involved in a variety of cellular-differentiation pathways. In the immune system, GATA3 is required for hematopoiesis, thymic development, and peripheral T cell effector functions (Ho et al., 2009). GATA3 is a critical regulator of the Th2 phenotype, and its elevated expression in T cells is required for both Th2 development and for maintaining the stability of Th2 memory cells (Farrar et al., 2001; Ouyang et al., 2000; Zheng and Flavell, 1997). Although GATA3 is expressed at basal levels in naive T cells, modest increases in GATA3 protein levels can promote Th2 commitment, even under a variety of conditions that drive other phenotypes (Lee et al., 2000). Moreover, early studies by Murphy and colleagues (Ouyang et al., 2000) demonstrated that ectopic expression of GATA3

via retroviral transduction led to the induction of GATA3 mRNA encoded by the endogenous gene. These data suggested a mechanism whereby GATA3 autoactivation could not only drive Th2 development and maintains the Th2 phenotype in the absence of further acute developmental signals, such as IL-4 (Farrar et al., 2002). Formal proof for the requirement of GATA3 in maintaining the Th2 program was demonstrated by deleting GATA3 in fully committed mouse and human Th2 cells (Pai et al., 2004; Zhu et al., 2004). Thus, GATA3 plays a dominant role in maintaining the stability of Th2 cells, and any pathway that suppresses its expression would be predicted to inhibit Th2 functions. Recently, work conducted in our laboratory by Dr. Jonathan Huber (Huber et al., 2010) and other investigators (Pritchard et al., 2012b) demonstrated that, unlike IL-12 or other innate cytokines, type I IFN (IFN-α/β) blocked IL-4-mediated Th2 development in human T cells and destabilized the Th2 phenotype by suppressing IL-4, IL-5, and IL-13 secretion. However, this effect was not observed in murine T cells (Huber et al., 2010; Wenner et al., 1996). Further, Dr. Huber found that the inhibition was mediated by suppressing GATA3 expression during Th2 development and in committed Th2 cells. As part of this study, I found that IFN-α/β suppressed GATA3 by selectively targeting the expression of the GATA3 gene at an alternative upstream exon (1a) used in response to IL-4 during Th2 commitment. The repression of exon 1a correlated with a condensed chromatin conformation of a conserved noncoding sequence-1 (CNS-1) region located 5 kb upstream of the alternative exon. Thus, epigenetic silencing of a putative enhancer of the Th2-specific GATA-3 exon 1a promoter is a potential basis for the induction of tolerance in atopic Th2 cells.

Results

Inhibition of GATA-3 expression by IFN-α/β

The GATA3 transcription factor is expressed during all stages of thymic development and remains expressed constitutively in resting peripheral naive $CD4^+$ T cells. Cells are prevented from committing to the Th2 lineage as a result of the stoichiometric expression of the ROG-1/FOG-1 repressors (Kurata et al., 2002; Zhou et al., 2001), which prevent GATA3 from driving Th2 differentiation. However, in response to IL-4, GATA3 levels are elevated sufficiently to overcome this repression. Work conducted by Dr. Huber recently demonstrated that IFN-α/β blocked the induction of GATA3 in response to IL-4 (Huber et al., 2010). This effect was confirmed in human $CD4^+$/$CD45RA^+$ T cells differentiated in response to IL-4 in the absence or presence of IFN-α (Figure 3.1A, 3.1B). Work performed by Dr. Hilario Ramos and I demonstrated by Western blot that IL-4 increased GATA3 protein levels 1.6-fold, which was completely suppressed by treatment with IFN-α (Figure 3.1A). Further, this suppression was seen at the RNA level, where as little as 100 U/ml of IFN-α significantly reduced IL-4-driven GATA3 mRNA induction (Figure 3.1B). During the early phases of innate priming, T cells divide rapidly in response to TCR and costimulatory signals. Innate cytokines drive their differentiation into effector cells, which occurs progressively at each cell division (Ben-Sasson et al., 2001; Bird et al., 1998; Farrar et al., 2001; Joshi et al., 2007; Ramos et al., 2009). During Th2 development, the ability to secrete IL-4 increases incrementally in daughter cells as they divide in the presence of IL-4. Because IFN-α inhibits the Th2 differentiation process by suppressing GATA3 (Huber et al., 2010), I assessed whether IL-4-driven GATA3 expression was inhibited

by IFN-α by slowing the progression of cell division or by direct repression of GATA3 in daughter cells. To test this, human $CD4^+/CD45RA^+$ cells were activated with anti-hCD3/anti-hCD28 and rhIL-2 in the presence or IL-4, IFN-α, or IL-4 + IFN-α, and GATA3 protein levels were quantified as a function of CFSE dilution (Figure 3.2A). At day 5 of activation, a similar proportion of divided cells at each division were observed, regardless of the innate cytokine priming condition imposed at the beginning of the culture. Furthermore, GATA3 expression was enhanced incrementally at each cell division (Figure 3.2B), and IFN-α completely inhibited the induction of GATA3 protein by IL-4. The inhibition of GATA3 by IFN-α correlated with a reduction in the percentage of $IL\text{-}4^+$ cells present upon restimulation (Figure 3.2C). Thus, IFN-α inhibited the induction of GATA3 by IL-4 without significantly altering cellular expansion in response to TCR stimulation. Because IL-4 signaling establishes a positive-feedback loop of GATA3 autoactivation (Farrar et al., 2001; Ouyang et al., 2000), there are several steps at which IFN-α could block this pathway. For example, IFN-α could suppress the initial induction of GATA3 by downregulating IL-4R or by interfering with STAT6 activation (Kim and Lee, 2011; So et al., 2000). However, the recent studies conducted by Dr. Huber ruled out this possibility (Huber et al., 2010), and the observation that IFN-α can repress GATA3 expression in fully committed Th2 cells make this scenario unlikely. Further downstream, IFN-α could disrupt GATA3 autoactivation through a variety of posttranslational mechanisms. First, GATA3 nuclear localization was examined by confocal microscopy in cells differentiated in vitro with IL-4 or IFN-α (Figure 3.3A). Dr. Huber conducted all confocal microscopy experiments. Although IFN-α decreased the total staining intensity of GATA3, all of the detectable GATA3 was co-localized with DAPI staining under all cytokine conditions, suggesting that IFN-α did not block GATA3 autoactivation by excluding GATA3 from the nucleus. Second, IFN-α could lead to proteasomal

degradation of GATA3 protein. However, when Dr. Huber treated cells with the general proteasome inhibitor, MG132, the cells were still suppressed by IFN-α signaling (Figure 3.3B). Other posttranslational modifications could interrupt the ability of GATA3 to bind DNA and regulate transcription. Dr. Huber and I tested the ability of IFN-α to alter GATA3 DNA binding activity by both EMSA and ChIP. First, IL-4 significantly enhanced binding of GATA3 to a consensus GATA3 target sequence, which was completely inhibited by IFN-α (Figure 3.4A, 3.4B). EMSAs performed in this chapter were conducted by Dr. Huber. Of note, the enhancement of GATA3 DNA-binding activity by IL-4 was greater in magnitude (0.3-fold) than what can be accounted for by a modest (1.5-fold) induction of total GATA3 protein, perhaps suggesting mechanisms of regulation in addition to simple increases in protein content. Further, the inhibition of GATA3 binding activity by IFN-α was consistant with an experiment conducted by Dr. Huber that showed a reduction in GATA3 bound to the canonical GATA3 site found within the first intron of the IL-4 gene (Figure 3.4C). Collectively, these data rule out the possibility that IFN-α blocks GATA3 expression either by preventing nuclear import or by proteosomal degradation. However, the reduction in GATA3 DNA-binding activity could be due to overall reductions in GATA3 protein content, which may be regulated transcriptionally rather than at the posttranslational level.

IFN-α/β-mediated transcriptional repression of an alternative GATA-3 distal exon

The GATA3 gene contains two independently regulated first exons (Asnagli et al., 2002; Scheinman and Avni, 2009), denoted 1a and 1b (Figure 3.5A). These exons are separated by ~10 kb; when transcribed, each first exon is spliced to exon 2, which contains the initiator codon.

Splicing of exon 1a with 1b has not been detected in any cell type, which may indicate that the two exons operate as distinct transcriptional units. In support of this, previous studies identified exon 1a to be selectively induced by IL-4 in murine peripheral $CD4^+$ T cells, leading to Th2 development (Asnagli et al., 2002). I tested whether induction of GATA3 exon 1a was selectively induced by IL-4 in human T cells. IL-4 increased total GATA3 mRNA content, as assessed by qPCR with primers that spanned across exons 5 and 6 (Figure 3.5B, top panel). As previously demonstrated, IFN-α inhibited total GATA3 mRNA, which correlated with the decrease in GATA3 protein content (Figure 3.5B, top panel). Further, IL-4 selectively induced exon 1a, but not 1b, and this induction was also blocked by IFN-α (Figure 3.5B, middle and bottom panels). In contrast, exon 1b was not affected by any cytokine condition and may be the main exon that contributes to constitutive low expression of GATA3 in naive T cells.

Dr. Huber and I further assessed GATA3 mRNA expression in dividing populations of cells undergoing differentiation in response to IL-4 or IFN-α. Cells were labeled with CFSE, activated with anti-hCD3/anti-hCD28 and rhIL-2 for 5 d, and then purified by sorting based on CFSE dilution, as indicated by the gates in Figure 3.5C. In parallel with the expression of GATA3 protein (Figure 3.1A, 3.1B, 3.2A), Dr. Huber's experiment demonstrated that GATA3 exon 1a was selectively induced by IL-4 at each incremental cell division (Figure 3.5C, middle and bottom panels), while exon 1b was not altered by cell division or by cytokines. In addition, Dr. Huber and I analyzed exon 1a and 1b expression in committed Th2 cells. Once induced by IL-4, GATA3 autoactivation stabilizes Th2 cells by uncoupling their phenotype from the initial signals that drove their development. Further, Dr. Huber's previous studies demonstrated that IFN-α could suppress GATA3 expression in fully committed Th2 cells, thus disrupting the overall Th2 program (Huber et al., 2010). As shown in Figure 3.5D, IL-4 induced the expression

of GATA3 exon 1a, which remained elevated in subsequent rounds of stimulation regardless of whether IL-4 was neutralized in the second week. However, in agreement with our previous findings, IFN-α suppressed GATA3 exon 1a expression in committed Th2 cells down to levels observed in cells that were activated under neutralizing conditions. In summary, I found that the induction of GATA3 mRNA by IL-4 was regulated exclusively at exon 1A, increased as a function of cell division, and was stabilized in differentiated Th2 cells. Importantly, this regulation was blocked by IFN-α, which even suppressed GATA3 exon 1a expression in committed Th2 cells.

Epigenetic modification of GATA-3 CNS-1 in human Th2 cells

Located upstream of exon 1a are several conserved noncoding sequences that have been identified previously by VISTA analysis (Scheinman and Avni, 2009), which demonstrates the percentage of sequence homology between different species. The most proximal CNS-1 region is positioned 5 kb upstream of exon 1a and is of particular interest because it contains multiple GATA3 consensus binding sites, which may be targets for GATA3 autoactivation. I was interested in determining how this genomic region was being regulated both positively by IL-4 and negatively by IFN-α/β. I assessed DNase I hypersensitivity of the region, as well as various primary chromatin modifications that directly impact transcription, including DNA methylation and histone acetylation/methylation. First, given that local increases in DNA methylation patterns often correspond with reductions in transcriptional activity, we proposed that CNS-1 could be a target of IFN-α-mediated recruitment of DNA methyltransferases (Yu et al., 2012). However, when Dr. Huber utilized the general DNA methylation inhibitor 5-azacytidine, it did

not prevent IFN-α from markedly suppressing GATA-3 exon 1a expression (Figure 3.6), suggesting that IFN-α was able to suppress GATA-3 mRNA expression independently of the DNA methylation status of the cells or the GATA-3 locus specifically.

Local chromatin compaction at both promoters and enhancers can significantly alter transcription rates. Such regions can be qualitatively compared by their accessibility to digestion with DNase I. As a first approach, I assessed relative DNase I hypersensitivity across the entire CNS-1/exon 1a region of the GATA3 gene as a function of cytokine treatment (Figure 3.6A, 3.8). Primary naive human $CD4^+$ T cells were differentiated *in vitro* in the presence of IL-4 or IL-4 + IFN-α and compared with cells differentiated under neutralizing conditions. DNase I hypersensitivity analysis was performed on these cells by interrogating tiled intervals across the CNS-1/exon 1a region by semi-quantitative PCR analysis (Figure 3.7A, 3.8). I identified two regions in which relative DNase I hypersensitivity was reduced in the presence of IFN-α. The first region mapped across tiling interval 1, which spanned the entirety of CNS-1. The second region spanned intervals 9 - 10, corresponding to the transcriptional start site and first intron of exon 1a (Figure 3.7A). We did not observe any cytokine-mediated changes in DNase I hypersensitivity within the 5' untranslated region of exon 1a spanning from the putative promoter (interval 8) through the most distal region adjacent to CNS-1 (interval 2). Because CNS-1 has been identified as a critical regulatory element of GATA3 expression in murine Th2 cells, I confirmed the DNase I hypersensitivity of CNS-1 in T cells isolated from multiple donors by qPCR assay. IL-4 enhanced the relative DNase I hypersensitivity of CNS-1 in T cells from four donors, whereas IFN-α suppressed this activity to levels comparable to the neutralized control (Figure 3.7B, 3.7C). Thus, IFN-α promoted a closed chromatin configuration of CNS-1 and potentially at the exon 1A transcriptional start site. These data suggest a role for IFN-α/β

signaling in suppressing the transcriptional accessibility of this region during human Th2 commitment.

Changes in specific histone modifications, such as acetylation and methylation, can often distinguish or predict regions of enhanced or suppressed transcriptional activity. Dr. Huber and I quantified various histone modifications by ChIP at the GATA-3 CNS-1 region, as well as local segments spanning the putative promoter region of exon 1A (Figure 3.9, 3.10). For these experiments, ChIP of total histone H3, along with H3K27me3, H3K9me2, and H4Ac, was performed in naive $CD4^+$ T cells isolated from four or five donors, depending upon the modification. The relative ChIP efficiencies for each donor were calculated and expressed as a percentage of the neutralized control. For H3K9me2 (Figure 3.9), none of the cytokine conditions led to a significant alteration in the density of these marks at any of the sites interrogated. The H4Ac modification was significantly increased at proximal promoter sites by IL-4, regardless of whether IFN-α was present during the priming, whereas this modification was not significantly altered at CNS-1 (Figure 3.9, bottom graph). However, the H4Ac modification pattern at the exon 1A promoter cannot explain the dominant effect of IFN-α in suppressing GATA3 expression in the presence of IL-4. In assessing repressive marks, I found that H3K27me3 was not significantly altered by cytokine activation at the exon 1a promoter. In contrast, I found that the H3K27me3 modification was significantly enriched at the CNS-1 site in response to IFN-α in both the absence and presence of IL-4, which correlates with the inhibition of exon 1a expression by IFN-α. The increased H3K27me3 modification was reflected by a significant increase in the total density of H3 in response to IFN-α at CNS-1, but not the exon 1a promoter (Figure 3.9, upper graph). Thus, the repressive marks at CNS-1 correlate with the reduction in DNase I hypersensitivity at this region, implicating IFN-α/β signaling in blocking

this potential enhancer element.

The CNS-1 region contains at least eight conserved GATA3 consensus-binding sites (Figure 3.10, upper diagram), which are potential targets for GATA3 autoactivation. Because IFN-α/β led to a closed confirmation of this region by DNase hypersensitivity and histone modifications, Dr. Huber and I wished to determine whether this site was bound by GATA3 and, if so, whether this binding was blocked by IFN-α/β treatment. To test this, Dr. Huber performed a GATA3 ChIP at CNS-1 in human naive $CD4^+$ T cells differentiated in the presence of IL-4 or IFN-α. IL-4 significantly increased GATA3 binding to both segments of the CNS-1 region spanning 650 bp, both of which contained consensus GATA3-binding sequences (Figure 3.10). Further, IFN-α inhibited IL-4-driven binding of GATA3 to CNS-1 in T cells from both donors. I also assessed GATA-3 binding at the exon 1a proximal promoter but was unable to detect any significant GATA-3 binding activity under any condition. Thus, the CNS-1 site may contribute to GATA3 exon 1a regulation by acting as an enhancer for GATA3 autoactivation. Further, IFN-α/β represses this activity, which correlates with epigenetic silencing of CNS-1, even in the presence of IL-4.

In summary, Dr. Huber and I found that DNA binding by GATA3 at the CNS-1 region increased in response to IL-4 but was inhibited by IFN-α. This activity coincided with enrichment of the suppressive H3K27me3 mark selectively at the CNS-1 site but not the exon 1a promoter. The reduction in DNase I hypersensitivity, the increase in the H3K27me3 mark, and, consequently, the increase in total H3 content selectively at CNS-1 demonstrate that this area becomes more compact and inaccessible in response to IFN-α signaling. Collectively, these data highlight the selective repressive activity of IFN-α on GATA3 exon 1a transcription in human $CD4^+$ T cells (Figure 3.11).

Discussion

In mouse $CD4^+$ T cells, various external stimuli, such as IL-4 and Notch, can promote elevated expression of GATA3, which is selectively enhanced through the use of the alternative first exon, exon 1a (Asnagli et al., 2002; Fang et al., 2007; Scheinman and Avni, 2009). To our knowledge, the present study is the first to demonstrate that IL-4 preferentially induces GATA3 exon 1a expression during human Th2 development. Furthermore, Dr. Huber and I show that the exon 1a transcript, but not the exon 1b transcript, remains elevated in Th2 cells in the absence of further IL-4 signaling, suggesting that GATA3 feedback in human Th2 cells preferentially maintains expression of the exon 1a transcript. Moreover, Dr. Huber and I identified the CNS-1 region as a potential GATA3 autoregulatory enhancer element that was bound by GATA3 in response to IL-4. Finally, we uncovered an IFN-α/β-dependent mechanism that suppressed Th2 development and stability by disrupting the GATA3-autoactivation loop. Dr. Huber systematically ruled out defects in nuclear localization or proteasome-mediated degradation while finding that overall GATA3 DNA binding activity was reduced. However, a reduction in GATA3 DNA binding activity could be due to reduced levels of GATA3 present in the nucleus of IFN-α-treated cells. Alternatively, IFN-α signaling could alter the function of the GATA3 protein by inhibiting the ability of GATA3 to regulate its own expression. However, this possibility is somewhat unlikely, because IFN-α also blocks the induction of GATA3 expression by IL-4 prior to the establishment of the autoactivation loop. Finally, IFN-α could inhibit transcriptional activation of the GATA3 gene, which is supported by this, data demonstrating a selective block in GATA3 exon 1A expression both in response to IL-4 and in fully committed Th2 cells.

IFN-α/β is a potent inducer of hundreds of IFN-sensitive genes that regulate the antiviral response, but very few genes are actually suppressed by IFN-α/β signaling. Of the few select genes that are repressed by IFN-α/β, some are involved in cell cycle regulation, such as cyclins (Krishnaswamy et al., 1996; Steen and Gamero, 2012). As such, Drs. Fatema Chowdhury and Ramos showed previously that IFN-α slows the progression of cell division in human $CD8^+$ T cells, thus preventing some cells from terminally differentiating into effectors (Chowdhury et al., 2011; Ramos et al., 2009). However, in this study, I found that IFN-α did not have this effect on cells in the naive $CD4^+$ T cell compartment. Rather, IFN-α suppressed GATA3 expression without significantly altering TCR-mediated proliferation, thus blocking IL-4-driven terminal differentiation of Th2 cells. Although cell division is not a "clock" that strictly controls cytokine production (Ben-Sasson et al., 2001), S phase offers the best opportunity to modify local chromatin architecture and alter its accessibility. In this regard, I observed a marked loss in DNase I hypersensitivity, which correlated with a significant increase in the total density of histone H3 specifically at CNS-1 in response to IFN-α. There are three forms of histone H3 (H3.1, H3.2, and H3.3), which are encoded by distinct genes. Although this study design did not distinguish between these H3 variants, recent reports suggested that H3.3, in particular, can be deposited at nucleosome-depleted gaps in chromatin (Schneiderman et al., 2012). H3.3 is usually associated with areas of active transcription, but it also has been found in repressed and poised regions (Delbarre et al., 2010; Goldberg et al., 2010). Furthermore, changes in nucleosome density have been reported as a mechanism that controls epigenetic modifications of the histones (Yuan et al., 2012), suggesting that nucleosome density could be regulated through a mechanism distinct from direct modification of the histones. In most cases, IFN-α/β signaling generally promotes transcription rather than repressing it, which is accounted for by specific deposition of

histones H3.3 at IFN-sensitive genes (Tamura et al., 2009). However, IFN-α/β selectively repressed expression of GATA3 in a manner that may be distinct from the modes of transcriptional regulation of IFN-sensitive genes.

Based on these findings, Dr. Huber and I propose a unique model of GATA3 regulation that accounts for both the induction by IL-4 and repression by IFN-α/β (Figure 3.11). The region encompassing CNS-1 displays different epigenetic patterns in response to cytokine activation than do the regions more proximal to the exon 1a promoter. The permissive mark H4Ac is increased by IL-4 but reduced by IFN-α in the absence of IL-4. Although IL-4 increases H4Ac even in the presence of IFN-a at the exon 1a promoter, this does not occur at CNS-1. Furthermore, IFN-α reduced H4Ac levels below baseline near exon 1A but not at CNS-1. This is in stark contrast to the repressive H3K27 mark, in which IFN-α treatment, even in the presence of IL-4, increased H3K27me3 at CNS-1 but induced no significant changes at the exon 1a promoter. That IL-4 signaling does not result in significant changes in permissive marks at CNS-1 is even more surprising considering that this region is bound by GATA3. GATA3 can complex with trithorax group proteins that normally increase chromatin accessibility (Kozuka et al., 2011; Nakata et al., 2010). As such, GATA3 binding to CNS-1 would be predicted to increase permissive marks on the histones and reduce nucleosomal density. However, IL-4 treatment only marginally increased DNase I hypersensitivity and did not reduce H3 density. Thus, the cause-effect relationship between GATA3 binding and H3K27me3 marking is still unclear. In summary, the IL-4-driven permissive H4Ac mark at the exon 1a promoter does not obviate the repressive effects of IFN-α that are imposed by the H3K27me3-silencing mark at CNS-1.

IFN-α/β signaling could inhibit exon 1a expression and promote chromatin modifications within the CNS-1 region by either direct or indirect mechanisms. Perhaps the most linear

pathway would involve direct recruitment of STAT2 to the GATA3 locus, which would place STAT2 in the position of a transcriptional repressor. A recent report found that STAT2 could recruit the histone methyl transferase, Ezh2, which is responsible for catalyzing the H3K27 trimethyl modification (Testoni et al., 2011a). Alternatively, IFN-α signaling, either through STAT2 or other signaling intermediates, may induce the expression of a downstream repressor. Achieving a complete understanding of GATA3 regulation is difficult because of the complexity of the GATA3 locus. Our data suggest that epigenetic modifications to the CNS-1 region may play an important role in the suppression of GATA3 by IFN-α, and our data suggest that these repressive modifications may block GATA3 binding and autoactivation at this site. Furthermore, there are likely additional regulatory regions involved in the induction of GATA3 by IL-4 and perhaps in the counter-regulation by IFN-α. It is noteworthy that murine $CD4^+$ T cells are completely resistant to the counter-regulatory effects of IFN-α/β to block IL-4-driven Th2 development. If the CNS-1 region regulates the suppression of GATA3 in human, but not mouse, $CD4^+$ T cells, it is likely that small sequence differences within this region may confer species-specific regulation. Alternatively, there may be unique IFN-induced genes that target CNS-1 for silencing in human T cells that are not expressed in mouse. Nonetheless, these data suggest that accessibility of CNS-1 in human $CD4^+$ T cells is integral to the IFN-α-mediated inhibitory effect on exon 1a, which would play an important role in driving permanent suppression of Th2 function in response to IFN-α/β.

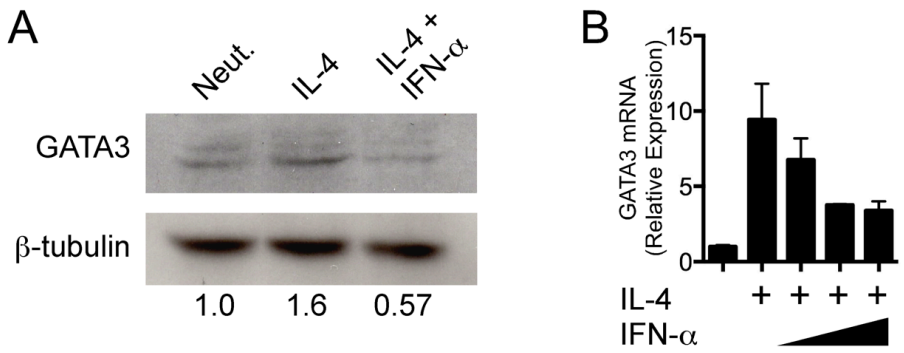


Figure 3.1: IFN-α/β suppresses IL-4-driven GATA3 expression. (A) Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 6 days with the indicated cytokine conditions. Cell lysates were assessed by Western blotting for GATA3 and re-probed for β-tubulin. Bands were quantified by densitometry, and ratios of GATA3/β-tubulin are listed beneath each lane. (B) Purified human $CD4^+/CD45RA^+$ cells were activated with IL-4 and increasing concentrations of IFN-α (10-1000 U/ml) for 3 days. GATA3 mRNA was quantified by qPCR, and data are expressed relative to the neutralized control. I performed all experiments in this Figure except for the detection of GATA3 in (A), which was performed by Dr. Hilaro Ramos.

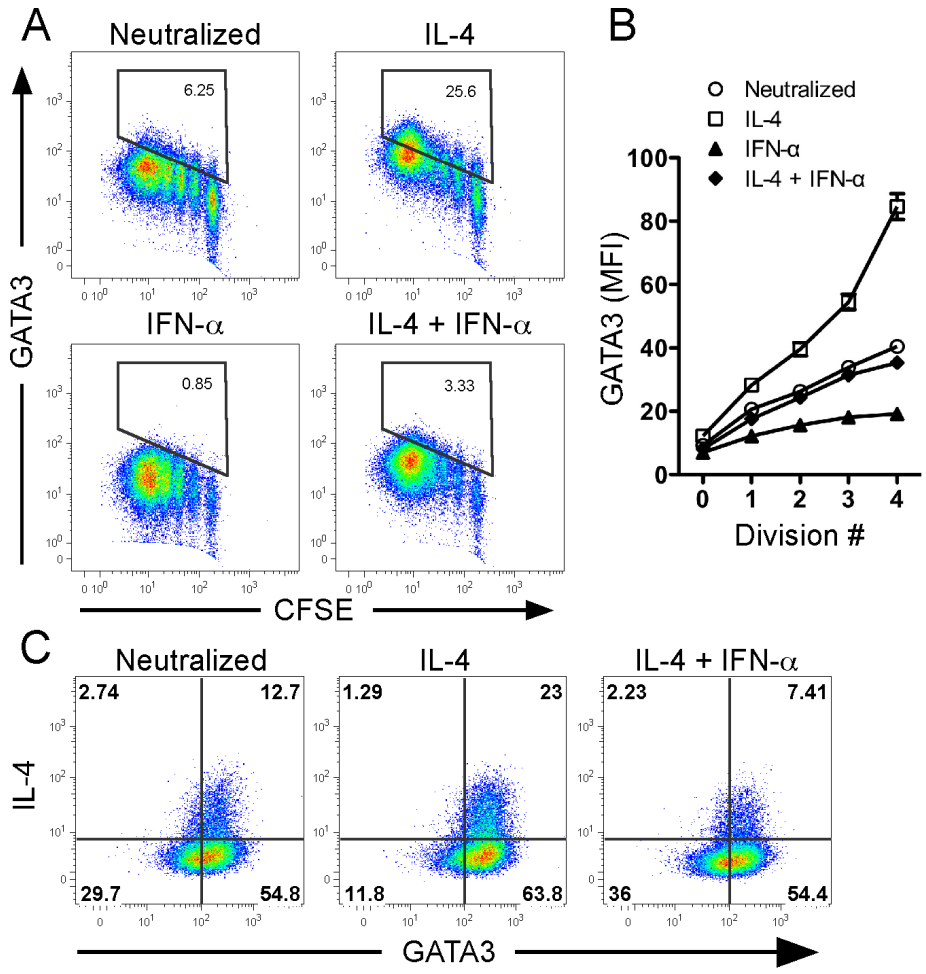


Figure 3.2: IFN-α/β suppresses IL-4-driven induction of GATA3 expression as a function of cell division. (A) Purified human $CD4^+/CD45RA^+$ cells were labeled with CFSE and activated with plate-bound anti-hCD3/anti-hCD28 for 6 days under the indicated cytokine conditions. Cells were restimulated with PMA/ionomycin in the presence of monensin and stained for intracellular GATA3 and IL-4. Data are gated on live cells, and the polygon gate indicates a threshold of GATA3 expression based on basal levels of GATA3 staining in the neutralized condition. (B) The mean fluorescence intensity (MFI) was calculated for each CFSE division peak. (C) Data from (A) were gated on CFSE division 4 and assessed for expression of GATA3 and IL-4. I performed the experiments shown in this Figure.

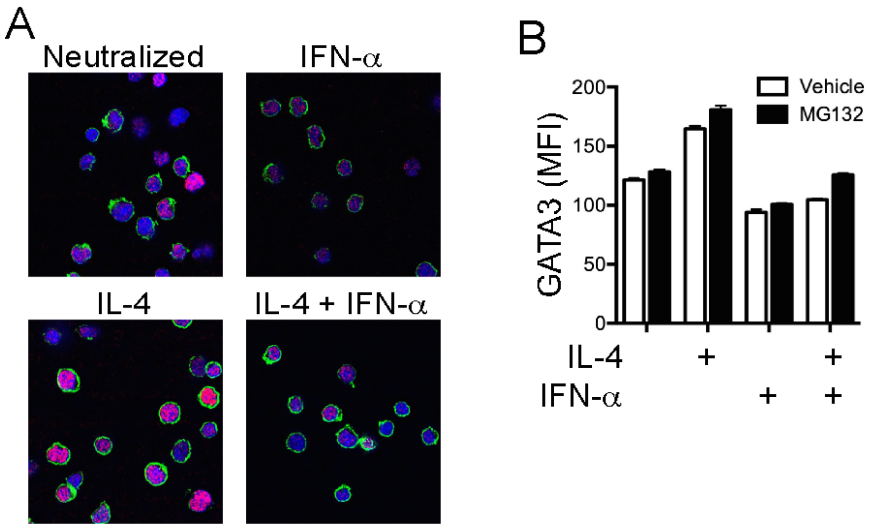


Figure 3.3: IFN-α/β-mediated suppression of GATA3 does not alter nuclei localization or stability of GATA3 protein. Purified human $CD4^+/CD45RA^+$ cells were activated for 5 days with plate-bound anti-hCD3/anti-hCD28 with the indicated cytokine conditions. (A) Cells were stained with DAPI (blue), anti-CD4 (green), and anti-GATA3 (red) Abs and visualized by confocal microscopy at an original magnification 40X. (B) Cells were incubated in the absence or presence of MG132, and intracellular GATA3 was quantified by intracellular staining. Dr. Jonathan P. Huber performed all of the experiments in this figure.

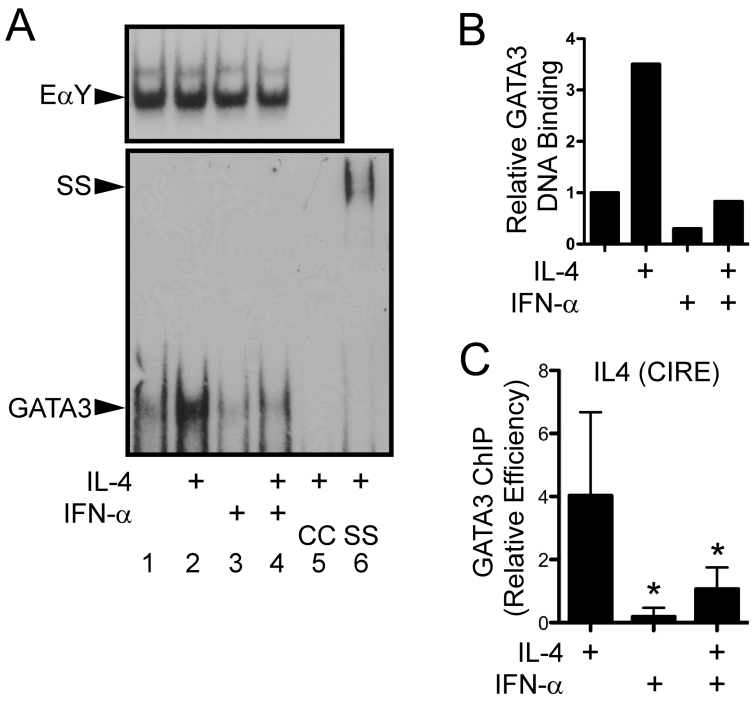


Figure 3.4: IFN-α/β inhibits GATA3 DNA-binding activity. Purified human $CD4^+/CD45RA^+$ cells were activated for 5 days with plate-bound anti-hCD3/anti-hCD28 with the indicated cytokine conditions. (A) GATA3 DNA-binding activity from nuclear extracts was measured by EMSA with a labeled consensus GATA3 DNA probe from the IL-4 conserved intronic regulatory element. Specificity controls included cold competitor (CC) and anti-GATA3 super shift (SS). The EaY box probe served as a loading control. (B) DNA-binding complexes in (A) were quantified by densitometric scanning. (C) GATA3 DNA binding at the IL-4 conserved intronic regulatory element was assessed by ChIP, and data are expressed relative to the neutralized control. *$p < 0.05$. Dr. Jonathan P. Huber performed both experiments presented in this figure.

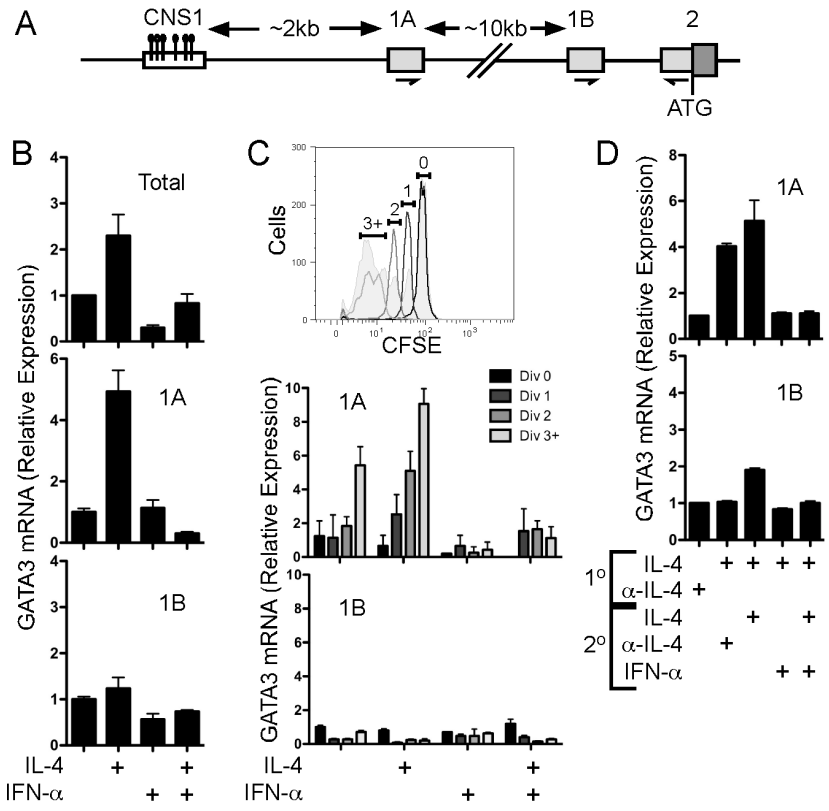


Figure 3.5: Progressive expression of GATA3 exon 1a and inhibition by IFN-α/β during cell division. (A) GATA3 transcription can proceed from two alternative first exons, 1a and 1b, each controlled by separate promoters. Arrows indicate the direction of primers to distinguish 1a from 1b by qPCR. The CNS-1 site is positioned ~5 kb upstream of exon 1a. (B) Purified human $CD4^+/CD45RA^+$ cells were activated for 3 days with plate-bound anti-hCD3/anti-hCD28 with the indicated cytokine conditions. GATA3 transcripts were quantified by qPCR with primers that measured total GATA3 spanning exons 5-6 (top panel) and with primers that distinguished exons 1a (middle panel) from 1b (bottom panel). (C) CFSE-labeled human $CD4^+/CD45RA^+$ cells were activated as above for 5 days, and individual cell divisions were sorted based on the gates shown (top panel). GATA3 mRNA transcripts derived from exon 1a (middle panel) and exon 1b (bottom panel) were quantified by qPCR and expressed relative to the neutralized Div 0 population. (D) Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 7 days under the indicated cytokine conditions for primary activation (1°). Cells were then washed and restimulated for an additional 7 days with the cytokine conditions indicated for secondary activation (2°). GATA3 exon 1a and 1b transcripts were quantified by qPCR. The experiments in this figure were performed by Dr. Jonathan P. Huber.

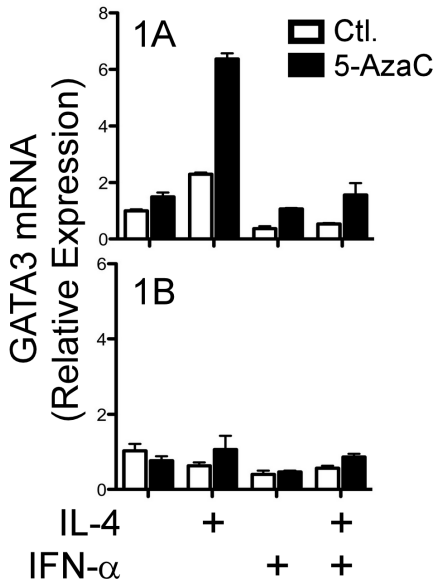


Figure 3.6: DNA methyltransferase activity does not mediate IFN-α/β-mediated GATA3 suppression. $CD4^+/CD45RA^+$ T cells were cultured for 5 days as above in the absence or presence of 5-Azacytidine (5-AzaC). GATA3 exon 1a (upper panel) and exon 1b (lower panel) mRNA transcripts were quantified by qPCR and expressed relative to the neutralized control without 5-AzaC treatment. Dr. Jonathan P. Huber performed this experiment.

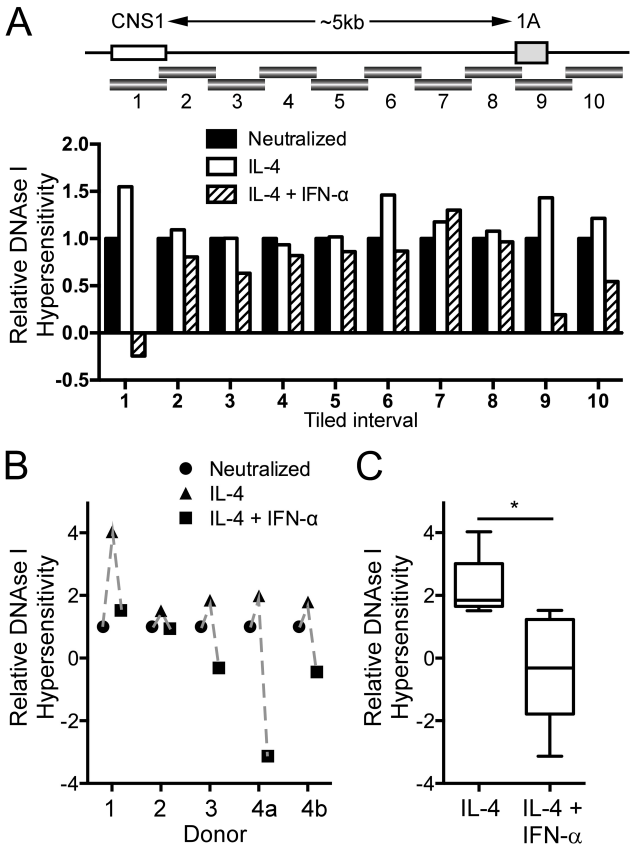


Figure 3.7: IFN-α/β signaling selectively decreases DNase I relative hypersensitivity at the GATA3 CNS-1 region and the exon 1a transcriptional start site. (A) Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. Cells were permeabilized and incubated with increasing concentrations of DNase I. DNA was purified, and semi-quantitative PCR analyses were performed with primers spanning 10 overlapping tiled intervals across the CNS-1/exon 1a region (gray bars). Amplicons were resolved by gel electrophoresis, and band intensities were quantified by densitometry using ImageJ software. The slope of the line formed by the reduction in amplicon as a function of DNase I concentration was calculated, and relative DNase I hypersensitivity was calculated as a ratio of slopes of neutralized versus cytokine treatment. (B) Quantitative DNase I hypersensitivity analysis of CNS-1 region was performed as described above from four healthy adult donors. Samples 4a and 4b are separate experiments performed on cells from the same donor. Relative hypersensitivity is referenced to the neutralized control of each donor/experiment. (C) Averaged data from (B) are expressed relative to the neutralized control. *$p < 0.05$. I performed all of the experiments presented in this Figure.

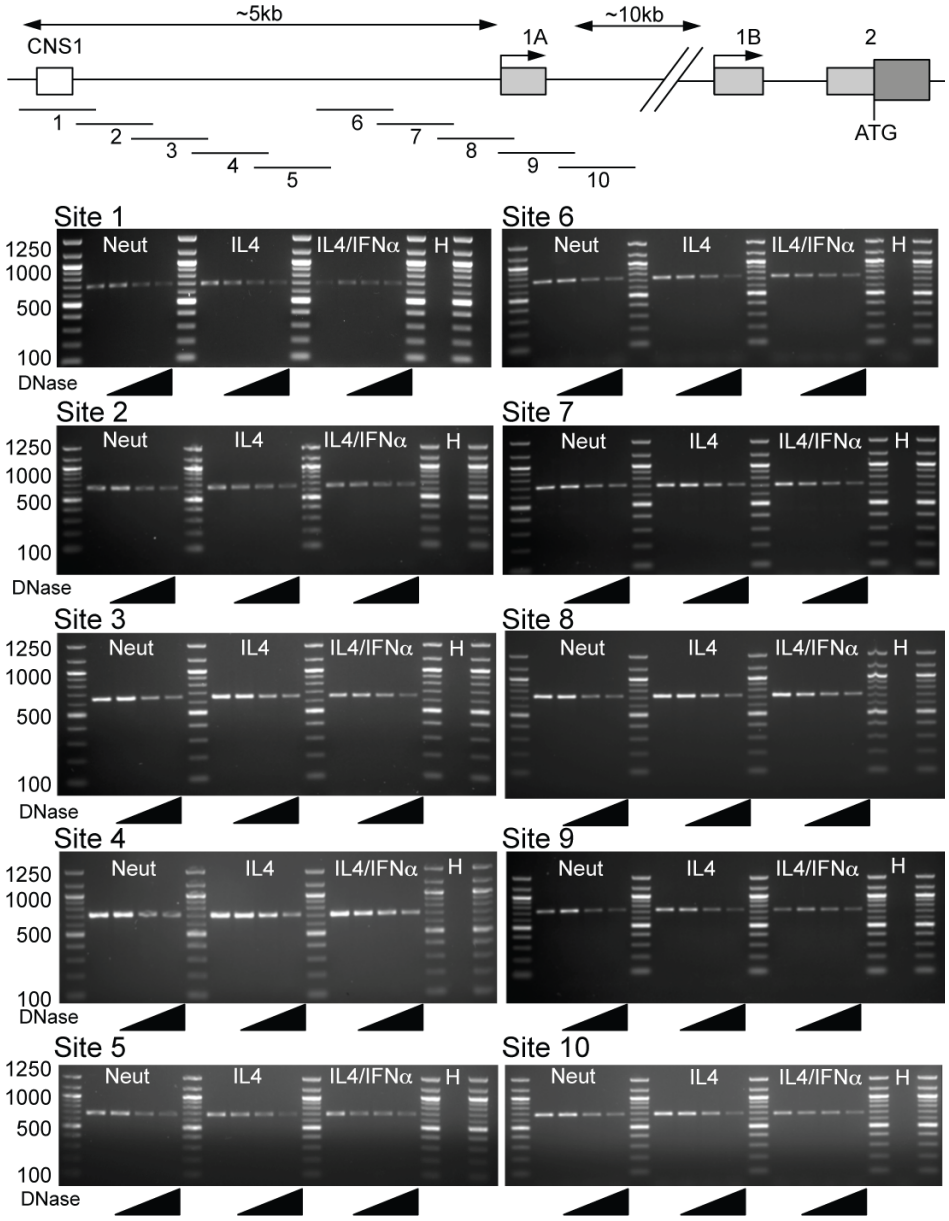


Figure 3.8: DNAse I hypersensitivity analysis of GATA3 CNS-1/exon 1a. Purified human $CD4^+/CD45RA^+$ T cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokines. Cells were permeabilized and incubated with increasing [DNase I]. DNA was purified, and semiquantitative PCR analyses were performed with primers spanning 10 overlapping intervals across the CNS-1/exon 1a region. Band intensities were quantified by densitometry. The slope of the line formed by the reduction of amplicon as a function of [DNase I] was calculated, and relative DNase I hypersensitivity was calculated as a ratio of slopes of neutralized versus cytokine treatment. Quantified data from this analysis are expressed in Figure 3.7A. I performed all of the experiments presented in this Figure.

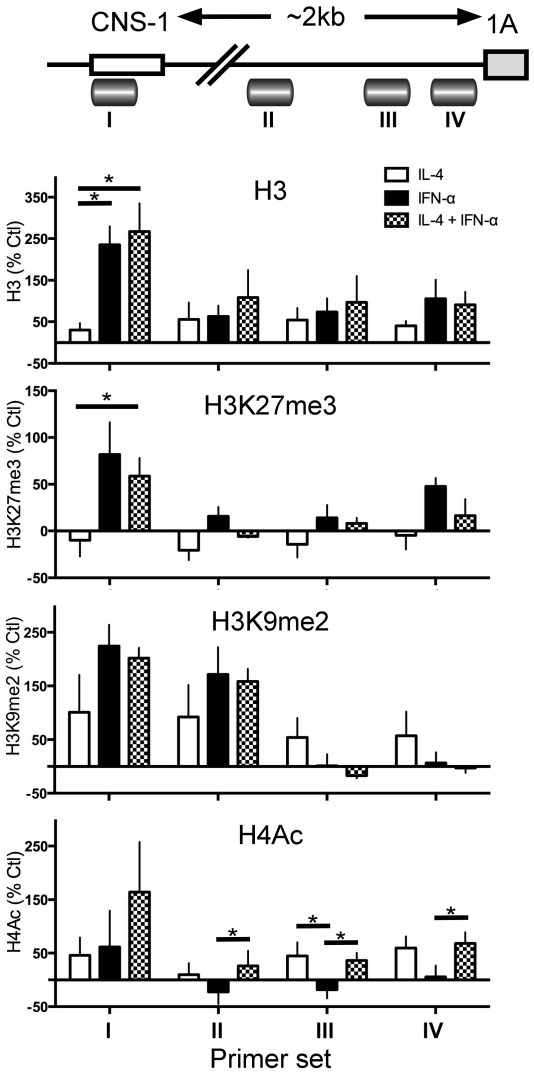


Figure 3.9: Epigenetic silencing of GATA3 CNS-1 in response to IFN-α/β. ChIP was performed with $CD4^+/CD45RA^+$ T cells activated for 5 days as described in the other figure legends, and the data are expressed as the percentage control of the ChIP efficiencies of each of the neutralized control samples. The relative density was assessed for total H3, H3K27me3, H3K9me2, and H4Ac within CNS-1 and three adjacent sites across ~1 kb of sequence upstream of GATA3 exon 1A. The four PCR primer sites are indicated by the gray bars beneath the diagram and are denoted I-IV. Data are averages from four or five independent healthy adult control blood donors. *$p < 0.05$. Both Dr. Huber and I performed the experiments presented here.

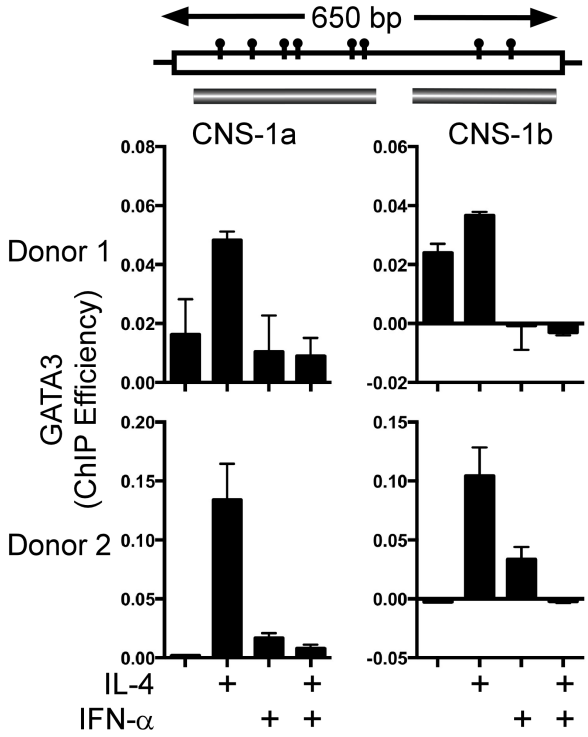


Figure 3.10: IFN-α/β signaling suppressed GATA3 CNS-1 binding. GATA3 DNA binding to the GATA3 CNS-1 region was assessed by ChIP in purified $CD4^+/CD45RA^+$ T cells activated for 5 days with the indicated cytokine conditions. The PCR primers spanned two adjacent regions (CNS-1a and CNS-1b) within the 650-bp CNS-1 site and are indicated by the gray bar in the upper diagram along with putative GATA3 consensus binding sites (flags). Experiments from two healthy adult blood donors are shown. *$p < 0.05$. Dr. Jonathan P. Huber performed the experiments shown.

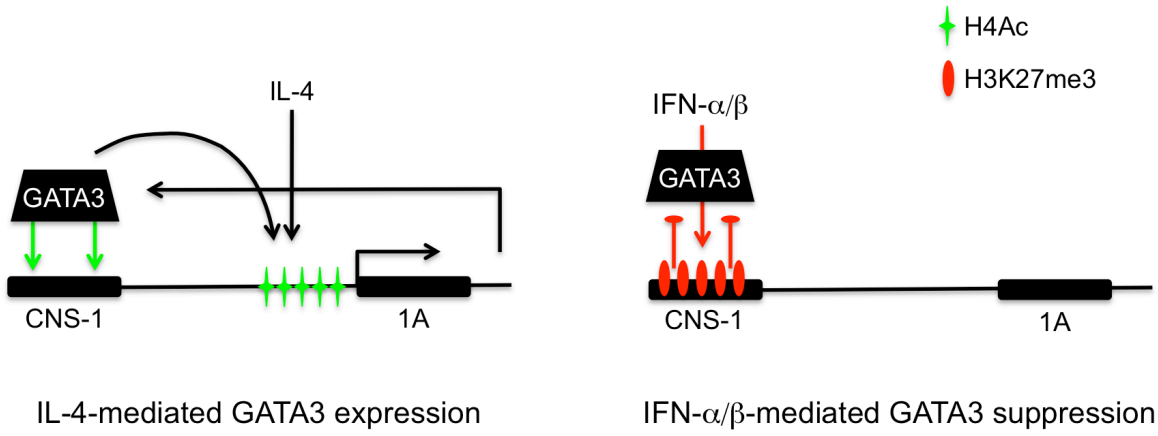


Figure 3.11: Positive and negative regulation of the GATA3 gene by IL-4 and IFN-α/β, respectively. IL-4 promotes H4 acetylation of the alternative exon 1a promoter region, whereas IFN-α blocks GATA3 accessibility to the CNS-1 region, which correlates with enhanced H3K27me3.

CHAPTER IV

ACUTE IFN-α SIGNALING ALTERS THE TRANSCRIPTOME PROFILE OF HUMAN TH2 CELLS

Introduction

Signaling cues driven by cytokines are critical in the instruction of how a T cell will respond to the environment. IFN-α/β signaling induces a specific transcriptome profile that is dominated by the upregulation of antiviral gene products that have been shown to play a role in controlling viral replication and enhancing peptide presentation (Crouse et al., 2015). In addition to effector molecules that act within the cell during a viral infection, IFN-α/β signaling also regulates the expression of other cytokines a cell produces; however, conflicting reports have been observed with regard to the effects of IFN-α/β on mediating cytokine production. In response to *in vitro* IFN-α/β and TLR stimulation, healthy human DCs have been shown to downregulate the expression of IL-1β, and IL-23p19, the specific subunit for IL-23, and upregulate the expression of IL-27, an IL-12-cytokine family member (Ramgolam et al., 2009). However, another study showed that IFN-α/β suppressed the production of IL-12p40 by human DCs as well as human $CD4^+$ T cell-expressed IFN-γ, TNF-α and LT in two week co-culture setting (McRae et al., 1998). The same study also demonstrated that IFN-α/β enhanced IL-10 production, but blocking IL-10 did not overcome the suppression of these T cell-produced cytokines, suggesting a more direct role for IFN-α/β-signaling in suppressing the expression of these cytokines, or the requirement of another secreted factor that remains unidentified in this

system (McRae et al., 1998). Despite these results, other groups have demonstrated the enhancement of both IL-10 and IFN-γ expression by $CD4^+$ T cells from healthy individuals, however, the enhancement of IFN-γ is acute and transient, especially compared to that of IL-12 treatment (Ramgolam et al., 2009; Schandené et al., 1996; Shibuya and Hirohata, 2005). Finally, the selective suppression of TCR-induced IL-5 and IL-13 expression has also been observed in response to IFN-α/β signaling in human PBMCs and purified $CD4^+$ T cells (Kaser et al., 1998; Pritchard et al., 2012b; Schandené et al., 1996; Shibuya and Hirohata, 2005). Collectively, these results have shown that *in vitro* treatment of human T cells or PBMCs with IFN-α/β selectively suppresses TCR-mediated IL-5 and IL-13 expression, while enhancing IL-10 and IL-4 expression. Despite enhanced IL-10 production, this mechanism was not mediated by autocrine signaling, as an IL-10 blocking antibody does not abrogate IFN-α/β-mediated IL-5 suppression (Schandené et al., 1996). Although these observations have demonstrated cytokine-specific regulation in response to IFN-α/β treatment, they have not looked specifically in memory Th2 cells, and have not characterized the transcriptional profile of these Th2 cells. Describing the effects of this cytokine on the Th2 population is important to more completely understand the *in vivo* effects of IFN-α/β during the treatment for disease.

The ability of IFN-α/β signaling to suppress the production of Th2 cytokines is documented in the literature, however, how this mechanism occurs is relatively unknown. The data I presented in Chapter 3 demonstrated this phenomenon through the negative regulation of the master transcription factor of Th2 cells, GATA3 (Huber et al., 2014). IFN-α suppressed IL-4-mediated GATA3 expression by selectively inhibiting the expression of the IL-4-sensitive splice variant induced by the distal non-coding exon 1a. This in turn suppressed the cells' ability to produce IL-4 upon restimulation. IFN-α treatment enhanced H3K27me3, and enhanced the

overall nucleosome content at an upstream conserved non-coding sequence within the GATA3 promoter (CNS-1). This induction of epigenetic silencing correlated with a reduction in GATA3 binding to the CNS-1 site in response to IFN-α, even when IL-4 was added to the cells. This study demonstrated that IFN-α signaling destabilizing human Th2 lineage commitment by preventing GATA3 auto activation during Th2 development.

As discussed in Chapter 1, naïve T cells require a distinct set of cues from the environment that lead to lineage-specific programming of the cell at the epigenetic level. I demonstrated in Chapter 3 how IL-4-mediated programming is altered in the presence of IFN-α signaling. In the current chapter, I demonstrate that IFN-α/β-mediated signaling discordantly suppresses the cytokine expression of human memory Th2 cells. Because these cells have already undergone lineage commitment and only require TCR stimulation to induce cytokine production, how they respond to IFN-α/β signaling is likely different compared to cells that have not completed STAT6- and GATA3-mediated programming when IFN-α is present. I hypothesized that IFN-α signaling alters the TCR-mediated cytokine expression of committed Th2 cells. To address this hypothesis, I utilized different pre-committed human Th2 populations to assess cytokine gene and protein expression in the presence of acute IFN-α treatment. These data demonstrate that IFN-α signaling mediates acute and robust effects on pre-committed human Th2 cells, which potentially parallels IFN-α-mediated epigenetic programming in a cell that is not yet committed to the Th2 fate.

Results

IFN-α/β selectively suppresses IL-5 and IL-13 expression

As discussed in the Introduction, a handful of reports have demonstrated that IFN-α is able to suppress protein expression of IL-5 and IL-13 from human $CD4^+$ T cells (Kaser et al., 1998; McRae et al., 1997; Schandené et al., 1996; Shibuya and Hirohata, 2005). As the majority of these studies looked at the effects of IFN-α on PBMCs, $CD4^+$/$CD45RA^+$ T cells or total $CD4^+$ T cells, I was interested in the effects within a pure population of $CD4^+$ T cells that have already been programmed in response to primary stimulation. As observed in the literature, I found that IFN-α treatment of PBMCs suppressed IL-5 and IL-13 protein and gene expression (Figure 4.1A, 4.1B). I was unable to detect acute IL-4 protein expression by ELISA from stimulated PBMCs, which has been reported previously (Pritchard et al., 2012b), however, there seemed to be no significant change in *IL4* gene expression in response to IFN-α treatment (Figure 4.1B). Further, IFN-α-mediated enhancement of *IFNG* expression was only seen at the mRNA level, but not at the protein level (Figure 4.1A and Figure 4.1B). It is important to note that the experiments depicted in Figures 4.1A and 4.1B were done with different donor cells, and there may be differential responsiveness to IFN-α that drives greater transient expression of *IFNG* in select donors.

It has been demonstrated that human $CD4^+$ T cells are relatively plastic in their ability to produce different cytokines (Ahmadzadeh and Farber, 2002; Geginat et al., 2014; Hegazy et al., 2010). To determine whether IFN-α-mediated suppression was specific to committed Th2 cells, I positively isolated human memory Th2 cells by Fluorescence Activated Cell Sorting (FACS)

from PBMCs and assayed TCR-mediated production of IL-5, IL-13, IL-4, and IFN-γ in the presence or absence of IFN-α. This population of memory Th2 cells was isolated using the prostaglandin D2 receptor, CRTH2, which is expressed exclusively on Th2 cells, ILC2, and granulocytes, and is a GATA3-sensitive gene (Mjösberg et al., 2011; Nagata et al., 1999a; 1999b; Quapp et al., 2007). Further, this population of cells has been shown to be enhanced in allergic diseases, including atopic dermatitis (Cosmi et al., 2000). IFN-α treatment suppressed TCR-mediated IL-5 and IL-13 protein expression 24 hrs-post stimulation of human $CD4^+CRTH2^+$ T cells, while enhancing IL-4 production (Figure 4.2). No detectible IFN-γ was produced from these cells (Figure 4.2). Alternatively, the non-Th2 $CD4^+$ memory cell population ($CD4^+CRTH2^-$) produced no detectible IL-5, and IFN-α did not alter the amount of IL-13 or IL-4 expression from this population of cells. Further, TCR-stimulated $CD4^+CRTH2^-$ cells produced all the detectable IFN-γ, however, I found that IFN-α had no effect on the production of this cytokine in the donors I utilized for these experiments (Figure 4.2).

Because human $CD4^+CRTH2^+$ memory Th2 cells from peripheral blood are less than one percent of the total $CD4^+$ T lymphocyte population, I utilized a larger population of cells to further interrogate this observation. I tested whether IFN-α treatment suppressed *IL5* and *IL13* gene expression in total memory $CD4^+$ T cells enriched using the maturation marker, CD45RO. Total human memory $CD4^+$ T cells were stimulated acutely with anti-hCD3 and rhIL-2 for 4 hrs in the presence or absence of IFN-α. In all our human donors, IFN-α suppressed *in vitro IL5* and *IL13* gene expression, while *IL4* expression was unaffected (Figure 4.3). Because this population contains different populations of $CD4^+$ memory cells, including Th1 and Th17 cells, and the fact that there could be contaminating APCs, I was interested in whether other cytokines were playing a role in suppressing *IL5* and *IL13*, which I was falsely attributing to IFN-α signaling. I

measured IFN-α-mediated gene suppression of *IL5*, *IL13*, *IL4* and *IFNG* in the presence or absence of neutralizing antibodies against IL-4, IL-12 and IFN-γ. Regardless of whether I neutralized these cytokines in the culture ('Neutralized') or left their ability to bind their respective receptors in tact ('Drift'), IFN-α was able to suppress *IL5* and *IL13* gene expression and had no affect on the expression of *IL4* (Figure 4.3). I measured *IFNG* expression in these cultures and found that IFN-α treatment modestly enhanced *IFNG* gene expression in the donors we utilized for these experiments, although it was only statistically significant in the 'Neutralized' conditions (Figure 4.3). Overall, the relative gene expression of the cytokines analyzed seemed to be higher in the cultures containing the neutralizing antibodies ('Drift' Figure 4.3, bottom panel). It may be the case that the other cytokines play a role in regulating overall TCR-mediated expression of these genes, but IFN-α is dominant over these other signaling cues, thus, you see the suppression of *IL5* and *IL13* expression regardless of whether the other signaling pathways are induced.

In addition to the IFN-α/β family, there are two other unique families of interferons, IFN-γ and IFN-λ. IFN-α/β and IFN-λ cytokines are both important in combating viral infections, and activate many of the same transcription factors, including the ISGF3, STAT1, STAT3, STAT4 and STAT5 (Dumoutier et al., 2004). Because of the similarities between signaling intermediates, and the promiscuity of STAT activation in response to each of these receptors, I wished to test whether IFN-γ or IFN-λ were able to suppress TCR-mediated *IL5* and *IL13* gene expression similar to IFN-α/β. By testing whether IFN-γ and IFN-λ could mediate *IL5* and *IL13* gene suppression, I could rule out the requirement for specific STAT species in mediating this regulatory mechanism. Interestingly, only IFN-α was able to negatively regulate IL-5 and IL-13 protein and gene expression; acute treatment of cells with IFN-γ or IFN-λ1 had no suppressive

effect on these cytokines (Figure 4.4A, 4.4B). As demonstrated previously, IL-4 protein and gene expression was not suppressed by IFN-α, and, interestingly, *IL4* gene expression was enhanced by all of the interferon types tested (Figure 4.4A, 4.4B). It was interesting that IFN-α, but not IFN-λ, induced gene suppression, because the signaling pathways induced by each of these cytokines is similar and classically dependent on the transcription factor STAT2. I measured myxovirus resistance A (*MXA*) gene expression, an interferon sensitive gene that is induced in response to IFN-α and IFN-λ in human hepatocytes (Doyle et al., 2006). IFN-λ1 treatment failed to enhance *MXA* expression (Figure 4.4C), while IFN-α potently enhanced gene expression. This experiment suggests that human memory $CD4^+$ T cells either lack a functional IFNLR, or in some fashion, do not induce signaling in response to receptor ligation. Indeed, reports have revealed that memory $CD4^+$ T cells are relatively unresponsive to IFN-λ as assessed by measuring the induction of ISGs, demonstrating the specificity of different cell types responding to the different interferon family members (Sommereyns et al., 2008; Witte et al., 2009). Together, these data demonstrate that only IFN-α/β is able to negatively regulate *IL5* and *IL13* expression, despite the similar signaling intermediates within each of the distinct families of interferons.

The human IFN-α/β family consists of 20 unique ligands, all of which bind the Interferon Alpha Receptor (IFNAR) that is expressed on all nucleated cells (Mogensen et al., 1999; Stark et al., 1998). This diversity of ligands is thought to be utilized in response to unique stimuli, as human pDCs stimulated with different PAMPs have been shown to alter the level of each of these ligands in a stimulation-dependent manner (Szubin et al., 2008). I wished to determine whether different IFN-α/β subtypes could induce *IL5* and *IL13* gene suppression similar to IFN-α(2a), the IFN-α/β subtype utilized in this study, as the IFNAR has varied affinity for the

different IFN-α/β ligands (Jaks et al., 2007; Kumaran et al., 2007; Thomas et al., 2011). Additionally, I was interested in the potency of these ligands in suppressing *IL5* and *IL13* gene expression. Human memory $CD4^+$ T cells were stimulated acutely in the presence or absence of three different IFN-α/β ligands, and *IL5*, *IL13*, *IL4* and *IFNG* gene expression was assessed by qPCR. Treatment of cells with IFN-α(2a), IFN-β and IFN-ω resulted in a similar negative regulation of *IL5* and *IL13* gene expression, and this suppression occurred in a dose-dependent manner (Figure 4.5). I found that all of the concentrations of different ligands we tested were able to suppress gene expression (10, 100, and 1000 U/ml, corresponding to 25.97, 259.7 and 2597 pg/ml, respectively), while enhancing *IFNG* expression and having limited effects on *IL4* (Figure 4.5). It has been shown that human pDCs produce abundant IFN-α/β in response to stimulation; for example, pDCs challenged with influenza virus *in vitro* produce up to 4000 pg/ml in a 36 hour period (Gill et al., 2010). Furthermore, IFN-α concentrations can reach up to 150 pg/ml in nasal lavage fluid 2 days post-experimental influenza A (H1N1) challenge in healthy individuals (Hayden et al., 1998). Thus, it may be the case that the concentration of IFN-α/β is relatively high at the immunological synapse, for example, during DC-T cell interactions. These data demonstrate that multiple IFNAR ligands are able to suppress TCR-mediated *IL5* and *IL13* gene expression, and this effect occurs in a dose-dependent manner.

The IFN-a-induced transcriptome profile of human in vitro polarized Th2 cells

The IFN-α/β-induced gene expression profiles of many different cell types have been documented and are widely available for analysis (Barrett et al., 2013; Schneider et al., 2014). For example, the Gene Expression Omnibus (GEO) database has 1063 available DataSets for

download when ‘interferon alpha’ is used to search all human database entries. Many different populations of cells have been used in these experiments, including cancer and normal cell lines, as well as primary myeloid, lymphoid and organ cells from diverse species. Yet, there is only one documented study in GEO that used microarray analysis to determine the effects of IFN-α treatment during human T cell polarization (Touzot et al., 2014). This study demonstrated that when IFN-α was present during Th1, Th2, or Th17 polarization for 5 days, the ISG profiles of each of the populations was unique, and Th2 and Th17 cells were less responsive at inducing the canonical ISGs (Touzot et al., 2014). To my knowledge, no studies have reported the acute effects of IFN-α on the transcriptome in pre-committed human Th2 cells. I was interested in determining what genes were differentially expressed in response to acute IFN-α/β, other than the classical ISGs, as well as how TCR stimulation modified this response. I performed mRNA sequencing analysis on RNA isolated from *in vitro* polarized Th2 cells from two individual healthy donors, one of each gender. *In vitro* polarized Th2 cells were acutely stimulated with anti-hCD3 and rhIL-2 in the presence of absence of IFN-α for 4 hours (Figure 4.6A). Four different treatments were assessed: 1) No TCR Drift, 2) No TCR +IFN-α, 3) TCR Drift, and 4) TCR +IFN-α. Of the 19,264 genes that had detectable FPKM values, genes with FPKM ≥ 8 and had at least a two-fold change between two or more treatments were utilized in a cluster analysis. All 5,002 genes within each treatment, as well as all treatments within each gene, were mean-centered and assessed by hierarchical clustering (Figure 4.6B). The data are represented on the heatmap using a log2 scale (Figure 4.6B). Genes in yellow are enhanced, while genes in blue are repressed. I observed that all ‘No TCR’ treatments from both donors clustered together, while the ‘TCR’ treatments from both donors clustered together (Figure 4.6B). Based on a cursory analysis of the heatmap itself, it seems that the transcriptome profile of cells that received IFN-α in the

absence of TCR stimulation are more dissimilar between the two donors compared to the transcriptome profile of cells that received IFN-α in the *presence* of TCR stimulation. Finally, as expected, there are also sets of clustered genes that seem to be enhanced/repressed specifically in response to TCR stimulation or IFN-α treatment (Figure 4.6B).

To determine which of the differences seen in the hierarchical clustering were statistically significant in both donors, I performed four individual analysis using CuffDiff: 1) 'No TCR Drift' versus 'TCR Drift', 2) 'No TCR +IFN-α' versus 'TCR +IFN-α', 3) 'No TCR Drift' versus 'No TCR +IFN-α', and 4) 'TCR Drift' versus TCR +IFN-α'. Based on a false discovery rate of < .05, the total number of differentially expressed genes that were significant in both donors within each analysis can be found in Table 4.1. Genes with at least a two-fold change in expression were further utilized for analysis (Table 4.2). Based on the statistics within the tables alone, it is apparent that there are a greater number of genes whose expression is altered in response to TCR stimulation than to IFN-α treatment. As expected, there are a greater number of genes that are enhanced in response to IFN-α treatment compared to genes that are suppressed, regardless of TCR stimulation. Finally, there are a larger number of genes regulated in response to IFN-α treatment when TCR stimulation is *absent* (1604 genes) compared to when TCR stimulation is *present* (371 genes) (Table 4.2). The fact that human T helper cell subtypes respond differently to IFN-α treatment in the presence or absence of TCR stimulation has been reported before, however, the experimental design was different than what I utlized in this study (Touzot et al., 2014). Due to this surprising difference in total genes regulated by IFN-α in the absence or presence of TCR stimulation, I was interested in which genes were similarly up- or down-regulated between these two scenarios. By plotting the data using a Venn diagram, I found that a number of genes were unique to each comparison (Figure 4.7). 117 upregulated and 42

downregulated genes were shared when 'No TCR-Drift vs. No TCR +IFN-α' genes were compared to 'TCR-Drift vs. TCR +IFN-α' genes. To get an idea of the global functions of these genes, I performed an ontology analysis using WebGestalt and assessed grouping patterns by Gene Ontology, KEGG, and Pathway Commons databases on all six of these groups: 1) up- and down-regulated genes that were shared, 2) up- and down-regulated genes that were unique to the 'No TCR' group, and 3) up- and down-regulated genes that were unique in the presence of TCR stimulation. The data presented in Figures 4.8 and 4.9 show the overall functions of the genes that are commonly up- and down-regulated by IFN-α in the presence or absence of TCR stimulation, respectively. As expected, the similar upregulated genes are involved host defense, IFN-α/β signaling, and innate-type responses to pathogens, and include *MXA*, *OAS1*, *ISG15*, and *STAT2* (Figure 4.8, Table 4.3). However, the shared genes that are downregulated in response to IFN-α seem to be more diverse in their biological functions. Based on Gene Ontology, KEGG, and Pathway Commons, genes involved in cell activation and different signal transduction pathways are suppressed in response to IFN-α, regardless of whether TCR stimulation is present (Figure 4.9). Examples of these genes include *SOX4*, *IRS2*, *CD27*, *CD101*, *CD5*, *IL16*, *CCR8* and *IL4RA*. The full lists of shared up- and down-regulated genes can be found in Tables 4.3 and 4.4, respectively.

In addition to the common genes that are regulated by IFN-α regardless of TCR stimulatory status, a number of genes are uniquely regulated in the presence or absence of TCR stimulation. Up- and down-regulated genes specific to the 'No TCR Drift vs. No TCR +IFN-α' are listed in Tables 4.5 and 4.6, while up- and down-regulated genes specific to the 'TCR Drift vs. TCR +IFN-α' treatment are listed in Tables 4.7 and 4.8, respectively. I wished to determine what sorts of functions these unique genes had, so I used the Gene Ontology database to define

the scope of function of these gene lists. Genes that were upregulated in response to IFN-α in the *absence* of TCR stimulation included genes involved in general pathways, including 'immune response', 'innate immune response, and 'apoptotic processes' (Figure 4.10, top panel), while downregulated genes fell into categories including 'cell signaling', 'cell communication', and migration signaling pathways (Figure 4.10, bottom panel). Comparatively, genes regulated by IFN-α only in the *presence* of TCR stimulation were categorized differently. Upregulated genes included those involved in 'cell proliferation', regulation of cellular processes and stimuli, as well as cytokine production (Figure 4.11, top panel), while downregulated genes were classified as those involved in the regulation of the immune response and immune system processes, as well as general immune responses (Figure 4.11, bottom panel). This qualitative analysis of the data demonstrates a unique role for IFN-α signaling in altering Th2 cell function in the presence or absence of TCR stimulation. In addition to the expected ISG enhancement, a number of genes have been identified that are not commonly associated with ISGs, and perhaps are specific to Th2 cells.

Targeted Analysis of Gene Expression Using RNA-Sequencing

In addition to utilizing a non-biased approach to look at the patterns of gene regulation in response to TCR stimulation and/or IFN-α treatment, I was interested in looking at a specific set of genes in a hypothesis-driven manner, and comparing these results to what I have seen in total human $CD4^+$ memory T cells treated with acute TCR and IFN-α stimulation (Figure 4.3). In the RNA-Seq experimental design (Figure 4.6A), I utilized *in vitro* polarized Th2 cells to obtain enough cells for both this experiment as well as a ChIP-Seq experiment described in Chapter 5.

Studies have observed differences between *in vitro* and *in vivo*-generated memory cells, and have hypothesized that signal strength and abundance of IL-2 may play a role in inducing these differences (Deenick et al., 2003; Fazekas de St Groth et al., 2004). As demonstrated earlier in this chapter, *in vivo*-generated total memory $CD4^+$ T cells, as well as purified $CRTH2^+CD4^+$ T cells treated with IFN-α produced less TCR-induced IL-5 and IL-13, with an enhancement of IFN-γ in bulk memory $CD4^+$T cells, and no change in IL-4 production (Figure 4.2 and 4.3). I wished to determine whether these genes behaved similarly in this *in vitro* polarized population of Th2 cells. I analyzed genes that fell into four categories 1) canonical ISGs, 2) TCR-enhanced genes and general activation markers, 3) Th2-associated genes, and 3) Th1-associated genes and other cytokine genes (Figures 4.12-4.15). Canonical ISGs, TCR-enhanced genes and general activation markers were utilized as controls within the experiment to verify the response to acute IFN-α treatment and TCR-mediated activation. Th1- and Th2-associated genes were analyzed to assess i) enhancement in response to TCR stimulation, and ii) how IFN-α treatment mediated expression. There was no expression of Treg- or Th17-associated genes (except IL-10 expression), thus, they were not included in this analysis. As expected, acute IFN-α treatment enhanced the expression of canonical ISGs, regardless of whether TCR stimulation was present (Figure 4.12). These representative genes demonstrate the potency of acute IFN-α signaling after 4 hours of treatment; the expression of these genes is at least 5-fold enhanced compared to cells that did not receive IFN-α (Figure 4.12). Enhanced ISG expression in response to acute IFN-α has also been observed in the experiments in which I utilized bulk memory $CD4^+$ T cells as well (Figure 4.4C), demonstrating the similar IFN-α-mediated antiviral response within different cell types. Next, I compared TCR- and IFN-α-mediated regulation of genes associated with T cell activation and global population status, for example, naïve, central memory or effector memory

populations. TCR stimulation enhanced *IL2*, *CD25*, *CD69* expression, but did not alter the expression of *CCR7*, *CD62L* or *CD44* (Figure 4.13A and 4.13B). Further, these cells expressed *CD25*, *CCR7*, *CD62L* and *CD44* prior to TCR stimulation, which is indicative of cues provided from primary stimulation and T cell polarization. In the presence of acute IFN-α treatment, *IL2* and *CD69* gene expression were enhanced (Figure 4.13A), which has been previously reported in the literature (Davis et al., 2008; Deblandre et al., 1992; Dondi et al., 2003). Finally, there was no change in *CCR7*, *CD62L* or *CD44* expression in response to acute IFN-α signaling (Figure 4.13B). The findings presented in Figure 4.13 demonstrate that these *in vitro* polarized Th2 cells responded acutely to TCR stimulation, with both donors responding in a similar fashion.

I was interested in how Th2-associated genes responded to acute IFN-α treatment in these *in vitro* polarized Th2 cells compared to that of the *ex vivo* memory $CD4^+$ T cells whose TCR- and IFN-α-mediated responses have been studied by qPCR (Figure 4.3-4.5). *GATA3* expression was suppressed by acute IFN-α treatment in the presence of TCR stimulation, while *IRF4* and *STAT6* expression was unchanged (Figure 4.14A). This suppression of *GATA3* expression in response to IFN-α was surprising because in total memory $CD4^+$ T cells, I have observed no change in gene expression by qPCR (Figure 4.16A). This finding suggests inherent differences might exist between the *in vitro* Th2 cells compared to the *ex vivo* population of Th2 cells, or that the population of total memory $CD4^+$ T cells is so diverse and has so few Th2 cells that I am unable to detect changes in GATA3 expression in response to IFN-α signaling. Further, I looked at the response of cytokine gene expression and other Th2-associated membrane-bound proteins, and found that IFN-α failed to suppress acute gene expression of *IL4*, *IL5*, *IL13* or *IL9* (Figure 4.14B). Additionally, there was relatively low enhancement of these genes in response to TCR stimulation, which might account for why these cells do not seem to be sensitive to IFN-α

signaling compared to *ex vivo* memory Th2 cells (Figure 4.2 and 4.3). This could be due several technical issues with the experimental design. First, it may be the case that only a fraction of the cells within the *in vitro* polarized cells became bona fide Th2 cells, which might reduce our ability to detect *IL5* and *IL13* gene suppression in response to acute IFN-α treatment. Further, it could be the case that these cells were incompletely rested prior to acute restimulation. On day 6, *in vitro* polarized cells were washed once and plated in fresh media containing no exogenous cytokines for 16 - 18 hours prior to restimulation. Although the gross morphology of the cells looked similar to 'resting' T cells, it may be the case that additional time was needed to see a more robust response to anti-hCD3/rhIL-2-mediated stimulation. In addition to the overall lack of Th2 gene expression in response to acute TCR stimulation, the abundance of transcripts was quite low. *IL4* transcript expression induced by TCR stimulation reached just above 3 FPKM for one donor, which is difficult to distinguish from background signal. Further analysis with more donors using qPCR will be important to test the reproducibility of this data. Finally, I was interested in other genes that play a role Th2 cell function, thus, I specifically looked at the effect of acute IFN-α signaling on *IL4RA*, *OX40*, *CCR4* and *CCR8*. *OX40* is has been shown to play a role in driving allergen-specific Th2 development and inflammation, while the expression of *CCR4* and *CCR8* have been reported to be expressed on different inflammatory Th2 populations (Burrows et al., 2014; Endo et al., 2014) Interestingly, *IL4RA* and *CCR8* expression were suppressed in response to IFN-α, regardless of whether TCR stimulation was present, however, the overall number of transcript reads of *CCR8* was relatively low (Figure 4.14B). *OX40* expression was only suppressed by IFN-α signaling in the presence of TCR stimulation (Figure 4.14B). Finally, there was no significant change in *CCR4* expression in response to TCR stimulation or IFN-α treatment (Figure 4.14B).

Based on the lack of TCR-mediated induction of the Th2 cytokine genes, I was interested in whether IFN-α signaling regulated Th1-associated genes in this cell population. Although there was no addition of exogenous IL-12, it has been observed that a unique GATA-3/T-bet double positive population of cells is produced from a Th2 population in response to IFN-α, IFN-γ, and IL-12 or *in vivo* LCMV infection that can co-produce Th1 and Th2 cytokines (Hegazy et al., 2010). Further, it is also that case that not every naïve $CD4^+$ T cell will respond to clonal TCR stimulation and IL-4 equally, and the accessibility of genes associated with non-Th2 cell types might be more accessible in response to TCR- and IFN-α-induced signals. Thus, I examined how acute TCR stimulation and IFN-α treatment regulated the expression *TBX21*, *STAT4*, *IL12RB2*, *IL10*, *IFNG* and *TNF* (Figure 4.15). Interestingly, there was relatively high induction of *TBX21* expression upon TCR restimulation as well as a modest but statistically significant enhancement of IFN-α-mediated enhancement in the absence of TCR stimulation (Figure 4.15). This was surprising because work from Dr. Ramos has demonstrated that IFN-α is insufficient at driving stable T-bet expression in human $CD4^+$ T cells (Ramos et al., 2007). Because there is very little expression of *TBX21* in the absence of TCR stimulation, enhanced gene expression seems to be in response to secondary TCR stimulation. Furthermore, *IL12RB2* expression was also enhanced in response to TCR stimulation and IFN-α treatment in both the absence and presence of TCR stimulation (Figure 4.15). However, there should be no IL-12 in these cultures, so it is unlikely that during the acute stimulation, exogenous IL-12 would be driving *TBX21* expression. It may be the case that this gene is accessible by TCR-mediated transcription factors, but the mRNA is only transiently expressed. In addition to enhancing *TBX21* and *IL12RB2* expression, acute TCR stimulation enhanced *IFNG*, *TNF* and *IL10* production, while IFN-α enhanced *IFNG* and *IL10* expression only in the presence of TCR

stimulation (Figure 4.15). IFN-α-mediated enhancement of *IFNG* and *IL10* gene expression has been reported by other groups (Aman et al., 1996; Brinkmann et al., 1993; Ziegler-Heitbrock et al., 2003). Taken together, this data suggest that acute IFN-α signaling acutely modifies the Th2-gene profile, skewing this heterogeneous population towards a more Th1/IL-10-positive phenotype. It is important to note that the RNA-Seq data presented here only represents the gene profiles of *in vitro* polarized Th2 cells from two individual human donors. It will be important that more donors be utilized for RNA-Seq and/or gene expression verification using qPCR.

Discussion

The data presented in this chapter demonstrate how human Th2 cells respond to acute IFN-α/β signaling. When I utilized PBMCs for the preliminary experiments to see whether I could repeat the observations in the literature, a similar trend was observed; IFN-α suppressed the expression of IL-5 and IL-13, but not IL-4 (Figure 4.1A and 4.1B). This observation can also be clearly observed in a purified population of memory Th2 cells, demonstrating that memory Th2 cells are directly responding to IFN-α (Figure 4.2). Because of how small of a population human purified Th2 cells are, I had technical difficulties obtaining enough cells to test more than two or three conditions as a time. Thus, I tested whether total memory $CD4^+$ T cells could be regulated in the same manner. I expected this to be the case, as I was able to see the same observation in whole PBMCs. Indeed, memory $CD4^+$ T cells, which contain the memory Th2 population, responded to IFN-α by suppressing the expression of IL-5 and IL-13, but not IL-4 (Figure 4.3).

Due to heterogeneity of the total memory $CD4^+$ T cell population, I measured the expression of *IFNG* expression, as we expected the majority of cells to be Th1 cells. Further, using *IFNG* as a control gene demonstrated the specificity of IFN-α to suppress specific genes, while enhancing others. I found that IFN-α enhanced *IFNG* expression, however, this upregulation was not consistently seen in all the donors (Figure 4.3). The enhancement of IFN-γ expression in response to IFN-α/β has been observed previously in response to STAT4 activation (Cho et al., 1996; Ramos et al., 2007; Rogge et al., 1998). The experiments presented here speak to the acute nature of IFN-α-enhanced *IFNG* expression. When we measured *IFNG* gene expression just 4 hours after treatment, we saw enhanced expression, however, when we waited

to measure IFN-γ protein 24 hours post-treatment, IFN-α treatment did not enhance IFN-γ production. It is likely the case that by the 24-hour time-point, STAT4 is no longer actively enhancing IFN-γ gene expression by binding to the *IFNG* promoter. When STAT4 phosphorylation is measured in bulk $CD4^+$ human memory T cells, phosphorylated STAT4 levels peak around 3 hours post-IFN-α treatment, which is completely abolished 6 hours post-IFN-α treatment (Ramos et al., 2007). Although this experiment looked at the effects of IFN-α in the absence of TCR stimulation, it still gives provides an example of the acute nature of IFN-α-mediated STAT4 phosphorylation.

It is surprising to me that IL-4 expression was not suppressed in response to IFN-α treatment. The Th2 cytokine genes exist within the Th2 cytokine gene locus that is approximately 140 kb in length. However, the *IL5* gene is separated from the *IL4* and *IL13* genes by the ~90 kb DNA repair gene, *RAD50*. It has been proposed that the active Th2 cytokine locus loops *RAD50* away from the transcriptional hub, whereby these genes can be coordinately regulated in trans (Lee et al., 2006; Spilianakis and Flavell, 2004). However, our findings and others demonstrate that the Th2 cytokine genes can be discordantly regulated (Bohjanen et al., 1990; Kelso et al., 1999; Sewell and Mu, 1996; Tanaka et al., 2011; Van Stry and Bix, 2011). Tanaka *et al* proposed an alternative model of transcriptional regulation whereby transcription factors, such as GATA3, independently regulate each gene by binding specific regulatory elements within each gene locus when activated to do so (Tanaka et al., 2011). It could be the case that both of these models can explain gene regulation; the Th2 cytokine genes can be coordinately regulated, such as in the context of TCR stimulation, or discordantly regulated, for example, in the context of acute cytokine cues, such as the observation I report here. Furthermore, work by Flavell and colleagues have demonstrated that interactions between distant

gene loci can impact the expression of the Th2 cytokine genes (Spilianakis and Flavell, 2004; Spilianakis et al., 2005). Further, Urwin *et al* demonstrated that a distant cis element 6.4 kilobase upstream of the human *IL5* gene is required for this gene to be maximally expressed in response to TCR stimulation (Urwin et al., 2004). Such long-distance chromosome interactions could play a role in IFN-α-mediated gene suppression, and might be important for how *IL5* and *IL13* are negatively regulated, but *IL4* is not.

Human populations of Th2 cells have been observed to be relatively heterogeneous, depending on the context of primary stimulation. IL-5-producing Th2 cells are thought to be the most differentiated and pathogenic in allergic diseases (Endo et al., 2014; Upadhyaya et al., 2011). Further, different programming cues can induce the development of Th2 cells capable of producing IFN-γ or IL-17 as well (Endo et al., 2014). A unique population of cell identified as 'super Th1 cells' has been reported to produce both IFN-γ and IL-13 in response to TCR stimulation and IL-18 (Nakahira and Nakanishi, 2011). We found that the $CRTH2^{-}CD4^{+}$ cells that produced IL-13 were unresponsive to IFN-α-mediated suppression (Figure 4.2). Thus, it could be the case that distinct populations of T cells are more permissive to IFN-α-mediated suppression, especially cells that have been epigenetically programmed to produce IL-5 in response to TCR stimulation. Furthermore, in the presence of TCR stimulation, IL-33 has been shown to act directly on human T cells and enhance the expression of IL-5 and IL-13, but not IL-4 (Kurowska-Stolarska et al., 2008). This mechanism of enhancement seems to be independent of STAT6 or GATA3 expression in mice (Kurowska-Stolarska et al., 2008). It is interesting that IL-5 and IL-13 are directly enhanced by the innate cytokine IL-33, and can also be suppressed by IFN-α. IFN-α-mediated suppression can be detected just 4 hours of the cells receiving treatment, thus, perhaps this mode of regulation is meant to suppress the 'innate' type responses that induce

swift *IL5* and *IL13* expression. It would be interesting to determine whether IL-33-enhancement is blocked by IFN-α, and whether IFN-α can suppress ILC2s from producing IL-5 and IL-13 as well.

The data presented in Figure 4.4 and 4.5 demonstrate that IFN-α/β, but not IFN-γ or IFN-λ, is able to induce *IL5* and *IL13* gene suppression. Further, multiple ligands that activate the IFNAR-mediated signaling pathway induce similar negative regulation of these genes in a dose dependent manner. It was interesting to observe that IFN-λ was unable to enhance *MXA* gene expression in total human memory $CD4^+$ T cells. Although most reports demonstrate that IFN-λ does not induce ISG expression in the $CD4^+$ T cell compartment (Freeman et al., 2014; Sommereyns et al., 2008; Witte et al., 2009), one group has observed that human memory $CD4^+$ T cells treated with IFN-λ in the presence of TCR stimulation have reduced IL-13 expression compared to cells that did not receive cytokine treatment (Dai et al., 2009). It is unclear how they observed this responsiveness, and the only obvious difference between my culture setup and their methods is the type of TCR stimulation utilized. In my experiments, I stimulated total memory $CD4^+$ T cells with anti-hCD3 and rhIL-2, while they utilized stimulatory beads containing anti-hCD2, anti-hCD3 and anti-hCD28. However, there is no indication in the literature that co-stimulation through CD2 or CD28 enhances IL-28Rα expression. An alternative explanation is that the cell purity is reduced in the Dai *et al* study, which could include myeloid-lineage cells that produce IFN-α/β. Despite the lack of IFN-λ-mediated *MXA* enhancement, I did see that *IL4* gene expression was enhanced above TCR-stimulated-levels in response to IFN-λ, similar to levels seen in response to IFN-α treatment (Figure 4.4B). It is unclear why *IL4* expression is enhanced in response to these antiviral cytokines. Perhaps the non-classical signaling pathways induced in response to IFN-α/β and IFN-λ enhance *IL4* gene expression. The proto-oncogene c-

Maf has been shown to enhance IL-4 expression in mouse $CD4^+$ T cells (Ho et al., 1996; Kim et al., 1999), and has been shown be enhanced by IFN-α treatment in total human memory $CD4^+$ T cells (Shibuya and Hirohata, 2005). It may be the case that IFN-α, and perhaps IFN-λ, is able to induce the expression of c-Maf, thereby enhancing *IL4* expression, while only IFN-α/β is also able to suppress *IL5* and *IL13* gene expression. It is not known how c-Maf is enhanced in response to IFN-α/β. If this process occurs through one of the non-classical signaling pathways that involve PI3K or p38 activation (Platanias, 2005), perhaps there is a lack of classical STAT activation by IFN-λ signaling, while other non-classical IFN signaling pathways are intact, but this remains to be proven.

To get a more broad idea of how IFN-α/β signaling was altering the transcriptome profile of human Th2 cells, we utilized RNA sequencing to identify all of the genes that are regulated in response to acute IFN-α treatment, and determine how TCR stimulation changes the way a Th2 cell responds to this antiviral cytokine. We identified many genes that were up- or down-regulated in response to IFN-α that were conserved or unique in the presence or absence of TCR stimulation. In general, commonly upregulated genes were those associated with the antiviral response, while commonly downregulated genes were associated with T cell activation (Figures 4.8 and 4.9). The genes that were uniquely up- or down-regulated to IFN-α treatment in the absence or presence of TCR stimulation are considerably more varied, and are described in Figures 4.10 and 4.11, respectively. It is important to note that this experiment was conducted with two donors, so it will be important to verify these changes in gene expression with more donors using qPCR and/or further RNA-Sequencing. Further, there seem to be differences between how the total memory $CD4^+$ T cell population responds to IFN-α compared to *in vitro* polarized Th2 cells. Both populations are heterogeneous, which means it is unclear if bona fide

Th2 cells are responsible for the gene trends we are seeing, or whether other T cell population are. For example, I showed that CRTH2$^+$CD4$^+$ T cell do not make an detectable levels of IFN-γ protein expression in response to TCR stimulation, whereas the CRTH2$^-$CD4$^+$ population made abundant levels of this cytokine (Figure 4.2). Thus, it may be the case that non-Th2 cells within the heterogeneous population are up- and down-regulating specific genes that are not regulated in Th2 cells. Additionally, it is difficult to compare the gene expression profiles from the RNA-Seq data to that of the memory CD4$^+$ T cell qPCR data because of the differences we see in response to TCR stimulation. The memory CD4$^+$ T cells are potently responsive to TCR-mediated cytokine production, for example, *IL5*, *IL4* and *IL13* were enhanced in response to TCR stimulation within 4 hours (Figure 4.3), whereas only *IL4* and *IL13* genes were enhanced above the 'No TCR - Drift' control in the RNA-Seq experiments (Figure 4.14B). Furthermore, *IL5* and *IL13* expression was not significantly suppressed in response to IFN-α in the RNA-Seq data, whereas all the donors we utilized in the qPCR experiments of total memory CD4$^+$ T cells were potently suppressed (Figure 4.3). It may be useful to alter the experimental design to either perform RNA-Seq on purified CRTH2$^+$CD4$^+$ cells from either peripheral blood or *in vitro* polarized Th2 cells. This population would respond more homogeneously to both TCR stimulation and IFN-α treatment, and would likely reduce the background of other genes that are not actually being regulated in Th2 cells.

It was interesting to see that so many more genes were differentially regulated by IFN-α in the absence of TCR stimulation compared to the presence of TCR stimulation. Perhaps the signals received through the T cell receptor override the innate signaling cues provided by IFN-α signaling. Once CD4$^+$ T cells are programmed during a primary challenge, they are able to do a finite number of effector functions to combat a specific 'non-self' target. Perhaps preventing a

comprehensive ‘antiviral’ response allows these cells to function more appropriately upon restimulation with cognate antigen in the manner in which they were programmed. Overall, it is clear that IFN-α/β-responsiveness is not the same in different cell types, including $CD4^+$ T cells that have undergone lineage commitment in response to primary stimulation. An additional example of this observation has been demonstrated in a recent study, in which human $CD4^+$ T cells polarized under Th1, Th2 or Th17 conditions in the presence or absence of IFN-α were probed to deteremine how the polarizing cytokines altered the antiviral gene profile (Touzot et al., 2014). Indeed, it was noted that Th2 and Th17 polarization in the presence of IFN-α upregulated fewer antiviral genes compared to Th1 cells.

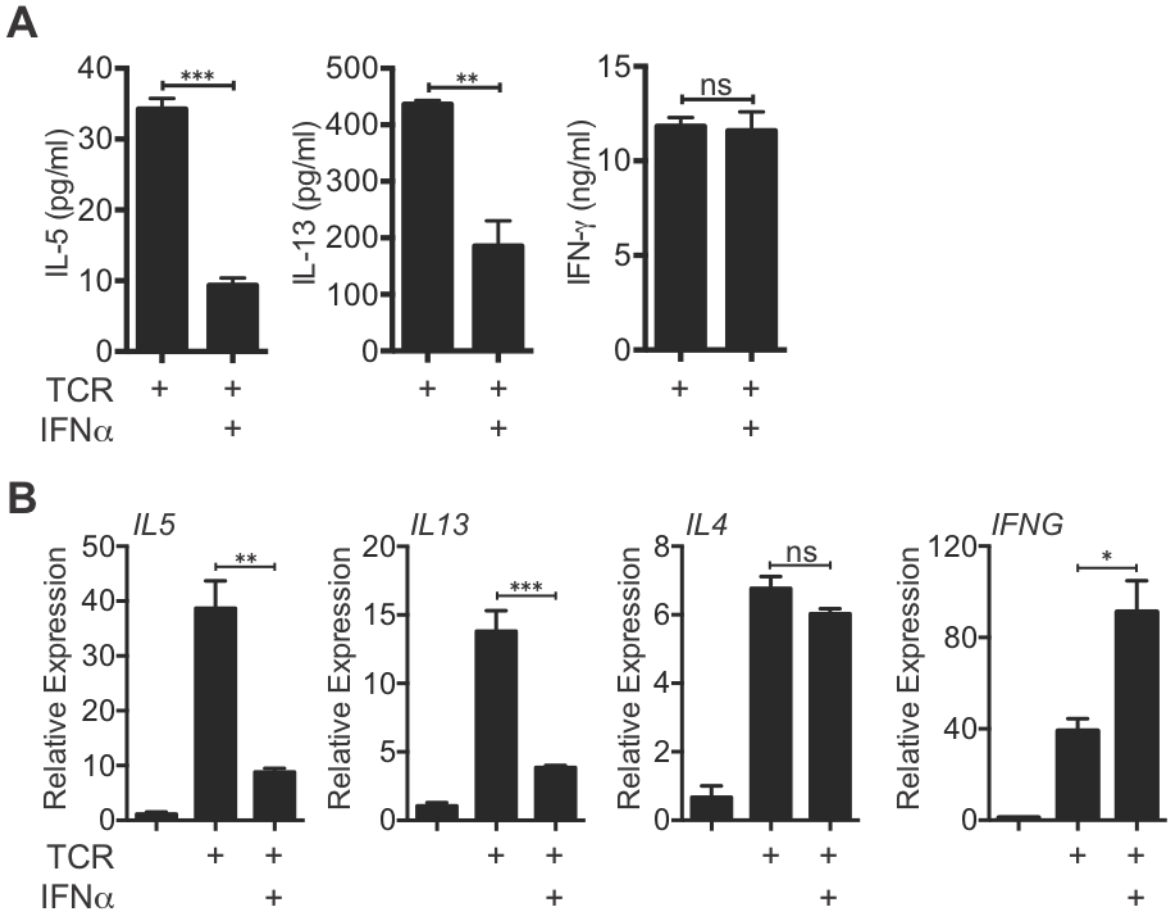


Figure 4.1: Acute IFN-α treatment suppresses IL-5 and IL-13 expression by human PBMCs. Human PBMCs were stimulated with plate-bound anti-CD3 (3 μg/ml) and rh-IL-2 (50 U/ml) in the presence or absence of IFN-α (1000 U/ml). (A) Secretion of IL-5, IL-13 and IFN-γ was quantified by ELISA 24 hrs post-stimulation. (B) Relative gene expression of *IL5, IL13, IL4* and *IFNG* quantified by qPCR 4 hrs post-TCR stimulation. Each gene expression was determined relative to the 'No TCR' control. *PPIA* was used as the reference gene for all qPCR experiments. Each experiment is representative of three individual experiments. Student's t test in (A) and one-way ANOVA with a Bonferroni post-hoc test (B) were used to determine statistical significance. * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, ns: not significant.

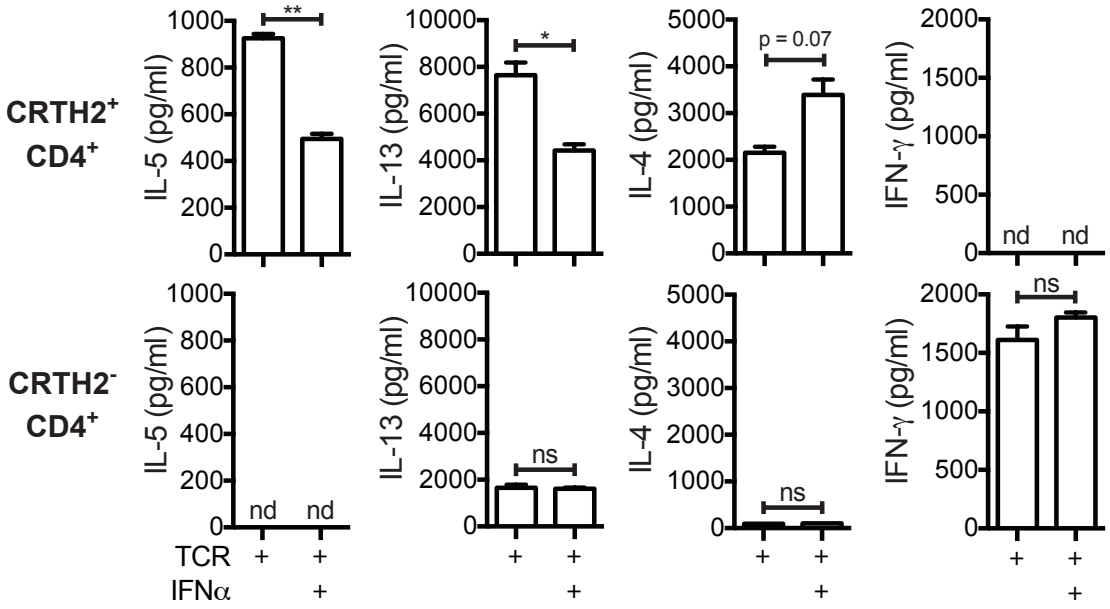


Figure 4.2: Human CRTH2$^+$/CD4$^+$ T cells are sensitive to acute IFN-α treatment. Purified human memory Th2 cells (CD4$^+$CRTH2$^+$) were stimulated for 24 hrs with plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) in the presence or absence of IFN-α (1000 U/ml) and ELISAs were performed on cell supernatants. Each graph represents 3 separate experiments. Statistics calculated by student's t-test; * ≥ 0.05, ** ≥ 0.01, ns: not significant.

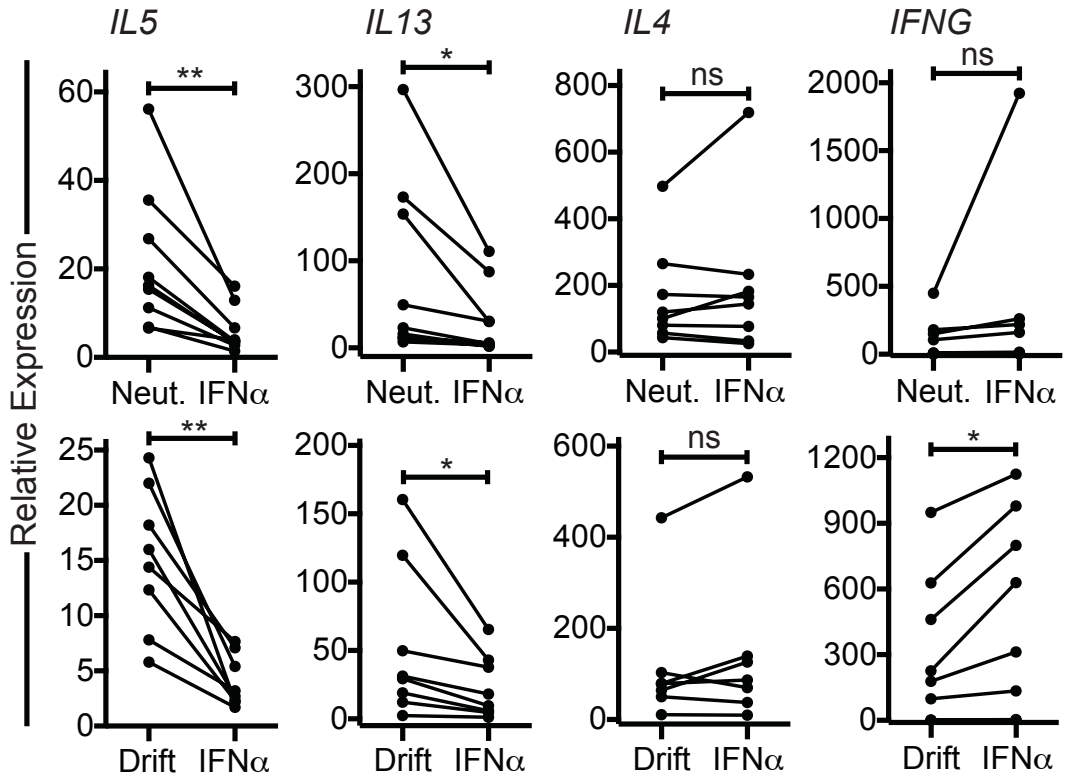


Figure 4.3: Human CD4$^+$/CD45RO$^+$ T cells express reduced *IL5* and *IL13* in the presence of acute IFN-α treatment. Purified human CD4$^+$CD45RO$^+$ T cells were stimulated with plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) in the presence or absence of IFN-α (1000 U/ml) for 4 hrs. RNA was isolated and used for quantitative PCR analysis. 'Neut,' 'Drift,' and 'IFN-α,' treatments are expressed relative to a 'No TCR' control, all of which are expressed relative to the reference gene, *PPIA*. Each line represents an individual experiment. Statistics calculated by student's t-test; * ≥ 0.05, ** ≥ 0.01, ns: not significant.

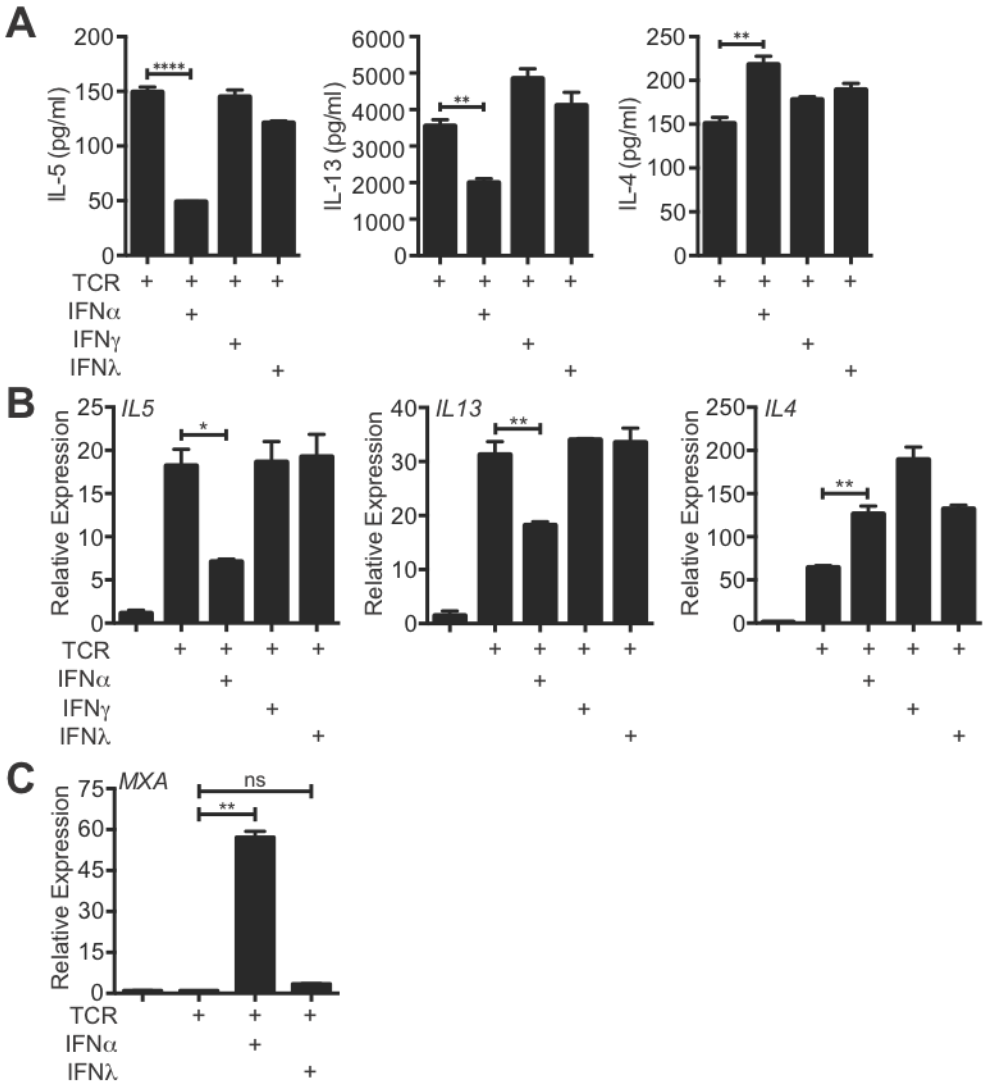


Figure 4.4: Type I, but not Type II or III interferons, suppress IL-5 and IL-13 expression. Human $CD4^{+}CD45RO^{+}$ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml) and rh-IL-2 (50 U/ml). (A) Cells were stimulated in the presence or absence of IFN-α (1000 U/ml), IFN-γ (5 ng/ml) or IFN-λ1 (100 ng/ml) for 24 hrs and cytokine secretion was quantified by ELISA. (B) Cells were stimulated in the presence or absence of IFN-α (1000 U/ml), IFN-γ (5 ng/ml) or IFN-λ1 (100 ng/ml) for 4 hrs, and relative gene expression was assayed by qPCR, where each treatment is relative to a 'No TCR' control. (C) Cells were stimulated in the presence or absence of IFN-α (1000 U/ml) or IFN-λ1 (100 ng/ml) for 4 hrs, and qPCR was utilized to determine relative *MXA* expression compared to a 'No TCR' control. All qPCR experiments were analyzed relative to the reference gene, human *PPIA*. In (A), (B), and (C) one-way ANOVA and a Bonferroni post-hoc test were used to determine statistical significance. * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

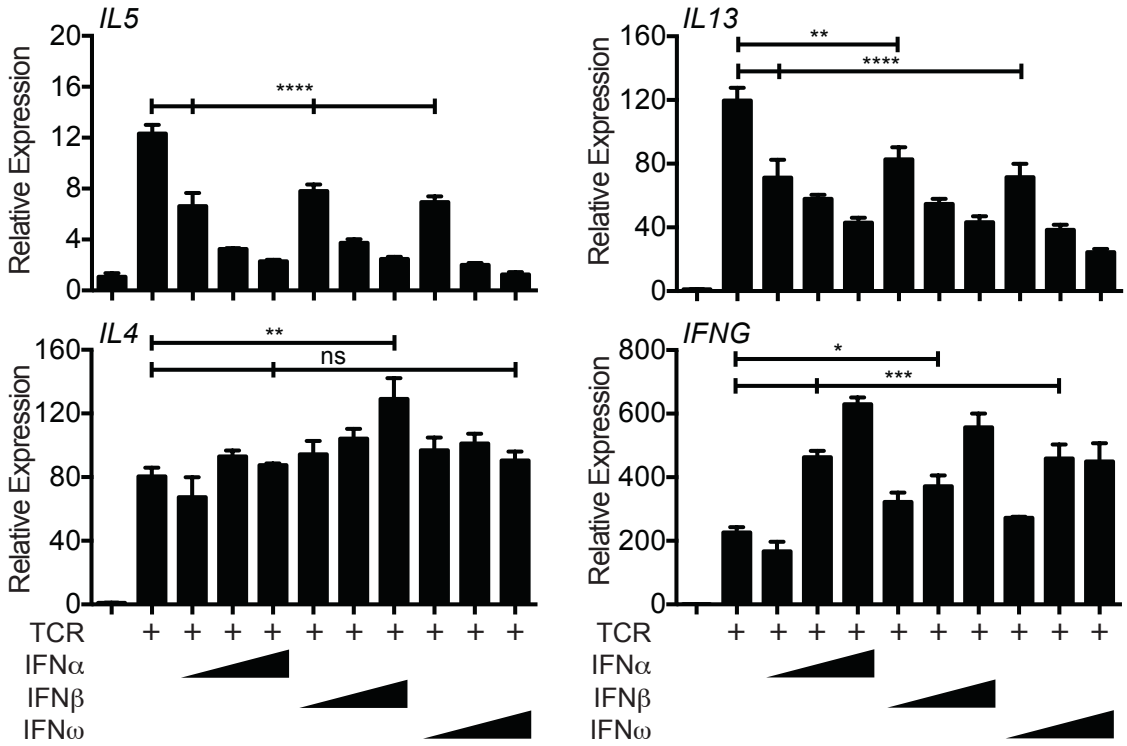


Figure 4.5: IFNAR-mediated suppression of CD4$^+$ memory T cells occurs in a dose-dependent manner in response to several IFN-α/β family members. Human CD4$^+$CD45RO$^+$ T cells were stimulated with plate-bound anti-hCD3 (3 μg/ml) and rh-IL-2 (50 U/ml). (A) Cells were stimulated in the presence or absence of increasing concentrations of IFN-α, IFN-β or IFN-ω (10, 100, 1000 U/mL) for 4 hrs and qPCR was utilized to determine relative gene expression using the reference gene, *PPIA*. Each gene is relative to a 'No TCR' control. One-way ANOVA and a Bonferroni post-hoc test were used to determine statistical significance. * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

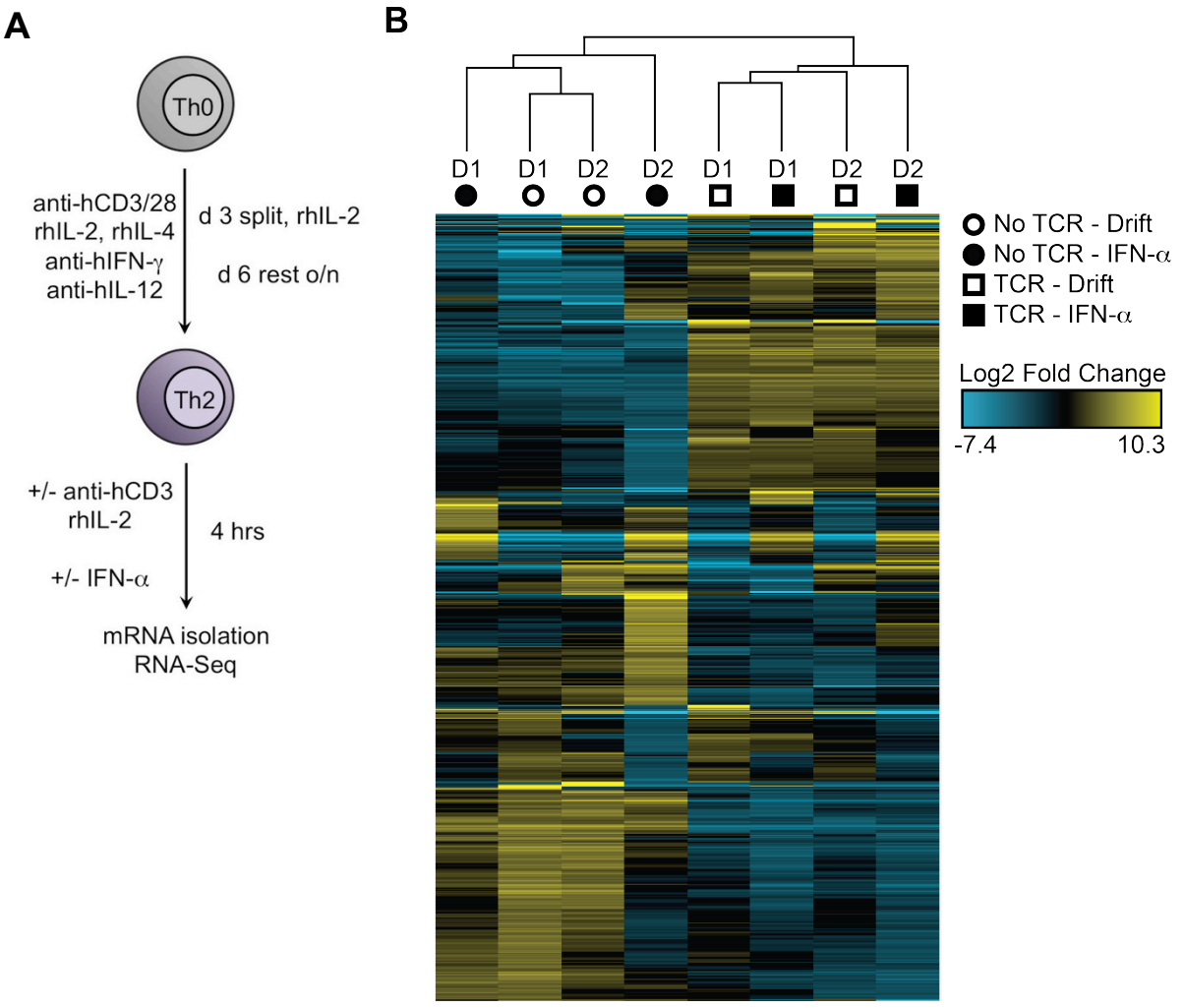


Figure 4.6: Human *in vitro* polarized Th2 cells alter their overall transcriptome profile in response to acute IFN-α signaling. Human naïve ($CD4^+CD45RA^+$) T cells were polarized under Th2 conditions for 7 days as described in Chapter 2. Rested *in vitro* polarized Th2 cells were acutely stimulated with anti-hCD3 (3 µg/ml) and rhIL-2 (50 U/ml) in the presence or absence of IFN-α for 4 hrs, and total RNA was isolated for subsequent RNA-Seq library preparation. Sequenced samples were analyzed using the UT Southwestern Molecular Biology Galaxy Cluster as described in Chapter 2. (A) Experimental design model. (B) Normalized FPKM values for each donor were log2 transformed and centered using Cluster, then visualized using Java TreeView. Genes presented in this figure had an FPKM value of 8 or greater and were differentially regulated 2-fold or greater.

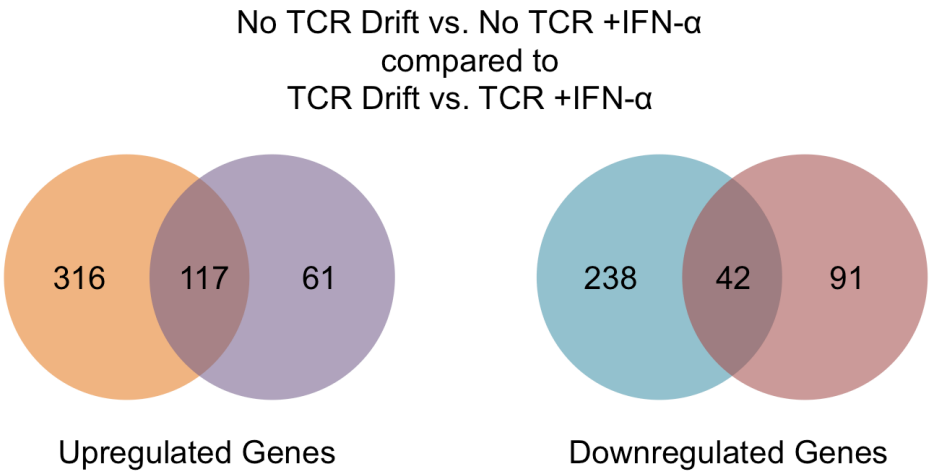


Figure 4.7: Differential comparison of up- and down-regulated genes of 'No TCR Drift vs. No TCR +IFN-α' compared to 'TCR Drift vs. TCR +IFN-α'. Genes up- or down-regulated at least 2-fold in any condition were utilized for this analysis. The web-based tool JVenn (http://bioinfo.genotoul.fr/jvenn/index.html) was utilized to prepare similar and unique gene lists and venn diagrams.

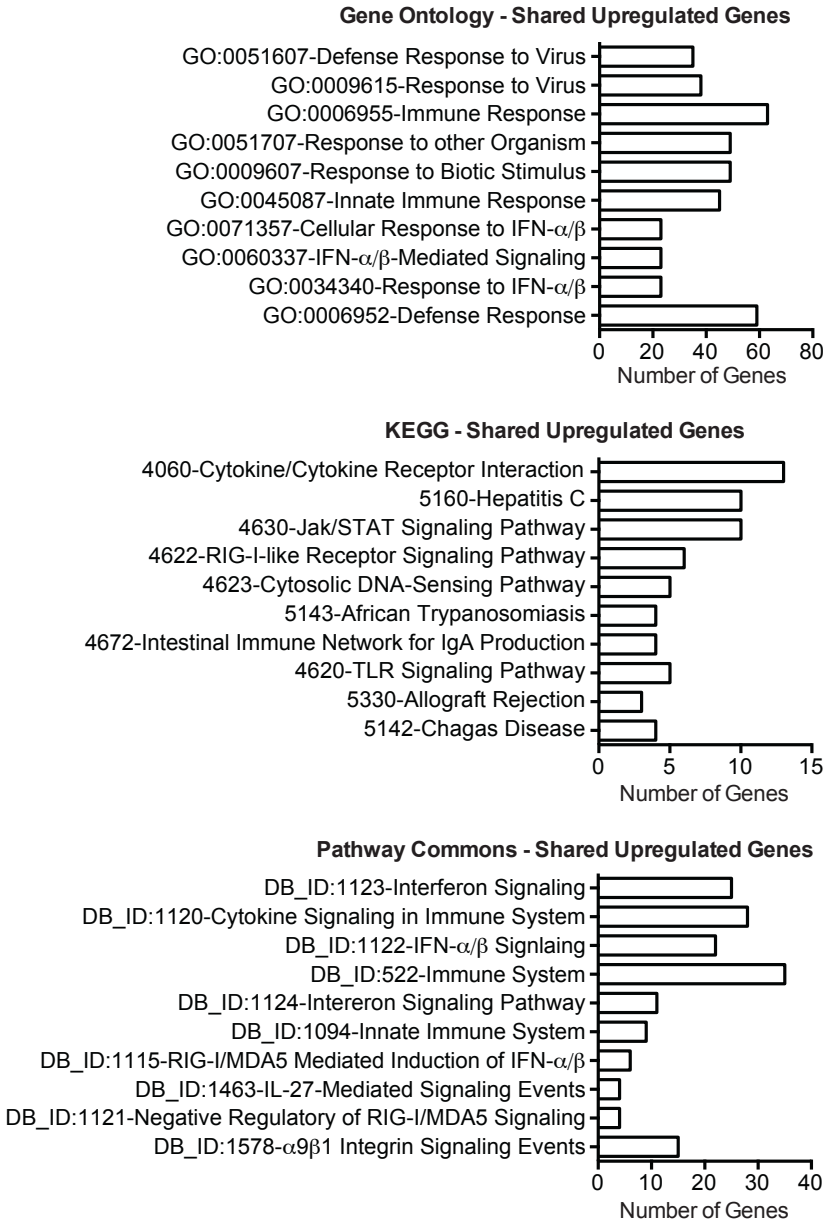


Figure 4.8: Gene characterization of commonly upregulated genes between 'No TCR Drift vs. No TCR +IFN-α' compared to 'TCR Drift vs. TCR +IFN-α'. Gene ontology analysis was performed using the web-based tool, WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/). Analysis was performed using the following databases: Gene Ontology, KEGG, and Pathways Commons Analysis, which only statistically significant grouping of genes considered with at least 3 genes per group were considered.

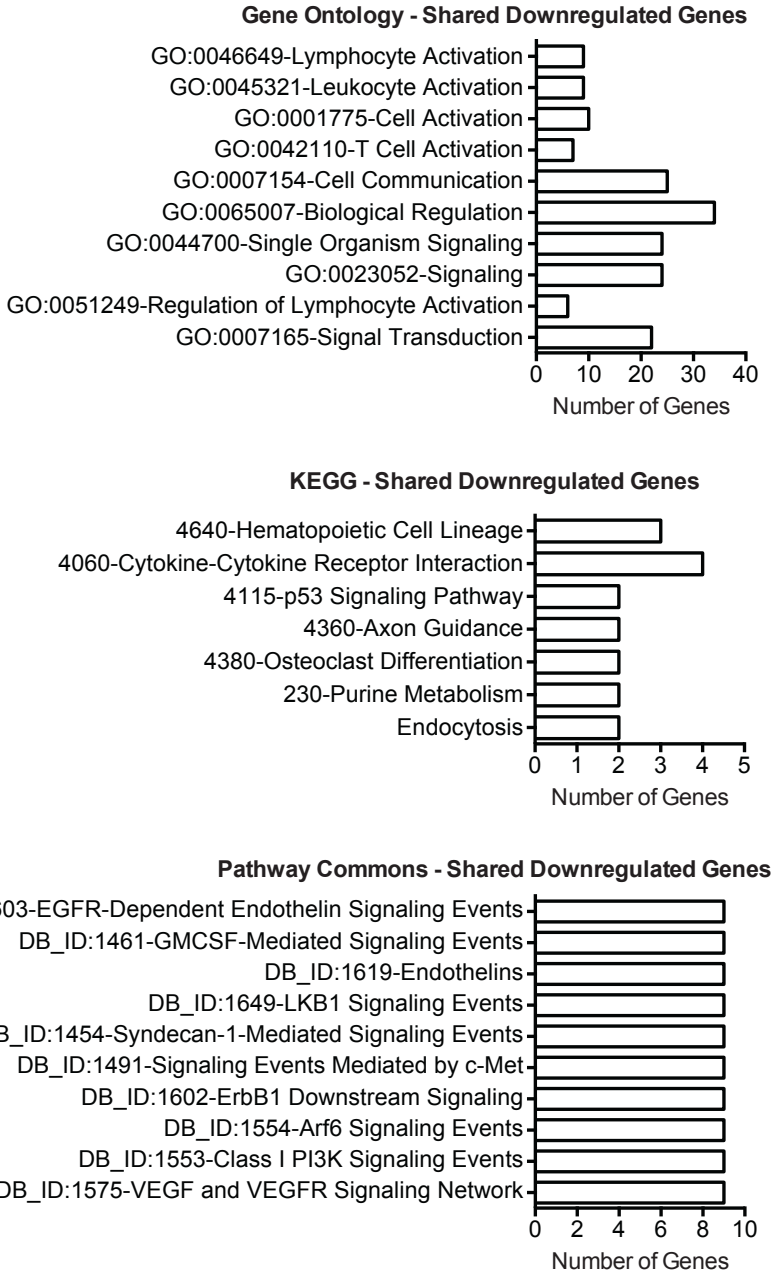


Figure 4.9: Gene characterization of commonly downregulated genes between 'No TCR Drift vs. No TCR +IFN-α' compared to 'TCR Drift vs. TCR +IFN-α'. Gene ontology analysis was performed using the web-based tool, WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/). Analysis was performed using the following databases: Gene Ontology, KEGG, and Pathways Commons Analysis, which only statistically significant grouping of genes considered with at least 3 genes per group were considered.

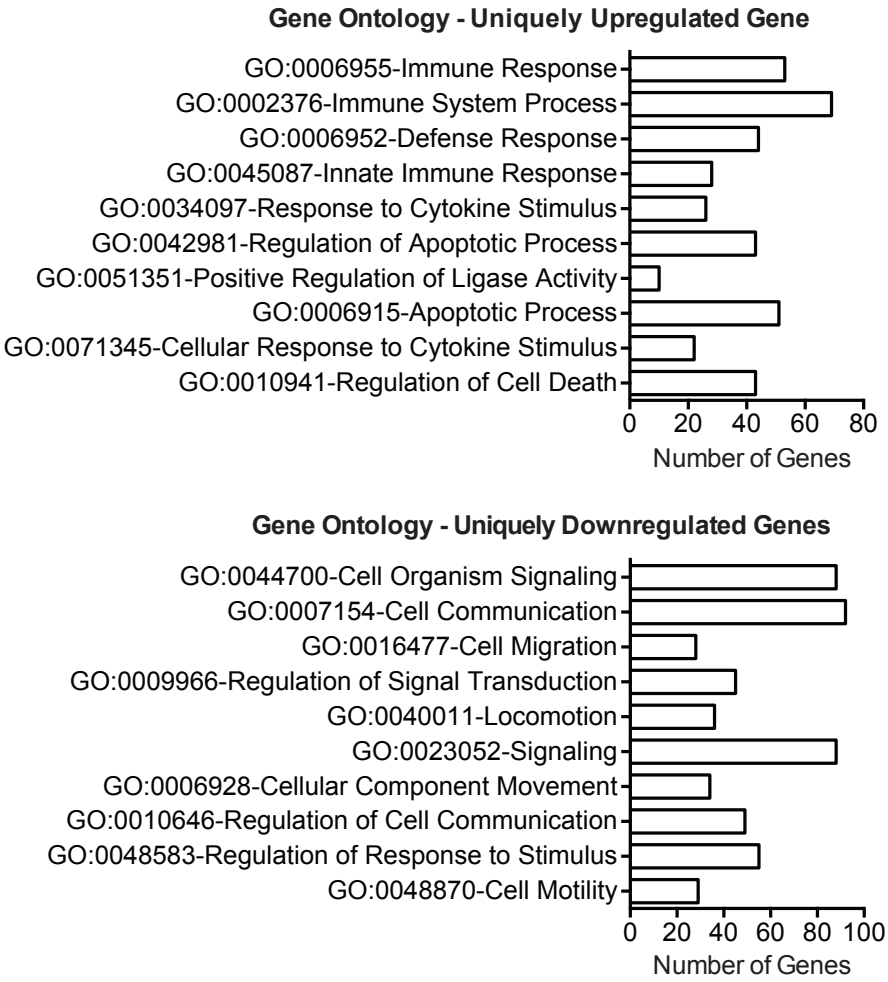


Figure 4.10: Gene ontology of unique up- and down-regulated genes within the 'No TCR Drift vs. No TCR +IFN-α' comparison. Gene ontology analysis was performed using the web-based tool, WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) using the Gene Ontology database tool. Only statistically significant grouping of genes considered with at least 3 genes per group were considered.

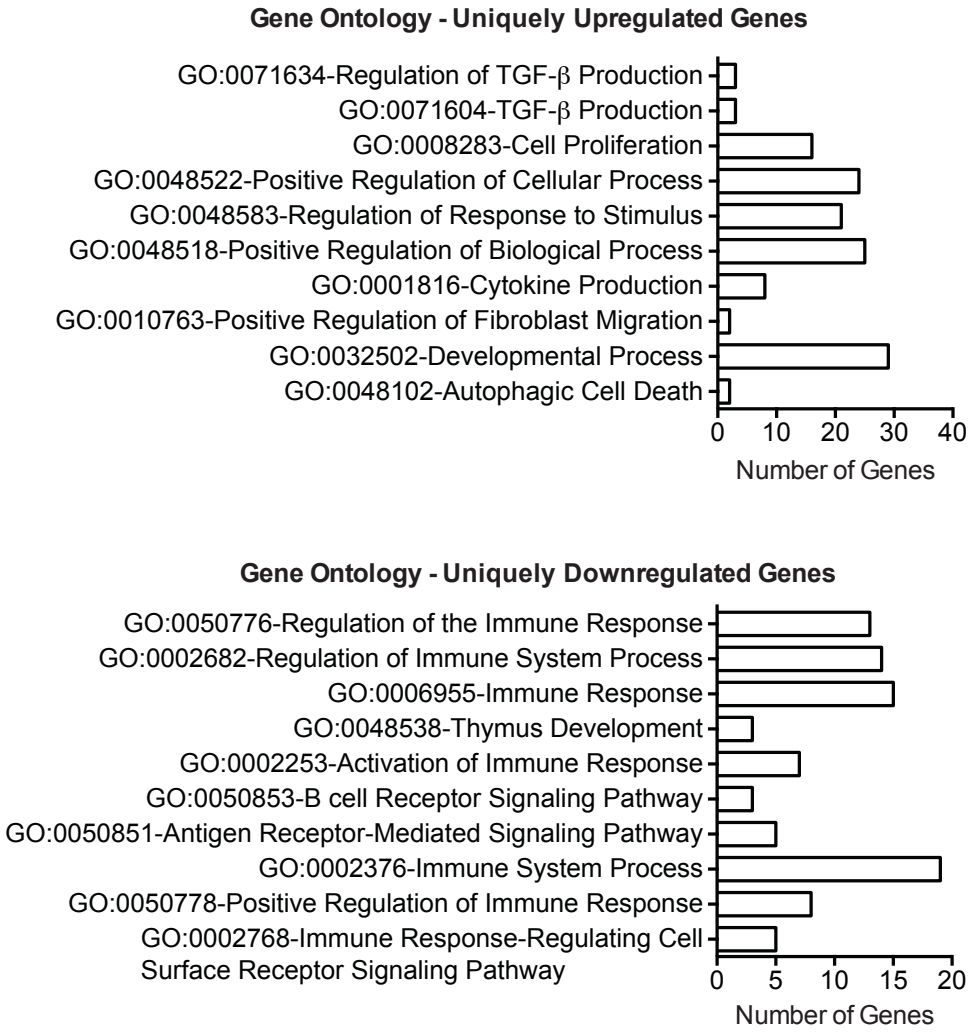


Figure 4.11: Gene ontology of unique up- and down-regulated genes within the 'TCR Drift vs. TCR +IFN-α' comparison. Gene ontology analysis was performed using the web-based tool, WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) using the Gene Ontology database tool. Only statistically significant grouping of genes considered with at least 3 genes per group were considered.

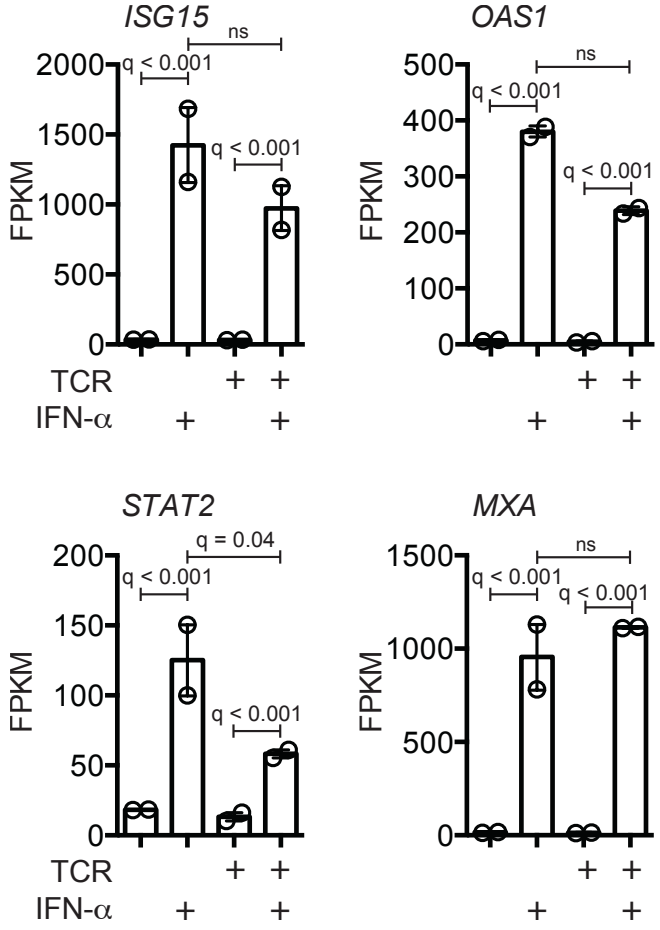


Figure 4.12: Acute IFN-α treatment enhances classical ISG expression in human *in vitro* polarized Th2 cells, independent of TCR stimulation. FPKM values of *ISG15, OAS1, STAT2,* and *MXA* were graphed, where individual donor levels are denoted by the open circles and the bar height represents the mean. Statistical significance was assessed using CuffDiff with a FDR < 0.5 considered significant. The q value (adjusted p value to consider the FDR) represents the differences only between two treatments, not all four conditions.

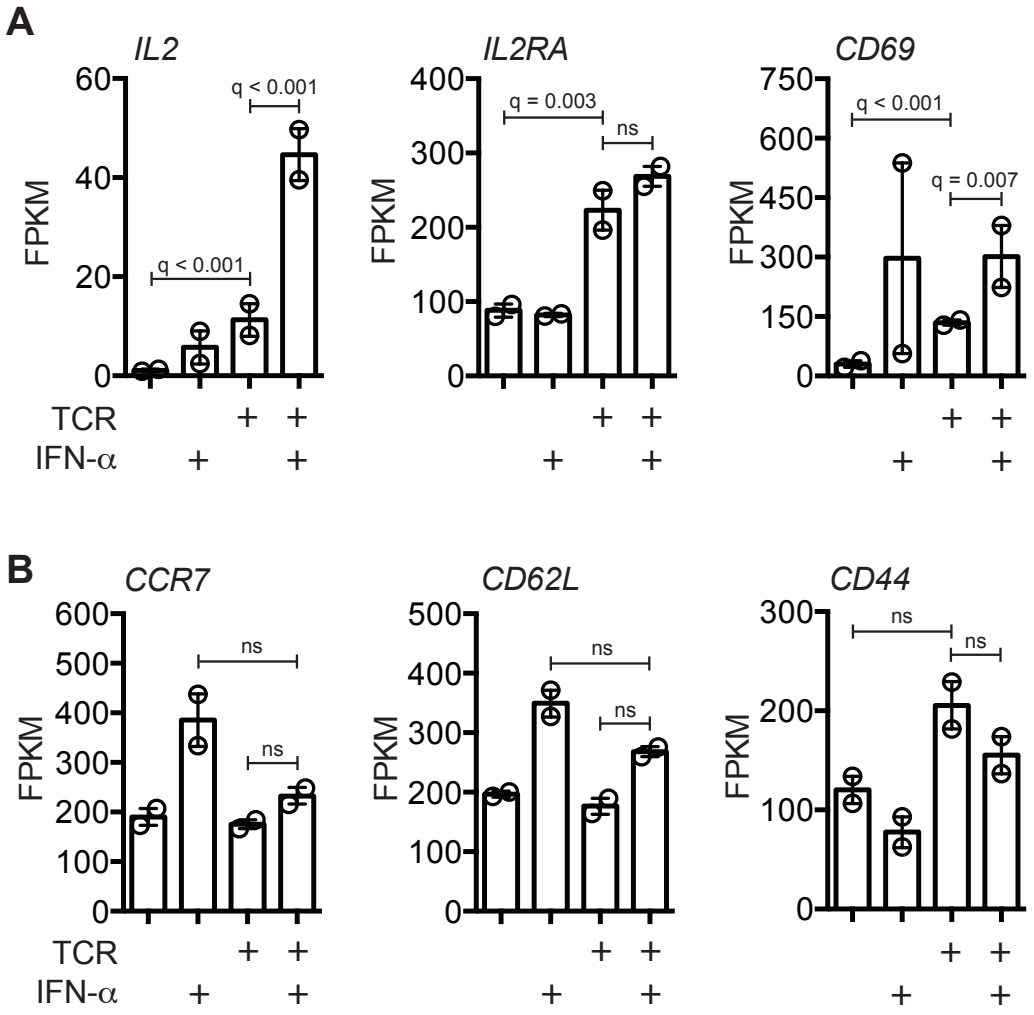


Figure 4.13: T cell activation markers and chemokines are regulated by acute TCR stimulation and IFN-α treatment. FPKM values of (A) *IL2, CD25, CD69,* and (B) *CCR7, CD62L,* and *CD44* were graphed, where individual donor levels are denoted by the open circles and the bar height represents the mean. Statistical significance was assessed using CuffDiff with a FDR < 0.5 considered significant. The q value (adjusted p value to consider the FDR) represents the differences only between two treatments, not all four conditions.

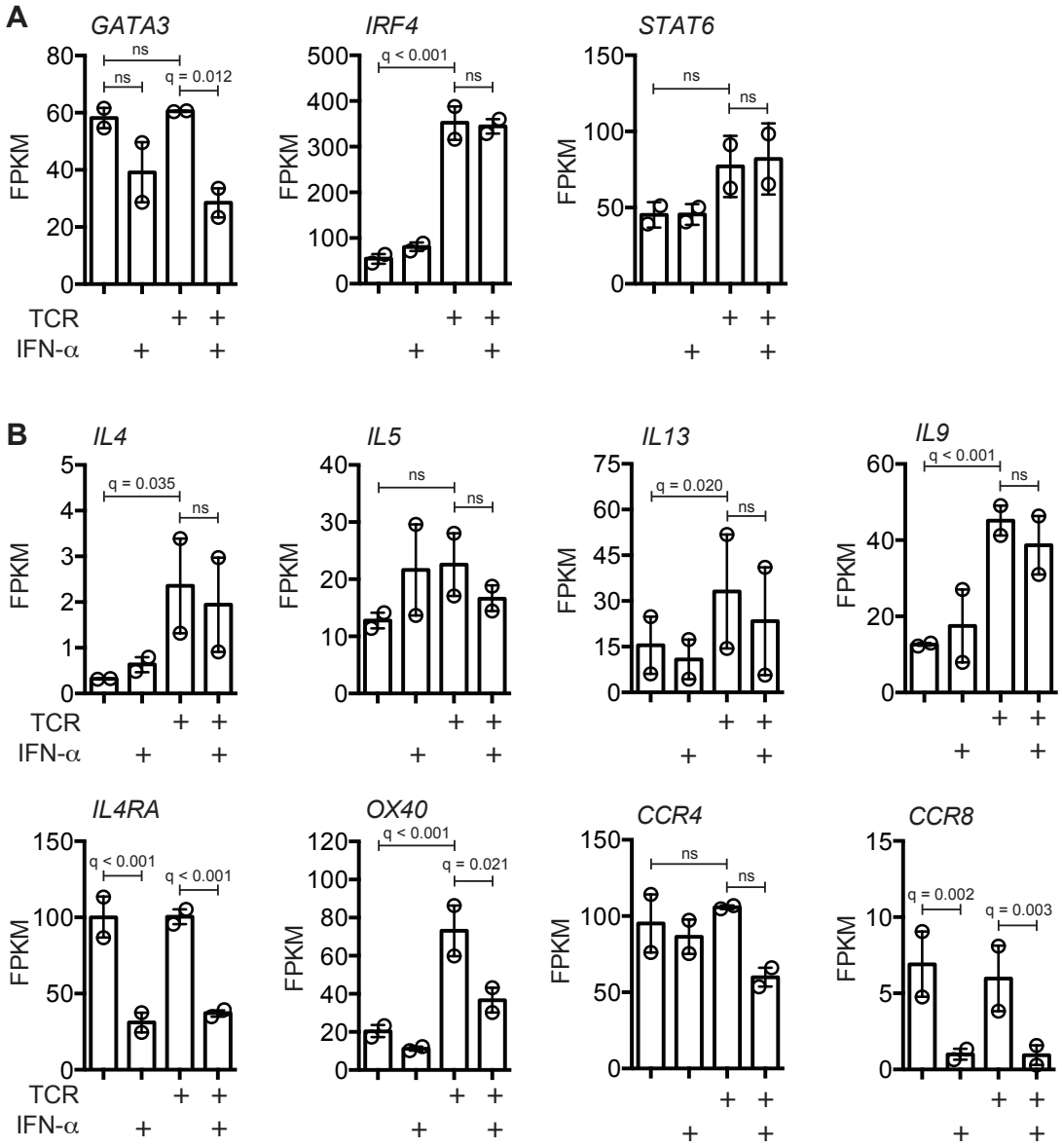


Figure 4.14: Acute IFN-α treatment discordantly regulates Th2-associated genes. FPKM values of transcription factors (A) *GATA3, IRF4, STAT6,* cytokine genes (B) *IL4, IL5, IL13* and *IL9,* and Th2-associated cell-surface proteins (C) *IL4RA, OX40, CCR4,* and *CCR8* were graphed, where individual donor levels are denoted by the open circles and the bar height represents the mean. Statistical significance was assessed using CuffDiff with a FDR < 0.5 considered significant. The q value (adjusted p value to consider the FDR) represents the differences only between two treatments, not all four conditions.

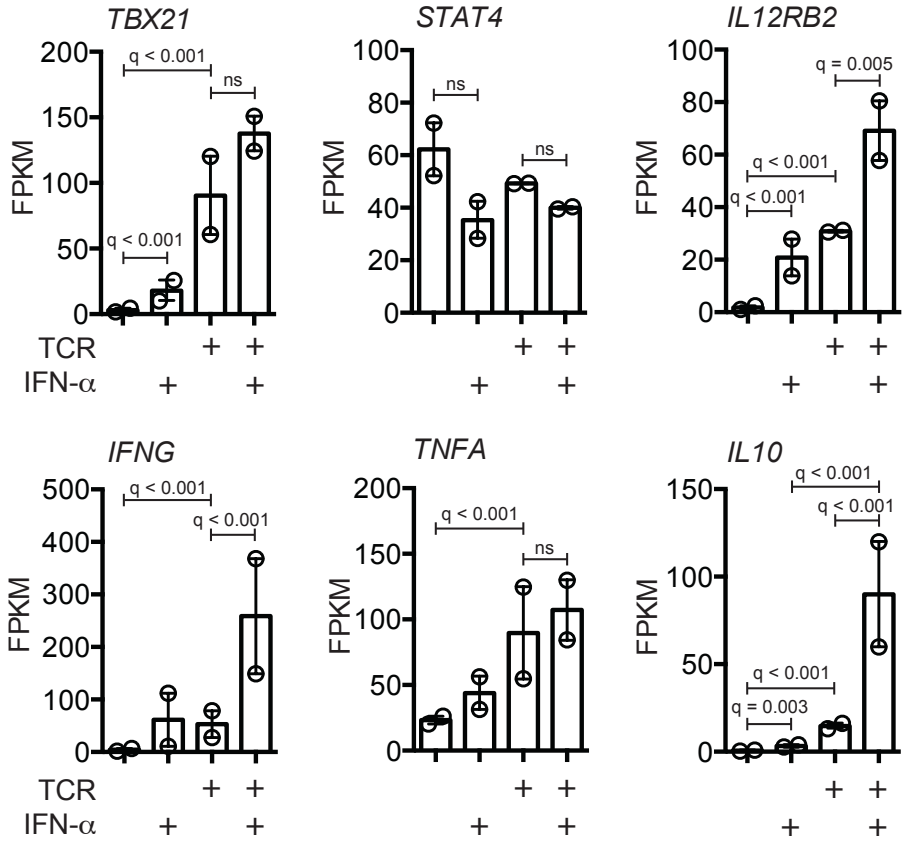


Figure 4.15: Acute IFN-α treatment mediates the enhancement of non-Th2-associated genes. FMKM values of Th1 associated genes (A) *TBX21, STAT4, IL12RB2,* and cytokine genes (B) *IFNG, TNFA,* and *IL10* were graphed, where individual donor levels are denoted by the open circles and the bar height represents the mean. Statistical significance was assessed using CuffDiff with a FDR < 0.5 considered significant. The q value (adjusted p value to consider the FDR) represents the differences only between two treatments, not all four conditions.

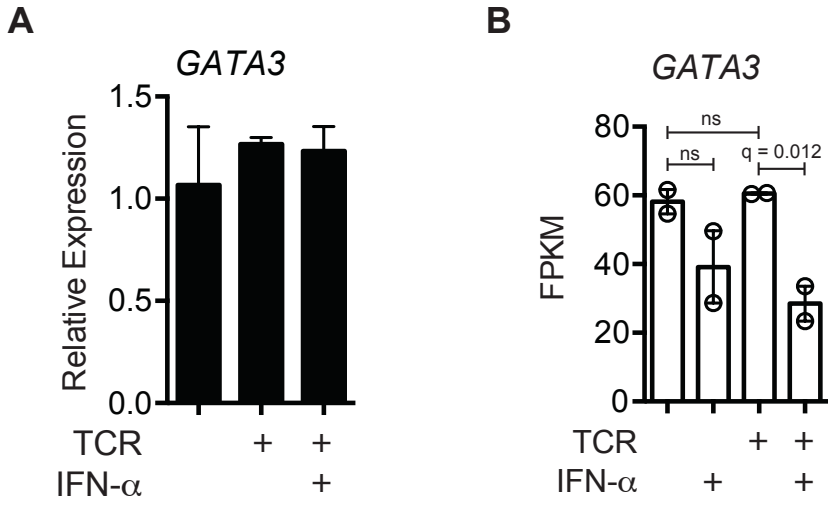


Figure 4.16: GATA3 in *ex vivo*-stimulated total memory CD4$^+$ T cells and *in vitro* polarized Th2 cells is differentially responsive to acute IFN-α signaling. A) Human CD4$^+$CD45RO$^+$ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml) and rh-IL-2 (50 U/ml) in the presence or absence of IFN-α (1000 U/ml) for 4 hrs, and qPCR was utilized to determine relative *GATA3* expression compared to a 'No TCR' control. All qPCR experiments were analyzed relative to the reference gene, human *PPIA*. (B) FMKM values of *GATA3* were graphed, where individual donor levels are denoted by the open circles and the bar height represents the mean. Statistical significance was assessed using CuffDiff with a FDR < 0.5 considered significant. The q value (adjusted p value to consider the FDR) represents the differences only between two treatments, not all four conditions.

Table 4.1: Total statistically significant genes within each comparison.

Comparison	Total No. Differentially Expressed Genes
No TCR Drift vs TCR Drift	2202
No TCR Drift vs No TCR IFN-α	955
No TCR IFN-α vs TCR IFN-α	1892
TCR Drift vs TCR IFN-α	443

Table 4.2: Statistically significant genes with a 2-fold change within each comparison.

Comparison	No. Genes 2-Fold Differentially Expressed	No. Genes Enhanced	No. Genes Suppressed
No TCR Drift vs. TCR Drift	1918	944	974
No TCR Drift vs. No TCR IFN-α	773*	280	493
No TCR IFN-α vs. TCR IFN-α	1604	804	800
TCR Drift vs. TCR IFN-α	371*	133	238

* Denotes the list utilized to determine which genes are shared or uniquely up- or down-regulated in Tables 4.3 - 4.8.

Table 4.3: Gene list of shared upregulated genes between 'No TCR Drift vs. No TCR +IFN-α' compared to 'TCR Drift vs. TCR +IFN-α'.

ANXA2R	GIMAP1	LY6E	SCLT1
ANXA3	GIMAP7	LY96	SCML4
APOL1	GLRX	MASTL	SIDT1
APOL6	GMPR	METRNL	SLC18B1
ATP10A	GNB4	MOV10	SLC25A12
BAK1	GPR171	MX1	SLC38A5
BCL2L14	HELB	MX2	SLFN12
BST2	HERC5	MYD88	SOCS3
C19orf66	HERC6	N4BP1	SP100
C4orf33	HSH2D	NARF	SP110
C5orf56	IFI16	NEXN	SPAG6
CARD17	IFI35	NMI	SPATS2L
CCR1	IFI44	NT5C3	SPSB1
CD164	IFI44L	NUB1	SSTR3
CD33	IFI6	NUDCD1	STAP1
CD38	IFIH1	OAS1	STAT2
CD69	IFIT1	OAS2	STK3
CHI3L2	IFIT2	OAS3	TDRD7
CHMP5	IFIT3	OASL	TLR7
CHST12	IFIT5	OSM	TMEM62
CMPK2	IFITM1	PARP10	TNFSF10
CXCL10	IFITM3	PARP12	TNFSF13B
CXCL13	IFNG	PARP14	TNIP3
CYB5R4	IL10	PDCD1	TOP1
CYP2J2	IL12RB2	PGAP1	TRANK1
DAPP1	IL15RA	PHF11	TREX1
DDX58	IL1RN	PI4K2B	TRIM14
DDX60	IL2	PLAC8	TRIM21
DDX60L	IRF7	PLSCR1	TRIM22
DHX58	IRF8	PML	TRIM38
DYNLT1	IRF9	PNPT1	TRIM5
EAF2	ISG15	PPM1K	TTC38
EHD4	ISG20	PRIC285	TTC39B
EIF2AK2	JMJD1C	PVRL3	UNC93B1
ENDOD1	KIAA0040	RBCK1	USP18
ENPP2	L3MBTL3	RBM11	VSIG10L
EPSTI1	LAG3	RBM43	WARS
ETV7	LAMP3	RNF213	XAF1
FAM184A	LAP3	RNF24	XRN1
FBXO6	LGALS17A	RSAD2	ZBP1
FLT1	LGALS3BP	RTP4	ZCCHC2
FRMD4B	LGALS9	SAMD9	ZNF107
FTSJD2	LIF	SAMD9L	
GCA	LOC643733	SAT1	
GCH1	LRRC3	SCARB2	

Table 4.4: Gene list of shared downregulated genes between 'No TCR Drift vs. No TCR +IFN-α' compared to 'TCR Drift vs. TCR +IFN-α'.

ABAT	MAML3
APBB1IP	NEK6
ARAP3	NELL2
BMF	OLFM2
CABLES1	PAG1
CCNG2	PDE4A
CCR8	PDE7B
CD101	PLEKHG2
CD27	PPP3CA
CD5	PTPN14
CHN2	SESN3
DIXDC1	SFXN3
FHOD1	SIRPG
IGSF9B	SLC35F3
IKZF4	SNX30
IL16	SOX4
IL4R	SPTBN1
IRS2	SRGAP3
KIAA1274	SUOX
KIT	TSC22D3
MAL	TTYH3

Table 4.5: Uniquely upregulated genes in the 'No TCR Drift vs. No TCR +IFN-α' comparison.

ABCD1	CCL4	ESF1	HSPA13	MRPS33	PRPS2	TAPT1
ADAR	CCNC	EXOC6	HSPB11	MT2A	PSMA2	TARBP1
ADPGK	CCR7	FAM18B1	ICOS	MTHFD2	PSMA3	TBK1
ADPRHL2	CD200	FAM26F	IDH3A	NAA25	PSMA4	TBX21
AIM2	CD274	FAM46A	IDI1	NABP1	PSMA6	TIPIN
AKAP2	CD2AP	FAM53A	IDO1	NAMPT	PSMB9	TLR5
ALPK1	CD68	FAM84B	IGF1	NAPA	PSMC6	TM2D1
ANKFY1	CDC73	FAR2	IL18R1	NBN	PTRH2	TMEM126B
ANKIB1	CENPQ	FAS	IL18RAP	NCOA7	RASGEF1B	TMEM140
ANKRD46	CFH	FASLG	IL8	NDUFA5	RBM7	TMX1
AP1AR	CISD1	FASTKD2	INTS12	NDUFAF2	RGS1	TNF
APOL2	CISD2	FCHSD2	IPCEF1	NDUFAF4	RGS13	TNFAIP8
ARHGAP27	CLDND1	FOS	IRF2	NDUFB3	RNF19A	TOMM5
ARHGAP8	CLEC2B	FOSB	IRS1	NDUFS4	RPF2	TPRKB
ARL5B	CLEC2D	FRMD3	ITGB3BP	NFIX	RPL22L1	TRAFD1
AZI2	CNDP2	GADD45B	JAK2	NFKBID	RPL23P8	TRIM69
B3GNT2	CNP	GALM	KBTBD8	NFYB	RPL26L1	TRMT10C
BAG1	COMMD10	GBP1	KIAA1033	NGDN	RPL31P11	TTC21A
BAZ1A	COMMD6	GBP2	KLF10	NIPSNAP3A	RPL9	TXN
BBX	COPS2	GBP3	KLF5	NMD3	RPS29	TXNDC9
BCL2A1	COPS4	GBP4	KPNA5	NR4A1	RPS3A	UBA7
BCL6	COX17	GBP5	KRR1	NR4A2	RPS6KA5	UBC
BET1	COX7A2	GBP6	LACTB	NR4A3	RSL24D1	UBE2D1
BLZF1	COX7B	GBP7	LARP7	NSMCE2	RWDD1	UBE2E1
BRIX1	CREM	GEMIN2	LIN52	NT5C1B	SBNO2	UBE2E2
BTLA	CSF1	GIMAP2	LOC100131733	NTNG2	SCO1	UBE2L6
C12orf23	CTSS	GIMAP4	LOC100133669	NXT2	SCOC	UCHL3
C15orf48	CXCL9	GIMAP6	LOC79015	ODF3B	SECTM1	UQCRB
C17orf96	CXorf21	GIMAP8	LSM3	OGFR	SERPINB9	WDFY1
C1GALT1	DNAJA1	GNL3	LSM5	OPN3	SF3B14	XCL1
C21orf91	DNAJC15	GPBP1	MAN1A1	OR52N4	SLC25A28	XKR9
C22orf28	DNAJC25	GPR155	MAP3K5	OTUD6B	SLIRP	XRCC4
C3orf14	DRAP1	GPR180	MAP3K8	OXR1	SMCHD1	YAP1
C4orf32	DTX3L	GSDMD	MKI67IP	PARP11	SNRPG	ZC3H15
C6orf57	DUSP2	GTF2B	MLKL	PARP8	SNX4	ZFYVE26
C8orf59	DYNLT3	GTPBP1	MMADHC	PARP9	SNX6	ZNF267
CACNA1A	ECE1	HAPLN3	MNDA	PDCD10	SOBP	ZNF593
CALM2	EGR1	HAT1	MORC3	PFDN4	SP140	ZNFX1
CAPZA2	EGR2	HBD	MRPL1	PIGY	SPATS2	ZNRF2
CARD16	EGR3	HDAC4	MRPL17	PIM3	SRFBP1	ZUFSP
CASP7	EGR4	HDX	MRPL32	PLSCR4	SRGN	
CBR1	EIF1AY	HEATR3	MRPL42	PMAIP1	SRSF7	
CBWD1	EMC2	HFE	MRPL47	POLR2K	STAT1	
CBWD2	ENC1	HIGD1A	MRPL50	POMP	STOM	
CCDC58	ERGIC2	HM13	MRPS17	PRDX4	SUB1	
CCL3	ERO1LB	HNRPLL	MRPS23	PRKAG2	TAP2	

Table 4.6: Uniquely downregulated genes in the 'No TCR Drift vs. No TCR +IFN-α' comparison.

ABCG1	CMIP	ITGB2	NEURL1B	SLCO3A1
ABR	CMTM3	JAZF1	NLRP3	SMPD1
ACACB	CNN2	KCNA6	NOG	SMPD3
ACCN2	CORO2A	KCTD21	NTSR1	SNTB2
ACSS1	CTSD	KCTD7	ORAI2	SOCS2
ACTN1	CXCR4	KIAA1609	ORAI3	SORL1
ADAMTS10	CYTH4	KIF21B	PAK1	SPOCK2
ADARB1	DAAM1	KLF7	PCYOX1	SPON1
ADORA2A	DACH1	KLHL3	PDE4DIP	SPRED2
AEBP1	DACT1	L1CAM	PDLIM2	STARD9
AFAP1	DBH	LAIR1	PDLIM7	STX3
AK5	DBP	LDLRAP1	PECAM1	STXBP1
AMPD3	DGKZ	LFNG	PELI2	SUN2
ANKH	DMWD	LIME1	PGPEP1	SVIL
ANPEP	DPYSL2	LIMS2	PIEZO1	SYTL1
APBA1	DUSP6	LINC00176	PIK3C2B	TBC1D2
APBB1	DYRK1B	LINC00294	PITPNM2	TBC1D2B
APOBR	EDAR	LINC00341	PLAU	TCEA3
AQP3	EEF2K	LINC00426	PLCB2	TECPR1
ARHGAP10	EEPD1	LOC100499466	PLCB3	TET1
ARHGAP9	ENO2	LOC100506776	PLCH1	TGFBR1
ARHGEF18	EPHB6	LOC283174	PLEC	TGFBR3
ARRB1	ERBB2	LOC642852	PPARD	TMEM200A
ATP6V0E2	ESYT1	LRRC2	PPP1R3E	TMEM63A
AXIN2	FAM101B	LTB	PREX1	TNIK
BCL2	FAM129B	LY9	PSTPIP1	TOX2
C10orf54	FAM213B	LYPD3	PTPN12	TPCN1
C11orf21	FAM46C	LZTS1	PTPN9	TRAF5
C14orf132	FBXO32	MANBA	RAB37	TRAM2
C14orf49	FGFR1	MAP1A	RAB3D	TRIM46
C16orf54	FMNL3	MATK	RALGAPA2	TRPM2
C17orf103	GAB2	MBNL2	RASSF2	TTC9
C1orf162	GAL3ST4	MEOX1	RGS14	TUBA4A
C1orf38	GALNT6	MGAT3	RXRA	TXK
C20orf112	GATSL3	MGAT5	SDK2	UCP2
CACNA1I	GDPD5	MGAT5B	SGK223	UNC13D
CALHM2	GIPC3	MICAL2	SEMA4C	USP35
CAMK2G	GJB6	MIR1909	SH3BP5	VASH1
CBX7	GJD3	MIR4458	SH3TC1	VIPR1
CCR2	GNG2	MIR4639	SIAH3	VSIG1
CD248	GNPTAB	MIR600HG	SIGIRR	XYLT1
CDC14B	GOLGA7B	MPP7	SIGLEC6	YPEL3
CDK6	GRAMD4	MRC2	SIGLECP3	ZBTB16
CDKN2B	HABP4	MTSS1L	SIRPB1	ZDHHC23
CELSR2	HIP1	MTUS1	SLC27A1	ZNF589
CHST2	IFFO2	MYBL1	SLC2A4RG	ZYX
CISH	IGF1R	MYO18A	SLC35E2	
CITED4	IL23A	MYO1F	SLC43A1	

Table 4.7: Uniquely upregulated genes in the 'TCR Drift vs. TCR +IFN-α' comparison.

ABCA1	IL7R
ADAM19	INPP4B
ADD2	KCNK15
AIM1	KIAA1671
ANK3	KLF6
BNIP3	LAMA2
C11orf10	LMO7
C3AR1	MIER1
CASK	MIR4639
CASP10	MIR937
CDKN2D	MPP5
CEACAM1	PIM1
CEBPB	PLK2
CPM	PRKCE
CRISPLD1	PROS1
DES	PSD3
DPP4	PTMS
DUSP5P	PTPN1
EMP1	RGL1
FAM126A	RP2
FOSL2	SERPINB1
FURIN	SLC2A1
GBP1P1	SLFN5
GCNT1	SRGAP2
GIPR	SUSD3
GTPBP8	THBS1
GZMB	TIMP2
GZMH	TSHZ3
HIF1A	UTRN
IGFBP4	ZBTB32
IL21	

Table 4.8: Uniquely downregulated genes in the 'TCR Drift vs. TCR +IFN-α' comparison.

AMICA1	GATA3	PRKD3
ANK1	GLCCI1	PTPN22
ANTXR2	GLIS3	QPCT
ARMC9	GPR55	RAB30
ATG2A	GPRIN3	RASAL1
ATP9A	HEATR5A	RFX2
ATXN1	HOMER2	RHBDD2
B7H6	HS3ST1	RNF125
BCL2L11	ID3	RORC
C15orf48	IER5L	RYR1
C16orf45	KIAA1324	SH2D3C
CD160	KIF26B	SHF
CDK19	KIFC3	SHISA8
CDKN2C	KLHL24	SLC26A6
CEP97	LAT	SLC9A3
COL6A1	LOC348761	SMAD3
COL6A2	LRIG1	SMOX
CR2	MAP1LC3A	SPIN1
CTDSP2	MAP3K1	SPTBN4
CYSLTR1	MIR24-2	TBL1X
CYTH3	MIR4749	TNFRSF9
DHRS3	MPZL3	TP53INP1
DUSP10	MYB	TSPAN14
EFNA5	NHS	UBASH3B
EPAS1	PER1	VAV3
FAM102A	PIK3IP1	WHAMMP2
FAM102B	PKIA	YPEL2
FAM53B	PLCL1	ZBTB7C
FBXO34	PLEKHA1	ZNF704
FCER2	PLEKHA6	
FLVCR2	PRDM8	

CHAPTER V

IFN-α SIGNALING MEDIATES STAT4-DEPENDENT SUPPRESSION OF THE HUMAN IL5 GENE

Introduction

The suppression of a gene can be mediated by a variety of mechanisms that occur pre- or post-transcriptionally. Mechanisms that suppress nascent transcription can occur either through the suppression of transcriptional enhancers, or the upregulation of a transcriptional repressor, both of which ultimately lead to gene suppression. For example, murine *Il5* expression in $CD62L^{low}CXCR3^{low}$ Th2 cells was shown to be regulated by the transcription factor Eomesodermin (Eomes) (Endo et al., 2011). Eomes protein bound directly to GATA3, which correlated with reduced GATA3 binding within the murine *Il5* promoter (Endo et al., 2011). Alternatively, mRNA can be targeted post-transcriptionally for degradation through AU-Rich Element (ARE)-mediated mRNA decay pathways or miRNA-mediated silencing pathways. Murine *Ifng* mRNA expression has been shown to be negatively regulated by the ARE-binding protein Tristetraprolin (TTP) (Ogilvie et al., 2009). In TTP-deficient cells, *Ifng* transcript half-life was enhanced almost 2-fold in response to TCR stimulation, compared to TTP-sufficient cells (Ogilvie et al., 2009). I wished to determine which of these general mechanisms was induced by IFN-α/β signaling to suppress human *IL5* and *IL13* expression.

Classical IFN-α/β signaling leads to the formation of the STAT2/STAT1 dimer, which is bound by IFR9 (Stark et al., 1998). This ISGF3 complex acts as an enhancer of hundreds of ISGs

by directly binding to ISREs, often found within the proximal promoters of ISGs. A number of these genes likely act as transcriptional repressors, which could play a role in mediating *IL5* and *IL13* gene suppression. In addition to classical STAT2-mediated signaling, other STAT dimers are activated in response to IFN-α/β treatment. STAT1, STAT3, STAT4, and STAT5 are also mobilized in response to IFN-α/β treatment (Farrar et al., 2000b; Ghislain and Fish, 1996; Matikainen et al., 1999; Uddin et al., 2003). Each of these STAT dimers binds to the GAS DNA motif, although there are subtle differences between each STAT binding site. In addition to the activation of an ISG with repressor activity, STAT proteins themselves could potentially bind directly to target genes to suppress expression. An example of this has been demonstrated in a study from Kanno and colleagues, which compared the transcriptome profiles of WT versus STAT4 or STAT6 knockout murine cells and correlated the expression patterns to STAT4 and STAT6 binding as well as epigenetic modifications during T cell development (Wei et al., 2010). In comparing WT or STAT-deficient cells, the study was able to define which STAT binding sites were required to induce epigenetic modifications in a specific gene loci, in cases of either transcriptional enhancement or repression. Notably, STAT6 deficiency led to a considerable enhancement in the silencing modification, H3K27me3, whereas the absence of STAT4 led to a reduction of the number of genes bound by the permissive modification, H3K4me3 (Wei et al., 2010). However, there were also clusters of genes that seemed to be suppressed in response to STAT binding, whose transcriptional activity was enhanced in STAT-deficient cells. Of all transcripts sequenced, 265 genes were bound and negatively regulated in the presence of STAT4, while 213 genes were bound and negatively regulated in the presence of STAT6. In particular, the Th2-associated gene *Ccr8* gene was suppressed in Th1 cells and was bound by STAT4 and associated with enhanced H3K27me3, but this binding was deficient in STAT4-knockout cells.

How STATs recruit histone-modifying enzymes to specific gene loci is unclear. There is one example in which IFN-α treatment of cells for 1 hour induces enhanced binding of Ezh2 and enhanced H3K27me3 at an ISRE site within the promoter of a p53 family member, DNp57 (Testoni et al., 2011a). Ezh2 is part of the Polycomb repressive complex 2 (PRC2), which enzymatically methylates H3K27me2, resulting in H3K27me3 (Margueron and Reinberg, 2011). This post-translational modification of H3K27 causes tightening of the histones and DNA, leading to reduced accessibility of cis regulatory elements that induce gene expression (Margueron and Reinberg, 2011). Although epigenetic modifications are critical in shaping daughter cell behavior, Ezh2 binding and enhanced H3K27me3 have been observed within an hour after IFN-α treatment (Testoni et al., 2011a). It may be the case that Ezh2 activation and enhanced H3K27me3 can occur quickly in response to the initial signaling events, but that this event may not be mediating acute gene suppression. Rather, these modifications could be important for continued gene suppression after the initial signaling events are no longer present.

In chapter 4, I described the observation in which IFN-α/β signaling potently suppresses *IL5* and *IL13* gene expression in human memory Th2 cells. This negative regulatory pathway was only induced by IFN-α/β signaling, as both IFN-γ and IFN-λ signaling failed to suppress *IL5* and *IL13* gene expression. Furthermore, I showed that acute IFN-α alters the transcriptome profile of *in vitro* polarized Th2 cells differently in the presence or absence of TCR stimulation. Next, I was interested in identifying the molecular pathway that mediates *IL5* and *IL13* gene expression. I employed a series of experiments to rule out a broad range of potential mechanisms, and identified how murine Th2 cells responded to acute IFN-α signaling compared to human Th2 cells. These results demonstrate how acute IFN-α/β signaling induces STAT4-mediated *IL5* suppression in a species-specific manner.

Results

IL-5 transcription is rapidly suppressed by IFN-α

The experiments presented in chapter 4 demonstrate that IFN-α signaling suppresses TCR-mediated *IL5* and *IL13* gene expression within 4 hrs of treatment (Figure 4.3). Further, the effects of IFN-α/β signaling can be seen quickly within the cell; STAT1, STAT2, and STAT4 phosphorylation occurs within minutes of the cell responding to receptor ligation, and the subsequent induction of ISGs can be detected within an hour of IFN-α/β treatment (Farrar et al., 2000b; Ghislain and Fish, 1996; Honda and Taniguchi, 2006). I was interested in how quickly IFN-α/β is able to negatively regulate TCR-induced gene expression in human T cells. Human bulk lymphocytes were stimulated with anti-hCD3 and rhIL-2 in the presence or absence of IFN-α given to cells at the time of TCR stimulation (Figure 5.1, white symbols), or 2 hrs post-TCR stimulation (Figure 5.1, orange symbols). *IL5* and *IFNG* gene expression were measured each hour for 8 hrs by qPCR. As expected, cells that received anti-hCD3 and rhIL-2 stimulation upregulated *IL5* and *IFNG* expression (Figure 5.1, blue symbols). Further, cells given IFN-α at the time of TCR stimulation showed a marked suppression of *IL5* expression, while *IFNG* was enhanced (Figure 5.1, white symbols). Interestingly, *IL5* expression in cells that received IFN-α treatment 2 hrs post-TCR stimulation showed similar transcript levels compared to cells that received IFN-α at the 0-hour time point (Figure 5.1, yellow symbols). This rapid response to IFN-α treatment was confirmed by measuring *IFNG* expression of these cells, in which we did indeed see a delay in the upregulation of *IFNG* expression when cells were treated with IFN-α 2

hrs post-TCR stimulation. This result demonstrates that IFN-α signaling rapidly suppresses *IL5* expression in response to acute IFNAR/IFN-α ligation.

The kinetic analysis in Figure 5.1 demonstrated that IFN-α/β signaling potently suppressed IL5 expression, and this observation could be detected within 2 hrs of IFN-α treatment. I wished to determine whether one or several ISGs mediated this swift negative regulation of *IL5* and *IL13* gene expression, or whether direct IFN-α signaling was responsible for mediating this effect. By preventing *do novo* translation using the translational inhibitor cycloheximide, I was able to determine whether the protein expression of IFN-α/β-induced ISGs were required to observe *IL5* and *IL13* gene suppression. Human memory $CD4^+$ T cells were stimulated with anti-hCD3 and rhIL-2 for 2 hrs, and then were treated with IFN-α in the presence or absence of cycloheximide or DMSO for an additional 2 hrs. The presence of cycloheximide did not prevent IFN-α-mediated suppression of *IL5* and *IL13* gene expression (Figure 5.2A). However, the presence of cycloheximide did suppress the expression of *IFNG* in both the presence and absence of IFN-α (Figure 5.2A). The changes in overall *IL5* gene expression in response to cycloheximide were not surprising, as it is known that robust *IL5* expression requires nascent protein translation (Schwenger et al., 2002; Umland et al., 1998). To verify that the concentration of cycloheximide was sufficient to halt *de novo* protein synthesis, I restimulated *in vitro* polarized human Th2 cells with PMA and ionomycin in the presence of monensin for 4 hrs and asked whether the same concentration of cycloheximide was able to prevent IL-13 protein expression. PMA and ionomycin enhanced IL-13 protein expression compared to the unstimulated control (Figure 5.2B, first bar from the left). Compared to the cells that received stimulation only (Figure 5.2B, second bar from the left), cells that were given cycloheximide the entire time showed almost no expression of *de novo* translated IL-13 (Figure 5.2B, dark grey

bar). Comparatively, in cells that received cycloheximide 2 hours post-stimulation, similar to the experimental design in Figure 5.2A, *de novo* IL-13 synthesis was suppressed by approximately 75% (Figure 5.2B, light grey bar). Although this system seems to allow some *de novo* protein synthesis to occur, the experiments conducted in Figure 5.2A utilized anti-hCD3 and rhIL-2 as a stimulus, not PMA and ionomycin. Thus, protein synthesis in Figure 5.2A is likely to be inhibited to a greater extent than the experiment depicted in Figure 5.2B because the stimulation is not as overwhelmingly strong as PMA and ionomycin. These results suggest that the initial steps that suppress *IL5* and *IL13* gene expression likely occur directly in response to IFN-α signaling, and do not require the nascent expression of ISGs to mediate this process.

The robust suppression demonstrated in Figure 5.1, and the lack of requirement for nascent protein synthesis to mediate *IL5* and *IL13* suppression (Figure 5.2A) led me to hypothesize that IFN-α directly reduces the amount of *IL5* and *IL13* transcripts, either through the initiation of an mRNA decay pathway or by the suppression of nascent transcription. To test which of these hypotheses explained our observation, I performed two experiments. The first was to determine whether IFN-α treatment enhanced the rate of *IL5* transcript decay. Actinomycin D was added to human lymphocytes in the presence or absence of IFN-α 2 hrs post-TCR stimulation, and *IL5* and *IFNG* transcripts were measured every hour for 5 hrs (Figure 5.3A). TCR stimulation enhanced *IL5* and *IFNG* production compared to the 'No TCR' control (Figure 5.3A, blue-filled squares) while IFN-α treatment suppressed *IL5* expression, and enhanced *IFNG*, respectively (Figure 5.3A, orange-filled triangles). To determine the rate of transcript decay in the presence or absence of IFN-α treatment, I performed a linear regression analysis on each of the treatments, using the relative gene expression at each time point from the addition of actinomycin D (2 hr) to the end of the assay (5 hr) to calculate the slope of the line.

Based on this analysis, I found that IFN-α treatment did not enhance the rate of *IL5* transcript decay compared to the cells that did not receive IFN-α treatment (Figure 5.3B, compare 'TCR + Actinomycin D' versus 'TCR + IFN-α/Actinomycin D'). Additionally, the presence of IFN-α did not seem to alter the rate of *IFNG* transcript decay as well (Figure 5.3B). The lack of enhanced transcript decay in response to IFN-α treatment led me to perform the second experiment, which was to determine whether the rate of nascent transcription of *IL5* and *IL13* was suppressed by IFN-α signaling. I performed nuclear-run experiments and utilized *in vitro*-polarized human Th2 cells that were stimulated acutely in the presence or absence of IFN-α for 2 hrs. TCR-stimulation markedly enhanced the rate of nascent transcription of *IL5*, *IL13*, *IL4* and *IFNG* (Figure 5.4). Actinomycin D was used to determine the relative background signal from this assay (background nascent transcription), and cells that were treated with this inhibitor were unable to respond to TCR- and IFN-α-mediated signaling, as detected by qPCR (Figure 5.4). IFN-α treatment enhanced the rate of nascent transcription of the canonical ISG, *MXA*, as well as *IFNG*, while reducing the rate of nascent transcription of *IL5* and *IL13*, but not *IL4* (Figure 5.4). Taken together, these experiments demonstrated that IFN-α reduces *IL5* and *IL13* gene transcription by suppressing the rate of nascent transcription of these genes. This observation is consistent with the acute nature of STAT activation in response to receptor ligation, and suggests that the Jak/STAT signaling pathway is capable of potently suppressing gene expression in addition to enhancing ISG expression.

A novel GAS/ISRE element within the human IL5 promoter is bound by IFN-α-activated STAT species

The observations I've presented thus far have demonstrated that signals emanating from the activated IFNAR lead to the suppression of TCR-mediated *IL5* and *IL13* expression. Further, I've shown that IFN-α/β signaling uniquely suppresses gene transcription, as treating cells with IFN-γ or IFN-λ does not inhibit *IL5* and *IL13* expression (Figure 4.4). Next, I was interested in determining which signaling intermediates were being utilized to induce *IL5* and *IL13* gene suppression. Classical IFN-α/β signaling leads to the formation of the ISGF3 complex, which binds the ISRE, which has been shown to potently enhance gene expression (Stark et al., 1998). As described in chapter 1, IFN-α/β-mediated IFNAR activation can also lead the activation of other STAT signaling pathways, including STAT1, STAT3 and STAT4 homodimers (Figure 1.5). I wished to determine whether there were any putative STAT binding sites within the *IL5* promoter that STAT proteins could potentially bind to and thus mediate gene suppression. Further, I chose to focus on the *IL5* gene because it is particularly sensitive to acute IFN-α signaling, thus I hypothesized that the mechanism of this pathway might be more easily detected with the tools I had available. To get a general idea for the relative abundance of putative STAT binding sites in the area, I performed an *in silico* analysis of the 1 kb human *IL5* promoter. Using the web-based tool Jaspar with a relative profile score threshold of 80 percent, I found 73 putative STAT binding elements present within the 1 kb promoter, however, only 4 of these sites were ISRE elements. Interestingly, an overlapping putative GAS/ISRE site was found approximately 150 bp from the *IL5* transcriptional start site. I was interested in whether STAT proteins were able to bind to this region of DNA in response to IFN-α treatment; thus, I

performed gel shift assays to address this question. Human PBMCs were stimulated in the presence or absence of IFN-α, and nuclear lysates were probed with a biotin-labeled human *IL5* GAS/ISRE DNA probe (Figure 5.5A). I found that nuclear proteins were able to bind to the DNA probe in the presence or absence of IFN-α treatment, although binding seemed to be slightly greater in the presence of IFN-α (Figure 5.5B). Using antibodies against the different STAT proteins, I found that STAT1 and STAT4, but not STAT2 and STAT3, were able to bind the ISRE/GAS probe in response to IFN-α treatment, as observed by the super-shifted protein complex in the presence of STAT1 and STAT4 antibody (Figure 5.5B). Although this assay demonstrated what STAT species were able to bind to this DNA probe in the presence of IFN-α, it did not address whether this site was bound in live cells. Because there were numerous putative sites throughout the *IL5* gene locus, I utilized a non-biased approach to identify where STAT2 and STAT4 were able to bind within the entire Th2 locus using ChIP-Seq. Although STAT1 was identified in the EMSA to be able to bind to the promoter region, it is also utilized by the IFN-γ signaling pathway, which I showed does not suppress *IL5* and *IL13* gene expression (Figure 4.4). This experiment would also give an indication as to whether the EMSA data was biologically relevant in a whole-cell system, and especially whether STAT4, but not STAT2, did indeed bind to that site in the presence of other transcription factors interacting with the proximal promoter. Due to the technical difficulties of isolating enough DNA that was bound to STAT2 or STAT4 in the absence of IFN-α treatment, I was unable to collect enough material in a 'No IFN-α' treatment to make a sequencing library, thus I do not have this control. Instead of asking where STAT2 and STAT4 bind in the presence or absence of IFN-α signaling, I designed the experiments to address two alternative questions: 1) where do STAT2 and STAT4 bind in response to IFN-α treatment, and 2) how does TCR stimulation alter IFN-α-mediated STAT2 and

STAT4 binding. Although any positive 'hits' could be present in the absence of IFN-α treatment as well, the data still have me an indication of where to look for cis regulatory elements that could be bound by STAT proteins in response to IFN-α treatment, which I then validated by ChIP-qPCR. To obtain enough DNA from the immunoprecipitated chromatin, I utilized *in vitro* polarized Th2 cells, and acutely treated them with IFN-α in the presence or absence of TCR-stimulation (Figure 5.6). I performed ChIP-Seq on samples from 1 donor for STAT4 and 2 donors for STAT2; cells from both of these donors were also utilized for the RNA-Seq experiments. As predicted, a number of genes known to be responsive to STAT2 or STAT4 were bound by their respective transcription factor. Such genes included the *OAS* gene locus bound by STAT2, as well as intron 1 of the *CD25* gene that was bound by STAT4 (Figure 5.7A and 5.7B, black arrows). However, other sites, including sites within the Th2 locus and *GATA3*, were more difficult to interpret because of the low abundance of transcription factor binding. This could be due to technical issues, including high noise in the control samples, or insufficient binding/detection of binding in the immunoprecipitated samples. Alternatively, this could be biologically relevant, and perhaps genes that are suppressed in response to IFN-α signaling are not bound as efficiently as sites that are enhanced by this cytokine. Despite the risk of false positives, I assessed potential binding sites within the Th2 locus and the *GATA3* gene locus (Figure 5.8A and 5.8B). I observed numerous sites of enhanced binding in both the STAT2 and STAT4 immunoprecipitated samples. However, I found no overlap between STAT2 sites in either donor. I did find that STAT2 and STAT4 binding to different sites seemed to be altered in response to TCR stimulation; however, the patterns were not consistent (Figure 5.8), except when looking at STAT2-sensitive ISGs, in which STAT2 binding was independent of TCR stimulation (Figure 5.7A). Interestingly, in one donor, I found that STAT2 bound to the *in silico*

identified ISRE site within the proximal *IL5* promoter (Figure 5.8A, red arrow, donor 1) that was assessed by EMSA (Figure 5.5B). Furthermore, when I looked at whether other STAT2-bound sites contained an ISRE, I found that another site approximately 7.5 kB upstream of the *IL5* gene also contained a putative ISRE (Figure 5.8A, black arrow, donor 2). Both of these STAT2-bound sites were present in the TCR-stimulated samples, but not in 'No TCR' samples, however, the sites were not conserved between both donors. Compared to the *IL5* and *RAD50* gene loci, the *IL4* and *IL13* gene loci were relatively unbound by STAT2 or STAT4 (Figure 5.8). However, because both *IL5* and *IL13* gene expression were suppressed in response to IFN-α, I was interested in the *IL13* 3'-bound sites as well (Figure 5.8A, black arrow, donor 1). The STAT2-bound site closest to the *IL13* gene contained a GAS motif, while the STAT2-bound site farther from the *IL13* gene contained no putative ISRE or GAS motifs. Furthermore, STAT4 bound to several sites within the *RAD50* gene, along with a couple sites within the *IL5* gene locus. Interestingly, the 3' IL5 locus site bound by STAT4 contained a putative ISRE site (Figure 5.8A, black arrow, orange line). Finally, I was interested in whether STAT2 or STAT4 bound to the *GATA3* locus in response to IFN-α signaling. There seemed to be binding in various regions across the GATA3 locus (Figure 5.8B), however, I did not perform a thorough validation of these sites because I determined previously that the transcript expression of *GATA3* does not change in response to acute IFN-α treatment (Figure 4.16A). Although these sites could be important for driving epigenetic modifications to prevent Th2 development (Chapter 3), they would likely not play a role in the acute mechanism of *IL5* and *IL13* gene suppression.

In addition to identifying potential binding sites and regions of interest within the Th2 cytokine and *GATA3* locus, I was interested in whether cytokine genes regulated in response to IFN-α in the RNA-Seq data were bound within their respective gene loci by either STAT2 or

STAT4. Using RNA-Seq to look at transcription patterns in response to TCR stimulation and IFN-α, I found that the enhanced expression of ISGs in the presence of IFN-α, such as *OAS1*, also showed abundant STAT2 binding within the gene promoter (Figure 4.12 and 5.7A). However, this correlation was not always as clear as this example. RNA-Seq analysis also showed that both *IL2* and *IL10* expression was enhanced by TCR stimulation in the presence of IFN-α (Figure 5.9A, compare 'No TCR + IFN-α' vs. 'TCR + IFN-α'). In the case of *IL2*, STAT2 binding in donor 2 was enhanced in the 'TCR +IFN-α' condition compared to the No TCR + IFN-α (Figure 5.9B). Additional STAT2 and STAT4 binding within the region were also observed, which might play a role in driving IFN-α-induced enhancement of *IL2* gene transcription (Figure 5.9A and 5.9B, (Davis et al., 2008)). In contrast, although *IL10* expression was enhanced by TCR stimulation and IFN-α treatment in the RNA-Seq results, there was very little STAT2 or STAT4 binding within the *IL10* gene locus (Figure 5.9A and 5.9C). It could be the case that STAT binding within this region is not required for enhanced *IL10* expression in response to IFN-α treatment. Alternatively, it could be that the time point in which I harvested the cells was not optimal to see STAT binding within this region, or that the levels of STAT binding are very low and are not considered 'significant'. Taken together, it seems that IFN-α-mediated STAT activation and DNA binding does not necessarily correlate with genes that are differentially regulated in response to this cytokine, although further experiments will need to be conducted in order to validate this claim.

Validation of IFN-α-mediated STAT2 and STAT4 binding within the Th2 locus

Because there was considerable variability in STAT2 binding sites within the Th2 locus between both ChIP-Seq donors, and the lack of replicates for STAT4 ChIP-Seq, I utilized ChIP-qPCR to validate certain 'hits' to determine whether STAT2 or STAT4 bound to the human *IL5* and *IL13* gene loci in response to IFN-α treatment. Human *in vitro* polarized Th2 cells were acutely stimulated in the presence or absence of IFN-α for 2 hrs, and chromatin lysates were immunoprecipitated with STAT2- or STAT4-specific antibodies. Because I did not have a 'No IFN-α' condition in my ChIP-Seq experiments, it was important to include that condition in the validation experiments to determine what whether STAT binding was changed in response to IFN-α compared to this control. Figures 5.10 and 5.11 shows the STAT2 and STAT4 ChIP-qPCR results of *in vitro* polarized Th2 cells from four individual donors, respectively. Each of the sites within the Th2 cytokine locus interrogated by ChIP-qPCR is indicated within the ChIP-Seq figure with an arrow (Figures 5.8A). Finally, the *MXB* promoter was utilized as a positive control for STAT2 binding in response to IFN-α treatment (Figure 5.10). Although the *MXB* promoter was bound by STAT2 in response to IFN-α treatment in the majority of donors, the relative STAT2 binding to the *IL5* and *IL13* sites varied from donor to donor. In all donors except Donor A, STAT2 binding to the *IL5* and *IL13* sites were either unchanged in response to IFN-α treatment, or actually reduced compared to STAT2 binding in the absence of IFN-α (Figure 5.10). Because Donor A is the same as Donor 1 in the ChIP-Seq experiment, I expected to see enhanced STAT2 binding to be validated by ChIP-qPCR, and it was (Figure 5.8A, blue peaks with arrows compared to Donor A in Figure 5.10). This donor-specific STAT2 binding in response to IFN-α treatment could be biologically relevant within an individual, but not

significantly enhanced in the entire population. However, many more donors would be necessary to determine whether STAT2 does indeed bind to this site in a subset of individuals, which is beyond the scope of this project. In addition to validating STAT2 binding sites, I interrogated the proximal *IL5* site that was bound by STAT4 by EMSA (Figure 5.5B), as well as the *IL5* 3' locus site bound by STAT4 in the ChIP-Seq data (Figure 5.8A, arrow: orange bar). In contrast to STAT2 binding, IFN-α-mediated STAT4 binding to the *IL5* proximal promoter element was enhanced in response to IFN-α treatment in all of the donor cells, while seemingly binding more weakly at the *IL5* 3' locus site (Figure 5.11). Finally, I utilized the *CD25* intron 1 site bound in the ChIP-Seq data as a positive control for these experiments, and in all 100% of the donors, IFN-α signaling enhanced STAT4 binding to this site (Figure 5.11, far right column).

Because STAT4 is activated in response to IFN-α treatment, and functionally binds within the human *IL5* gene locus, I was interested in whether IL-12-induced STAT4 activation was also able to suppress *IL5* and *IL13* gene expression. Total memory $CD4^+$ T cells were acutely stimulated in the presence or absence of IFN-α treatment, and *IL5*, *IL13*, *IFNG* and *MXA* transcription was measured by qPCR (Figure 5.12) IL-12 treatment was insufficient at suppressing *IL5* and *IL13* gene expression, while it did enhance *IFNG* expression similar to acute IFN-α treatment (Figure 5.12). Finally, IL-12 treatment enhanced the expression of the ISG *MXA*, which was unexpected. This might be explained by promiscuous activation of STAT proteins in response to Jak/STAT activation mediated by IL-12. Further, some ISGs contain GAS elements in addition to ISREs, thus, acute activation of this gene might occur in the absence of IFN-α-mediated ISGF3 activation. Taken together, these results indicate that STAT4 binding within the *IL5* gene locus is consistently enhanced in response to IFN-a treatment. This observation correlates with the suppressed rate of nascent transcription of *IL5* and *IL13* in

response to acute IFN-α signaling, which is consistent with how the Jak/STAT pathway is known to regulate gene expression.

STAT4 is necessary for IFN-α-mediated IL5 suppression

The EMSA and ChIP results in Figures 5.5B an 5.11 demonstrated that STAT4 binds to the proximal *IL5* promoter in response to IFN-α in human Th2 cells, and this site contains overlapping putative ISRE/GAS elements (Figure 5.5A). Next, I was interested in whether STAT4 was necessary to induce IFN-α-mediated suppression. To test this hypothesis, I utilized a reporter gene driven by the 1.8 kb human *IL5* promoter to ask whether STAT4 was required to suppress reporter activation. T Ag-expressing Jurkat cells transfected with the hIL5pro-Fluc failed to upregulate firefly expression in response to TCR stimulation (Figure 5.13, Group 1). When I co-transfected a GATA3-GFP construct into the cells, the hIL5pro-Fluc reporter was expressed (Figure 5.13, Group 2), and the requirement of GATA3 to induce *IL5* gene expression has been observed previously (Kaminuma et al., 1999; Schwenger et al., 2001). However, this combination was relatively insensitive to IFN-α-mediated gene suppression. Only when I co-transfected STAT4-GFP into the cells was human *IL5* promoter activity suppressed in response to IFN-α treatment (Figure 5.13, Group 3), demonstrating that STAT4 is required for IFN-α-mediated suppression to occur. To further verify the requirement for STAT4 in this process, I tested whether STAT4 protein containing the point mutation R599K could ameliorate IFN-α-mediated suppression. This mutation has been shown to disrupt phosphotyrosine interactions within the SH2 domain of STAT4, preventing dimerization of activated STAT4 (Farrar et al., 2000a; Mayer et al., 1992). When I co-transfected cells with the STAT4 R599K mutant and

stimulated cells in the presence of IFN-α, I found that IFN-α-mediated suppression was reversed, and that the hIL5pro-Fluc gene was insensitive to IFN-α-mediated suppression (Figure 5.13, Group 4). This observation demonstrates that IFN-α-mediated activation of the STAT4 dimer acts in trans within the 1.8 kb *IL5* promoter to mediate gene suppression.

IFN-α does not induce epigenetic modifications to suppress IL5 and IL13 gene expression

It has been previously demonstrated that STAT binding at specific genes is required for the post-translational modification of histones, including the silencing mark, H3K27me3 (Wei et al., 2010). Furthermore, it has demonstrated that acute IFN-α treatment enhances both EZH2 binding and H3K27me3 modification at a promoter ISRE, which correlates with enhanced STAT2 binding (Testoni et al., 2011a). I was interested in whether a similar mechanism exists in Th2 cells that could be important for long-term suppression of these genes in response to IFN-α signaling. Furthermore, although post-translational modifications of histones and DNA methylation are important for stabilizing the transcript profile of daughter cells, I was interested in whether these modifications could be mediating acute IFN-α-mediated gene suppression. First, I used two inhibitors to determine whether histone deacetylase (HDAC) or DNA methyltransferase (DNMT) activity were required to suppress *IL5* and *IL13* gene expression. Trichostatin A (TSA) is a reversible HDAC inhibitor that mediates the suppression of all HDAC family members that utilize zinc in their catalytic site, which includes HDAC families I, II and IIa, but not III (Dokmanovic et al., 2007). Total memory $CD4^+$ T cells were pretreated with TSA for 45 min, and then stimulated in the presence or absence of IFN-α for 4 hrs, and gene expression was assessed by qPCR (Figure 5.14A). The presence of TSA inhibited IFN-α-

mediated enhancement of *IL4* and *IFNG* expression, however, IFN-α treatment still suppressed *IL5* and *IL13* gene expression (Figure 5.14A). Next, I asked whether preventing DNA methylation would ameliorate IFN-α-mediated gene suppression, so, I utilized the nucleoside analog 5-azacytidine (5-AzaC) to prevent DNA methylation. In order for 5-AzaC to function as an inhibitor, it must be incorporated into the DNA during cell replication, in which case it replaces the nucleotide cytidine (Christman, 2002). Thus, I polarized naïve $CD4^+$ T cells under Th2 conditions in the presence or absence of 5-AzaC, then acutely stimulated these cells in the presence or absence of IFN-α (Figure 5.14B). As demonstrated in Figure 5.14B, daily 5-AzaC treatment had no effect on IFN-α-mediated gene suppression. However, I expected that 5-AzaC treatment would enhance TCR-mediated gene expression, as preventing global DNA methylation should allow genes to be more sensitive to signals driving gene expression. 5-AzaC is extremely unstable and has a half-life of less than 12 hrs in aqueous solution at room temperature; thus, it may be the case that the concentration that I used was insufficient at integrating into the genome, and DNA methylation was actually unchanged.

In order to use a simpler culture system that was not prone to the non-specific effects of inhibitors, I next asked whether EZH2 binding was enhanced at different sites within the *IL5* and *IL13* gene loci in response to acute IFN-α treatment. *In vitro* polarized Th2 cells were stimulated in the presence or absence of IFN-α for 2 hrs and relative EZH2 binding was interrogated by ChIP-qPCR (Figure 5.15). In the four donors utilized for these experiments, IFN-α did not seem to consistently enhance EZH2 binding at the proximal *IL5* promoter, however, there was a trend of enhanced EZH2 binding at the distal *IL5* promoter site, as well as at the IL13 3' locus site (Figure 5.15). I used two different ISG promoters at an attempt to find a site that would theoretically have reduced EZH2 binding in response to IFN-α signaling (*MXB* and *ISG54*).

However, only in one donor was EZH2 binding significantly suppressed in response to IFN-α signaling (Figure 5.15, last column). Perhaps a more global approach needs to be taken in order to get a clear indication of the EZH2 binding patterns in response to IFN-α. It could be the case that I am simply not looking at the correct sites by qPCR, and other regions of the gene loci might be affected by IFN-α signaling and have enhanced or reduced EZH2 binding. It is interesting, however, that in Donor B, IFN-α treatment enhanced EZH2 binding at the *IL13* 3' locus site, but this did not correlate with enhanced binding of STAT2 at this site (Figures 5.15 and 5.10). There are likely STAT-independent signals that drive epigenetic changes at these genes that control Th2 cytokine expression, which has been described previously with regard to Th1 and Th2 development (Wei et al., 2010). Taken together, this data indicates that histone deacetylase activity, and possibly DNA methyltransferase activity, are not required for acute IFN-α-mediated gene suppression. More in depth experiments will be necessary to determine whether IFN-α-signaling mediates EZH2 activity in this system, similar to other reports in the literature (Testoni et al., 2011a).

Murine Th2 cells are unresponsive to IFN-α-mediated IL5 gene suppression

The data in Figure 5.5B demonstrate that STAT4 is able to bind to the ISRE/GAS cis regulatory element present within the proximal human *IL5* promoter, which has been validated in a whole-cell system using ChIP-qPCR (Figure 5.11). I was interested in whether this observation could be phenocopied in the murine system. It has been reported previously that mouse Th2 development is enhanced in response to IFN-α, whereas human Th2 polarization is potently suppressed in response to this cytokine (Huber et al., 2010; Szabo et al., 1997; Wenner et al.,

1996), but there are no reports indicating the role of acute IFN-α signaling in committed murine Th2 cells. I performed an *in silico* analysis to compare the murine and human *IL5* gene loci, and identify potential STAT binding motifs within the region (Figure 5.16A and 5.16B). The murine and human *IL5* gene promoters are relatively non-conserved, with the exception of a handful of sites that are conserved noncoding sequences (CNS), which reach 70 percent or greater sequence homology. Within the ~2.5 kilobase promoter of the *IL5* gene, there are numerous putative STAT binding sites, including both GAS elements and ISREs, although there are considerably less ISRE than GAS sites within the region (Figure 5.16A). Conservation of a fraction of these putative sites can be seen especially for STAT1, STAT3 and STAT4 binding sites, however, even fewer ISREs are conserved between the two species (Figure 5.16A). Interestingly, despite the relatively abundant sequence conservation between the human and mouse sequences just upstream of the transcriptional start site, the GAS and ISRE sites within this region were not conserved between the two species as determined using Jasper (Figure 5.16B). Although many canonical activation elements are relatively well conserved between both species (NFAT, AP-1/Ets, GATA3 sites), the ISRE/GAS element present within the human *IL5* promoter is disrupted in the murine sequence, with seven nucleotide additions (Figure 5.16B; in red, lowercase) and nine nucleotide differences (Figure 5.16B; in red, uppercase). Based on these species-specific differences, I was interested in determining whether IFN-α-mediated gene suppression was absent in the murine system.

First, I asked whether murine Th2 cells were sensitive to IFN-α, similar to human Th2 cells. Murine $CD4^+$ T cells were polarized under Th2 conditions, and then restimulated in the presence or absence of IFN-α for 24 hrs. Murine Th2 cells were responsive to IFN-α; however, IL-5 protein expression was enhanced in response to acute IFN-α signaling, while there was no

change in IL-4 or IFN-γ production (Figure 5.17). This observation demonstrates that IFN-α signaling is insufficient to suppression IL-5 expression in murine Th2 cells. In addition to this phenotypic difference, I wished to determine whether there was biological significance to the disrupted ISRE/GAS cis element within the mouse Il5 promoter (Figure 5.16B). I assessed IFN-α-mediated STAT binding in murine splenocytes by gel retardation assay using the murine Il5 promoter probe equivalent to the human ISRE/GAS sequence (Figure 5.16C). Although murine nuclear lysate proteins bound to the EαY probe, no binding was detected using the murine Il5 probe, even when film was exposed 10 times longer than the time sufficient to detect protein binding to the control probe (Figure 5.18A). This result suggests that there are insufficient IFN-α-mediated STAT proteins in the nucleus to bind to the murine Il5 probe, or that the murine sequence does not bind STAT proteins. To validate the EMSA results, I performed ChIP-qPCR using murine Th2 cells to determine whether IFN-α signaling was able to enhance STAT2 or STAT4 binding to the murine Il5 promoter in a whole-cell system. Murine splenocytes were polarized under Th2 conditions and then acutely stimulated in the presence or absence of IFN-α for 2 hrs. Compared to TCR-stimulation, IFN-α-treatment did not enhance STAT2 or STAT4 binding within the mouse Il5 promoter, although STAT2 did bind to a canonical ISRE located within the promoter of the antiviral gene, Oas1b (Figure 5.18B, (Pulit-Penaloza et al., 2012)). Furthermore, I tested whether a known IL-12-induced, STAT4-sensitive gene could be bound by STAT4 in response to a IFN-α signaling (O'Sullivan et al., 2004). The murine Cd25 promoter failed to be bound by STAT4 in response to IFN-α signaling. This observation is consistent with the reports demonstrating that the IFN-α signaling pathway in mouse T cells is insufficient at activating STAT4 compared to IFN-α signaling in human T cells (Berenson et al., 2004; 2006; Farrar et al., 2000b).

Our ChIP-qPCR findings demonstrate a species-specific binding pattern within the proximal human *IL5* promoter in response to IFN-α signaling. Specifically, human STAT4 and potentially STAT1 bind the human *IL5* ISRE/GAS regulatory element (Figure 5.5B and 5.11), while mouse STAT4 (and STAT2) do not bind to the aligned murine sequence (Figure 5.18). Based on this observation, and the fact that there are a number of differences within the *IL5* promoter between the two species, we wished to determine whether human STAT proteins activated in response to IFN-α could bind the murine Il5 promoter sequence, and whether any was any IFN-α-induced STAT4 activated in the murine system could bind to the human *IL5* promoter probe. We utilized EMSA to address this question, and compared the binding patterns of human and mouse nuclear lysates to both the human and mouse IL-5 probes. This experiment address two questions: 1) is the murine Il5 probe able to bind available STAT proteins, and 2) can murine STAT proteins activated in response to IFN-α bind the human *IL5* probe. Although both human and mouse nuclear protein lysates from cells stimulated in the presence or absence of IFN-α bound to the EαY control probe, we found a defect in the ability of murine lysates to bind both the murine and human IL5 probes (Figure 5.19). Human lysates from cells stimulated in the presence of IFN-α were able to bind to the human *IL5* probe, but not the murine Il5 probe, suggesting that the sequence differences are sufficient to prevent protein binding to this region (Figure 5.19). Furthermore, murine lysates from cells stimulated in the presence of IFN-α were unable to bind to the human *IL5* probe, which indicates that IFN-α signaling in murine cells fails to induce STAT4 (or other proteins') translocation to the nucleus, despite reports demonstrating that the murine IFN-α-signaling pathway can induce some STAT4 activation, although very minimally compared to human cells (Berenson et al., 2004).

In addition to this apparent STAT4-dependent regulation reported in Figure 5.13, I was interested in whether disruption of the human GAS/ISRE sequence within the human *IL5* promoter would abolish IFN-α-mediated suppression of the human *IL5* gene. To test this, I created five human IL5pro-Fluc mutants, each of which contained one or more human-to-mouse mutations of the proximal GAS/ISRE/GAS elements within the human *IL5* promoter (Figure 5.16B). I co-transfected each mutant or wild-type reporter into T-Ag-expressing Jurkat cells co-transfected with GATA3-GFP and STAT4-GFP expression plasmids and acutely stimulated cells for 6 hrs in the presence or absence of IFN-α. Interestingly, none of the mutants were able to reverse IFN-α-mediated suppression (Figure 5.20). However, mutant 5 seemed to drive enhanced expression compared to the WT or other mutant IL5pro reporters, although this could be due to differences between transfection efficiencies rather than functions of the specific DNA sequences. This result indicates that the human proximal GAS/ISRE elements are not required for IFN-α-induced suppression of TCR-mediated human *IL5* promoter activity. It could be the case that either this site is not the cis regulatory element required to suppress gene expression, or there are multiple and perhaps redundant elements whose binding contribute to IFN-α-mediated gene suppression in the human system.

Discussion

In this chapter, the data demonstrate that IFN-α-mediated *IL5* and *IL13* gene suppression occurs by suppressing nascent transcription of these genes (Figure 5.4). Furthermore, IFN-α induces STAT4 signaling, which was confirmed by EMSA and ChIP-qPCR (Figure 5.5 and 5.11). Using a reporter assay, I found that STAT4 is necessary for IFN-α-mediated suppression of the human *IL5* gene, and a STAT4 mutant that prevents STAT4 dimerization prevents IFN-α-mediated suppression (Figure 5.13). Further, I found that the *IL5* promoter is bound by STAT4 in a species-specific manner, and murine cells both fail to induce STAT4 mobilization, and are unable to bind STAT proteins to the proximal Il5 promoter (Figures 5.18 and 5.19). However, this disruption of the human *IL5* promoter ISRE/GAS element in the mouse sequence does not ameliorate IFN-α-mediate *IL5* gene suppression in the human system (Figure 5.20). To my knowledge, this is the first report to demonstrate that IFN-α-mediated STAT4 activation directly acts to suppress gene expression in human Th2 cells.

By using ChIP-Seq to identify potential binding sites of STAT2 and STAT4 in response to IFN-α treatment in Th2 cells, I was able to look at specific sites that might be utilized to induce gene suppression. However, there are several caveats to the experimental approach of the ChIP-Seq experiments. First, based on the binding patterns between the two donors for STAT2 and the apparent lack of shared binding sites within the Th2 locus, I was concerned about how to interpret the observed STAT binding sites (Figure 5.8). Although there is likely donor-to-donor variability in STAT binding sites due to single-nucleotide polymorphisms, or the overall responsiveness to IFN-α signaling, it will be important to use more donors to determine whether any of these bound STAT sites are conserved across a population of individuals. Many factors

could play a role in whether a donor's Th2 cells are responsive to IFN-α-mediated regulation. Although our donors did not have severe allergies or asthma, and were chosen based on the general health criteria listed in Chapter 2 'Human Donors,' a full health history was not documented. Future experiments should utilize more donors for ChIP-Seq to look at STAT4, EZH2 and H3K27me3 modulation in response to acute IFN-α signaling in pre-committed Th2 cells. As with the RNA-Seq data, the population of cells utilized for the ChIP-Seq experiments were heterogeneous, with only a fraction of the final cell population being Th2 cells, as determined by CRTH2 surface expression. The results of these experiments would be more interpretable if Th2 cells were sorted by CRTH2 positivity, and only these cells were acutely stimulated in the presence or absence of IFN-α. Further, it will be important to use a more sensitive library preparation kit so that the small amount of DNA bound by STAT4 in the absence of IFN-α treatment can be utilized for sequencing experiments.

The data in Figure 5.13 demonstrate the role of STAT4 in IFN-α-mediated gene suppression (Figure 5.13). Although this experiment demonstrates the requirement for activated STAT4, it would be useful to demonstrate that human Th2 cells require STAT4 to be sensitive to IFN-α-mediated gene suppression. Utilizing a siRNA-mediated knockdown of STAT4 could demonstrate the requirement of STAT4 in driving *IL5* and *IL13* gene suppression. Because the STAT proteins are expressed in the absence of specific signaling cues, the siRNA-mediated approach could be ineffective, in which case a CRISPR/Cas9-mediated knockdown could be utilized, as reagents to knockdown STAT4 using both methods exist. Alternatively, the overexpression of a dominant negative STAT4 protein, perhaps the STAT4-R599K mutant, would also demonstrate the requirement for this signaling pathway in suppressing gene expression. The presence of phosphorylated STAT4 in response to IFN-α has been detected in

human Th2 cells previously (Rogge et al., 1998), and the data in Figure 5.11 demonstrate that it is functionally able to bind chromatin in response to IFN-α treatment. However, in the same report, they found that human Th2 cells are unresponsive to IL-12 treatment, and phosphorylation of STAT4 is absent in response to acute IL-12 treatment (Rogge et al., 1998). Thus, it is likely that the results in Figure 5.12 can be explained by the lack of the IL-12 receptor expressed on Th2 cells.

The biological significance of why mouse Th2 cells are insensitive to IFN-α-mediated gene suppression is unknown, but our lab and other groups have provided evidence for why this mechanism is species-specific. The presence of the IFN-α/β signaling pathway is conserved between humans and mice, however, there are differences in the IFNAR chains and the STAT2 protein that make these three proteins relatively unconserved between the two species (Park et al., 1999; Paulson et al., 1999; Tyler et al., 2007). STAT2 is 69% conserved between mouse and human, with the C-terminal end sharing only 29% homology between the two species (Farrar et al., 2000a). This species-specific difference is in part due to a minisatellite insertion within the C-terminus of the mouse STAT2 protein that is absent in the human protein (Farrar et al., 2000a). As the C-terminus has been shown to be critical in recruiting STAT4 to the IFNAR/STAT2 complex, it is possible that this difference alone could account for the lack of IFN-α-mediated STAT4 activation in murine cells (Farrar et al., 2000a; 2000b). However, a murine transgenic STAT2 protein containing the human C-terminus was unable to restore IFN-α-mediated STAT4 activation in mouse cells (Persky et al., 2005). In addition to the requirement of STAT2 in recruiting STAT4, the IFNAR2 chain itself was shown to play a role in STAT4 recruitment (Tyler et al., 2007). The cytoplasmic domains of the human and murine IFNAR1 and IFNAR2 chains share only 38% and 48% sequence homology, respectively (Tyler et al., 2007).

Although it was demonstrated that none of the cytoplasmic phosphorylated tyrosine residues of the human IFNAR chains interacted with the SH2 domain of the STAT4 protein (Farrar et al., 2000b), work by our laboratory demonstrated that the N-terminal domain of STAT4 pre-associates with the IFNAR2 cytoplasmic chain in a species-specific manner (Tyler et al., 2007). The biological requirement for this N-terminal STAT4 interaction has been shown previously with regard to IL-12 signaling, whereby a STAT4 transgenic protein lacking the N-terminal domain failed to induce IL-12 mediated STAT4 phosphorylation and subsequent IFN-γ production (Chang et al., 2003). Taken together, these findings demonstrate two species-specific differences that play a role in preventing IFN-α-mediated STAT4 activation in the murine system.

In Figure 5.19, I tested whether murine lysates were able to bind to the human *IL5* probe (Figure 5.19). Because STAT4 is not activated in response to IFN-α treatment in murine cells, I expected to see very little binding to the human probe, which is what was observed. However, it would be interesting to determine whether the murine STAT4 protein is able to bind to this site, as well as the aligned site in the mouse Il5 promoter, despite the disruption of the GAS/ISRE element (Figure 5.16B and 5.16C). This experiment would unambiguously demonstrate the sequence differences between the two species result in differential STAT binding to this region. This experiment could be conducted *in vivo* utilizing a murine transgenic animal containing the human Th2 locus using a yeast artificial chromosome. Such systems have been utilized previously to identify conserved mammalian sites of the Th2 locus that coordinately regulate the Th2 cytokine genes (Loots et al., 2000). This system, paired with overexpression of STAT4 *in vitro*, could tell us whether the lack of STAT4 activation is what is solely responsible for the absence of this regulatory system in murine cells, or if there are also cis regulatory differences

within the murine system that provide additional insurance to prevent STAT4-mediated suppression of these genes.

Although the data in Figure 5.13 demonstrate that STAT4 regulates the human *IL5* promoter, we did not interrogate the *IL13* or *IL4* promoters in this study. It would be interesting to determine whether there are cis regulatory elements in each of these promoters, or gene loci, that are responsible for the discordant regulation of these cytokine genes, or if there is a single locus control region-type element that suppresses both *IL5* and *IL13*, but not *IL4*. It is apparent that the cis regulatory region required to suppress the *IL5* promoter exists within the 1.8 kb promoter utilized in Figure 5.13, or IFN-α treatment would not have suppressed the expression of the reporter gene. Despite my attempt to disrupt a single site with the mutation of the human-to-murine sequence, a better approach would be to truncate the promoter in sections, which would indicate which cis-regulatory element is required, and whether there are redundant elements within the promoter that can work in concert to suppress gene expression in response to IFN-α signaling.

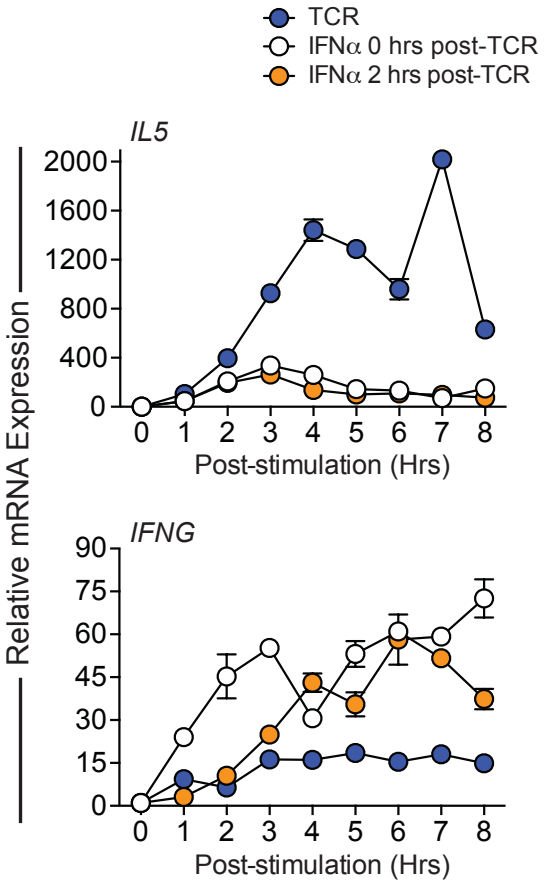


Figure 5.1: IFN-α induces robust suppression of human *IL5* expression. Bulk human lymphocytes were stimulated with plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml), and IFN-α (1000 U/ml) was added either with TCR stimulation (white), or 2 hrs post-TCR stimulation (orange). Cells were harvested every hour for qPCR analysis. Each treatment is relative to its own 0-hr time point and analyzed relative to the reference gene, human *PPIA*.

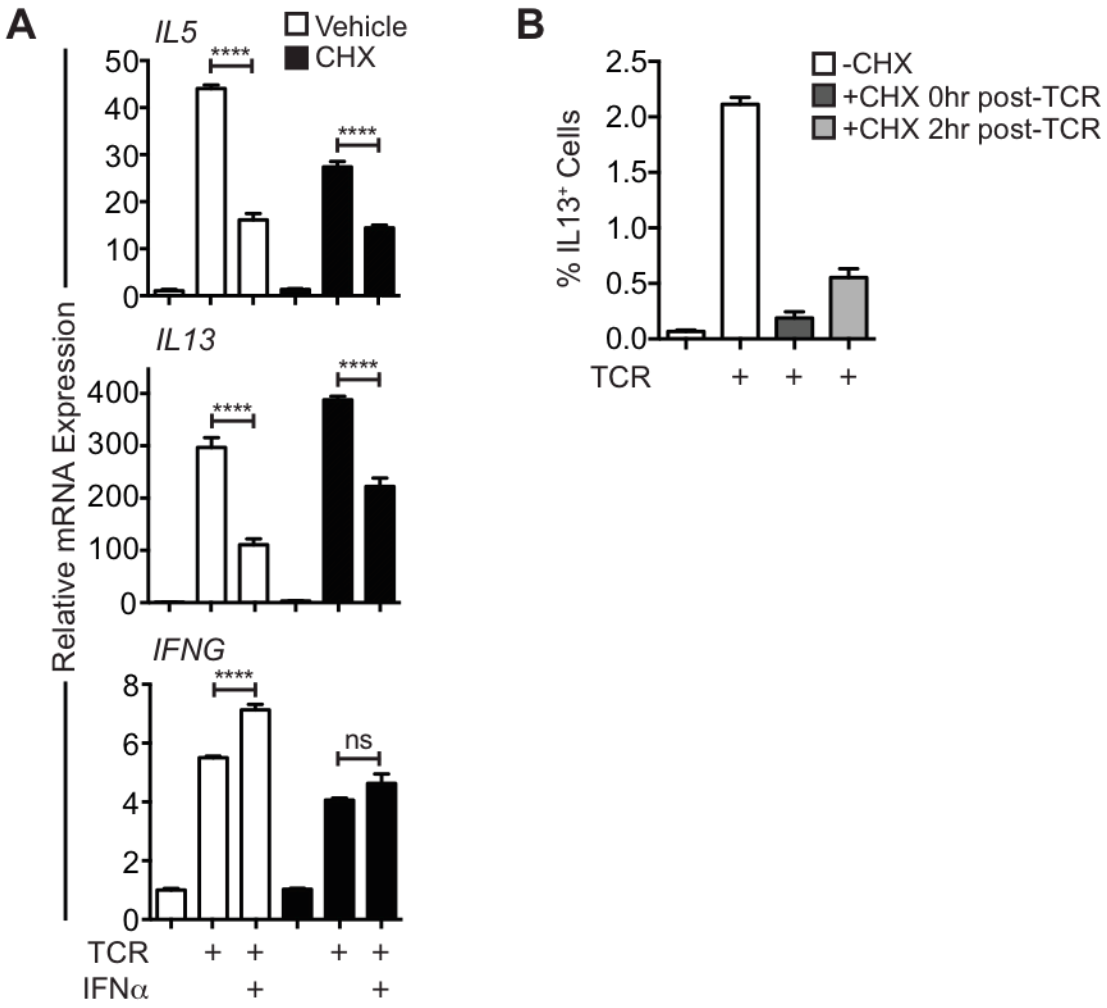
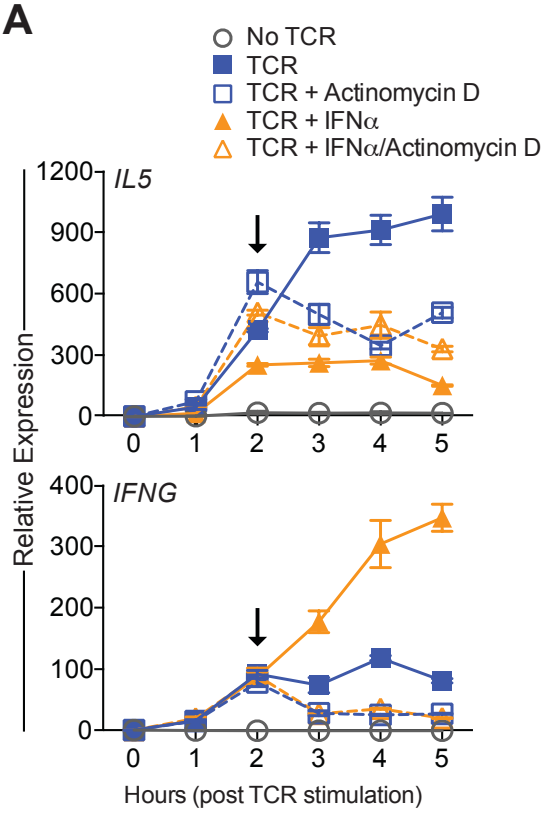


Figure 5.2: IFN-α-mediated suppression of *IL5* and *IL13* gene expression does not require the nascent translation of an ISG. Human $CD4^+CD45RO^+$ T cells were stimulated using plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) for a total of 4 hrs. IFN-α and CXH was added 2 hrs post-TCR stimulation. Cells were harvested for qPCR analysis. Vehicle or CHX samples are analyzed relative to their respective 'No TCR' controls by comparing to the reference gene, *PPIA*. Two-way ANOVA with a Bonferroni post-hoc test was used to determine statistical significance. * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

A



B

IL5	
Treatment	**Slope and y intercept**
TCR	y = 173.91x + 363.7
TCR + ActinoD	y = -59.98x + 652.3
TCR + IFN-α	y = -29.06x + 308.0
TCR + IFN-α/ActinoD	y = -47.49x + 538.9

IFNG	
Treatment	**Slope and y intercept**
TCR	y = 1.19x + 88.5
TCR + ActinoD	y = -15.52x + 78.9
TCR + IFN-α	y = 90.48x + 2.2
TCR + IFN-α/ActinoD	y = -19.98x + 93.5

Figure 5.3: IFN-α-mediated gene suppression is not mediated through enhanced transcript decay. Bulk human lymphocytes were stimulated for a total of 5 hrs with plate-bound anti-hCD3 (3 µg/ml) and rhIL-2 (50 U/ml). 2 hrs post-TCR stimulation, IFN-α (1000 U/ml) and/or actinomycin D (10 µg/ml) were added to the cultures (black arrow). Cells were harvested every hour for qPCR analysis. (A) Each treatment is compared relative to its own 0-hr time point and analyzed relative to the reference gene, human *PPIA*. (B) Linear regression was performed for each treatment using relative expression from the 2 hr time-point through the 5 hr time-point. The slope of a linear regression analysis was used to determine whether the rate of mRNA decay was changing in response to IFN-α treatment.

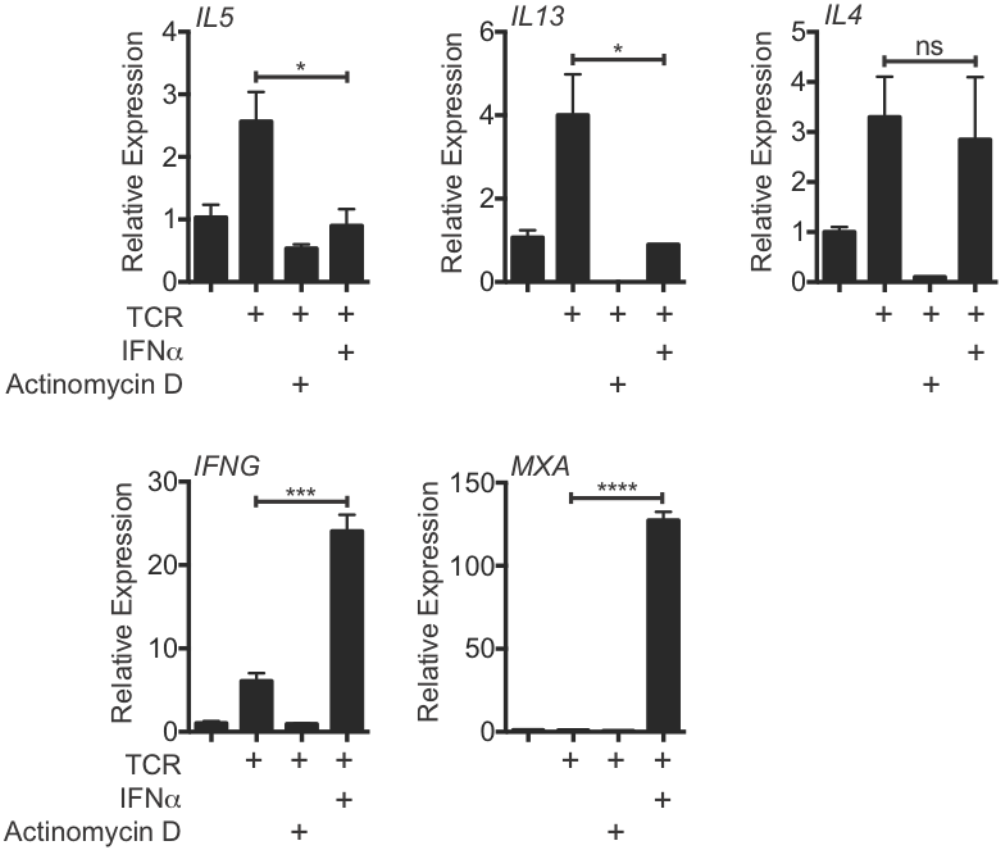


Figure 5.4: IFN-α signaling reduces nascent transcription of the *IL5* and *IL13* genes. *In vitro* polarized human Th2 cells were restimulated with plate-bound anti-hCD3 (3 µg/ml) and rhIL-2 (50 U/ml) for 2 hrs in the presence or absence of IFN-α (1000 U/ml) and actinomycin D (10 µg/ml). Nuclei were subjected to *in vitro* transcription to label all nascent transcripts with biotin-UTP. qPCR was used to analyze the rate of nascent transcription in response to IFN-α treatment. Each treatment is relative to the 'No TCR' control. All qPCR experiments were analyzed relative to the reference gene, human *PPIA*. One-way ANOVA with a Bonferroni post-hoc test were used to determine statistical significance. * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

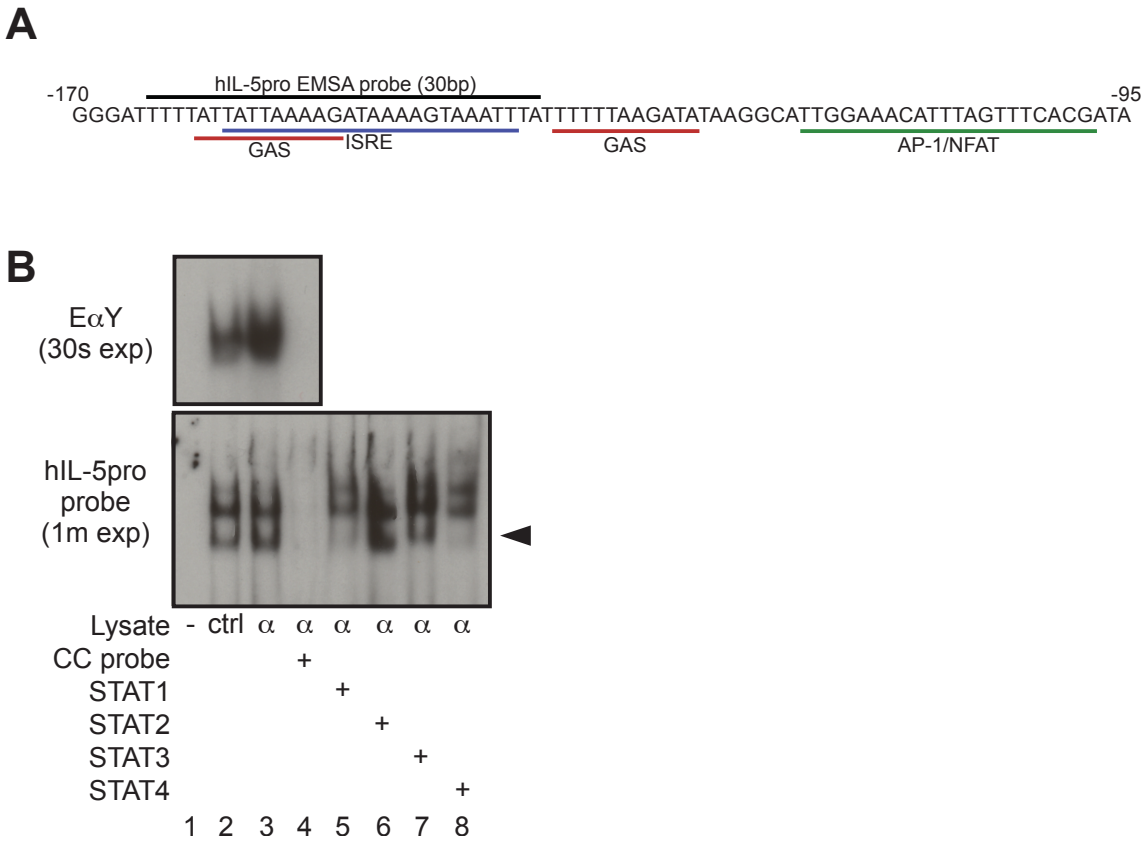


Figure 5.5: STAT1 and STAT4 bind the proximal human *IL5* promoter cis regulatory element. (A) The human sequence of the *IL5* proximal promoter and the DNA probe utilized for EMSA experiments. (B) Whole human PBMCs were stimulated with plate-bound anti-hCD3 (3μg/ml) and rhIL-2 (50 U/ml) for 2 hrs in the presence (α; lanes 3-8) or absence (ctrl; lane 2) of IFN-α (1000 U/ml). Nuclear lysates were utilized for *in vitro* binding assays to the EαY or hIL-5pro probe in the presence or absence of STAT proteins (lanes 5-8) or a cold competitor probe (CC, lane 4). Arrows indicate super-shifted proteins.

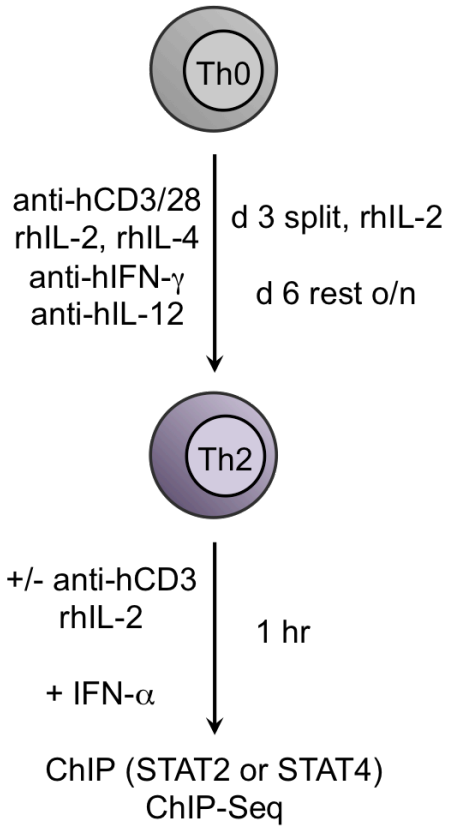


Figure 5.6: Experimental design of the ChIP-Seq experiments. Naïve $CD4^+$ T cells from two donors were polarized in the presence of the indicated cytokines, and Th2 cells were treated with IFN-α in the presence or absence of TCR stimulation for 1 hr. ChIP using STAT2 or STAT4 antibodies were used, and sequencing libraries were created in house for Illumina sequencing.

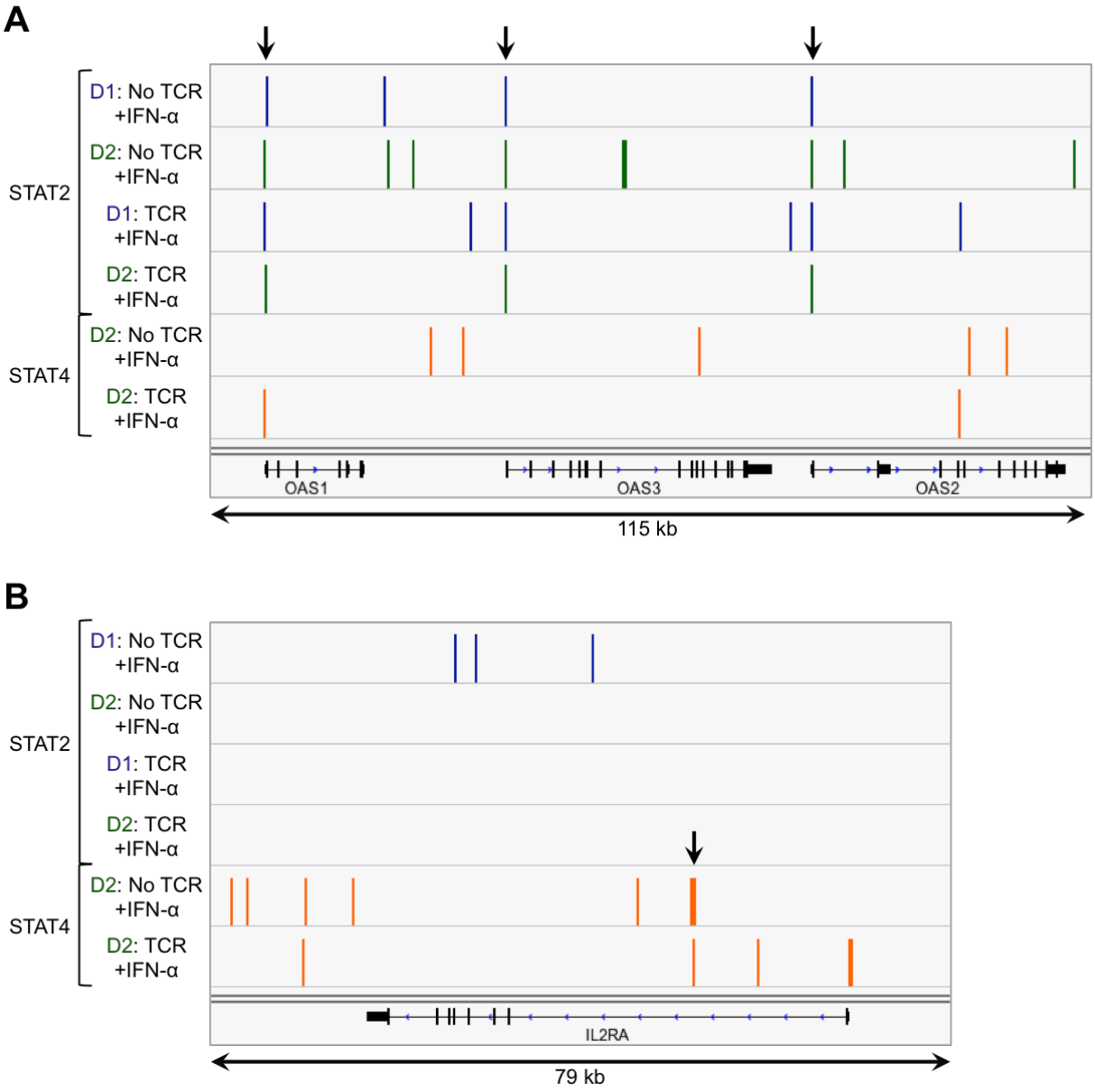


Figure 5.7: STAT2 and STAT4 binding patterns within the human *OAS* and *CD25* gene loci. DNA from immunoprecipitated chromatin was prepared as described in Figure 5.6 and Chapter 2 '*Next Generation Sequencing of Chromatin Immunoprecipitated DNA (ChIP-Seq)*'. Sequenced DNA was aligned to the hg19 genome using Bowtie, and MACS14 was used to create a peak model of all sequence reads to define enriched regions of protein binding. Blue bars correspond to Donor 1 STAT2 peaks, while green bars correspond to Donor 2 STAT2 peaks. Orange bars correspond to Donor 2 STAT4 peaks. All peaks are displayed on the same scale. (A) The human *OAS* gene locus is bound by STAT2 in all samples, while (B) the *IL2RA/CD25* gene is bound by STAT4 in all samples. Arrows indicate binding of interest.

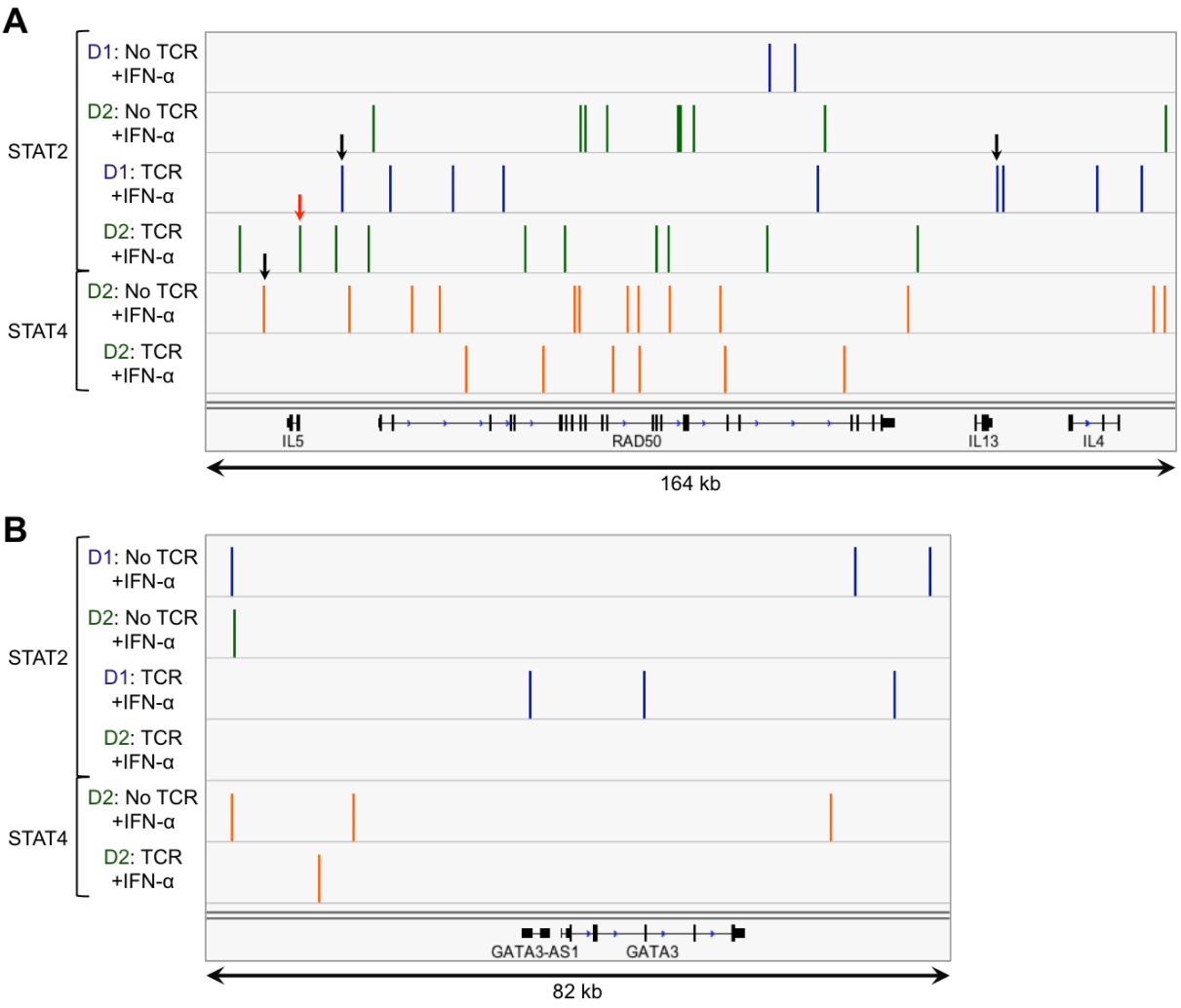


Figure 5.8: STAT2 and STAT4 binding patterns within the human Th2 cytokine and GATA3 gene loci. DNA from immunoprecipitated chromatin was prepared as described in Figure 5.6 and Chapter 2 '*Next Generation Sequencing of Chromatin Immunoprecipitated DNA (ChIP-Seq)*'. Sequenced DNA was aligned to the hg19 genome using Bowtie, and MACS14 was used to create a peak model of all sequence reads to define enriched regions of protein binding. Blue bars correspond to Donor 1 STAT2 peaks, while green bars correspond to Donor 2 STAT2 peaks. Orange bars correspond to Donor 2 STAT4 peaks. All peaks are displayed on the same scale. (A) The Th2 gene locus and (B) the GATA3 locus with respective binding for STAT2 and STAT4. All arrows (black and red) indicate sites validates by ChIP-qPCR, while the red arrow indicates the site interrogated by EMSA in Figure 5.5B.

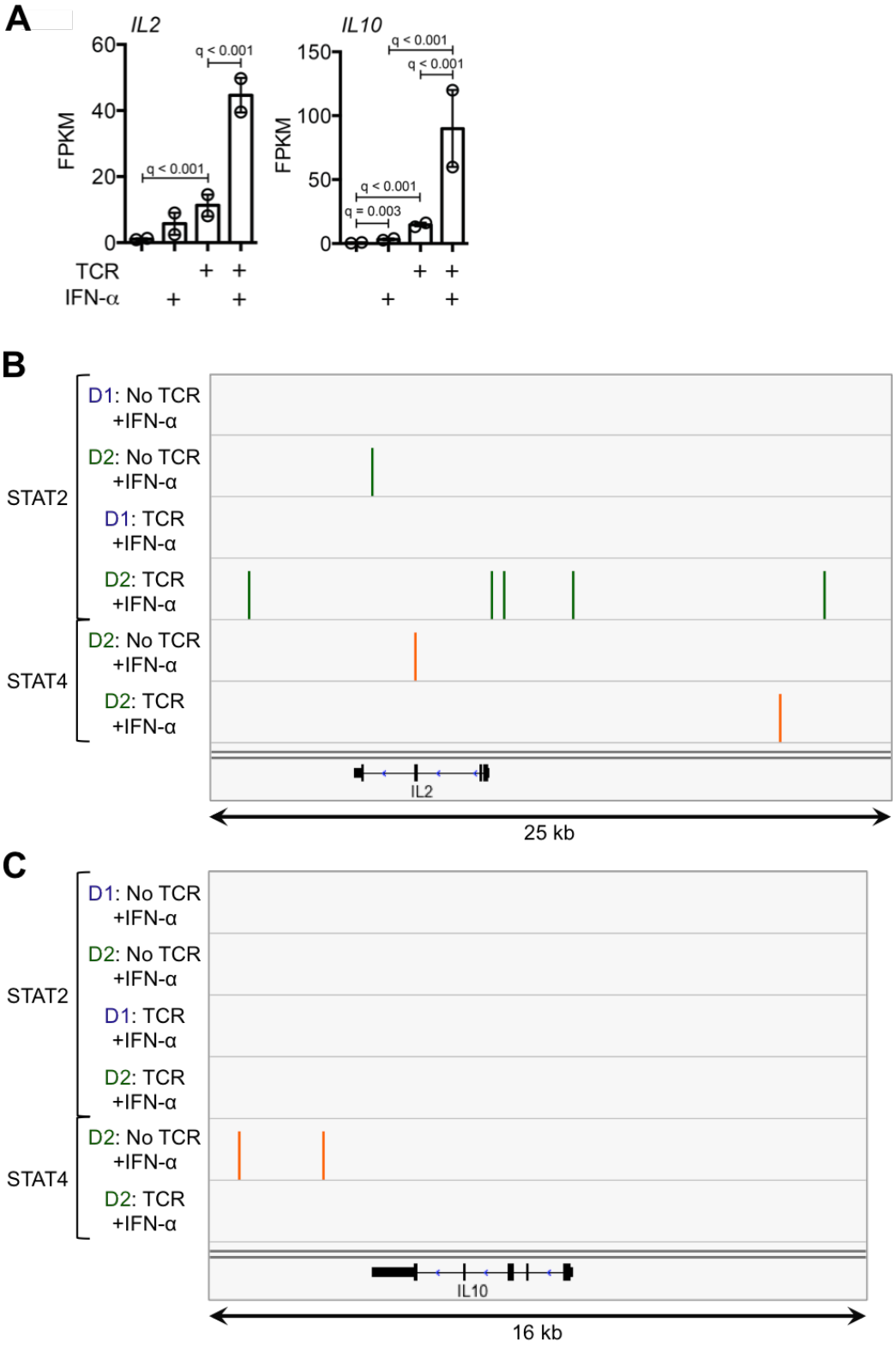

A
IL2
IL10
FPKM
60
40
20
0
150
100
50
0
q < 0.001
q < 0.001
q < 0.001
q < 0.001
q = 0.003
TCR
IFN-α
+
+
+
+
+
+
B
STAT2
STAT4
D1: No TCR +IFN-α
D2: No TCR +IFN-α
D1: TCR +IFN-α
D2: TCR +IFN-α
D2: No TCR +IFN-α
D2: TCR +IFN-α
IL2
25 kb
C
STAT2
STAT4
D1: No TCR +IFN-α
D2: No TCR +IFN-α
D1: TCR +IFN-α
D2: TCR +IFN-α
D2: No TCR +IFN-α
D2: TCR +IFN-α
IL10
16 kb

Figure 5.9: Correlation of IFN-α-sensitive transcription of *IL2* and *IL10* with STAT2 and STAT4 binding by ChIP-Seq. DNA from immunoprecipitated chromatin was prepared as described in Figure 5.6 and Chapter 2 '*Next Generation Sequencing of Chromatin Immunoprecipitated DNA (ChIP-Seq)*'. Sequenced DNA was aligned to the hg19 genome using Bowtie, and MACS14 was used to create a peak model of all sequence reads to define enriched regions of protein binding. Blue bars correspond to Donor 1 STAT2 peaks, while green bars correspond to Donor 2 STAT2 peaks. Orange bars correspond to Donor 2 STAT4 peaks. All peaks are displayed on the same scale. (A) Transcript expression from the RNA-Seq experiment in Chapter 4. STAT-bound sites within the (A) *IL2* and (B) *IL10* gene loci.

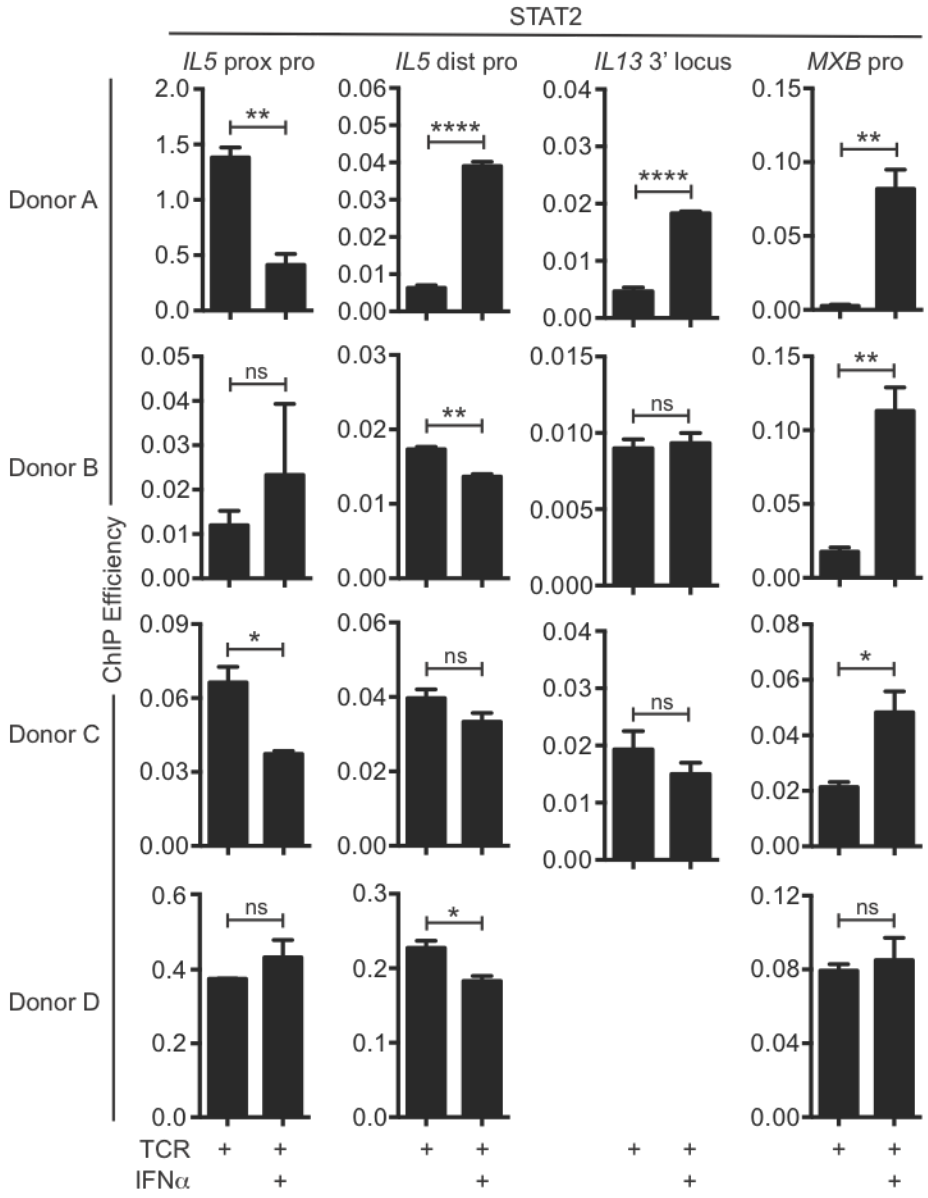


Figure 5.10: IFN-α-mediated STAT2 binding in multiple donors by ChIP-qPCR. *In vitro* polarized human Th2 cells were restimulated with plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) for 2 hrs in the presence or absence of IFN-α (1000 U/ml), and ChIP lysates were prepared and probed for STAT2 binding to DNA. qPCR was used to analyze the human *IL5* proximal and distal sites, the *IL13* 3' locus, and the human *MXB* promoter. ChIP efficiencies were determined as described in the methods section (Chapter 2). The four donors shown are representative of all the donors performed for this experiment. Student's t-test was used to determine significance; * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

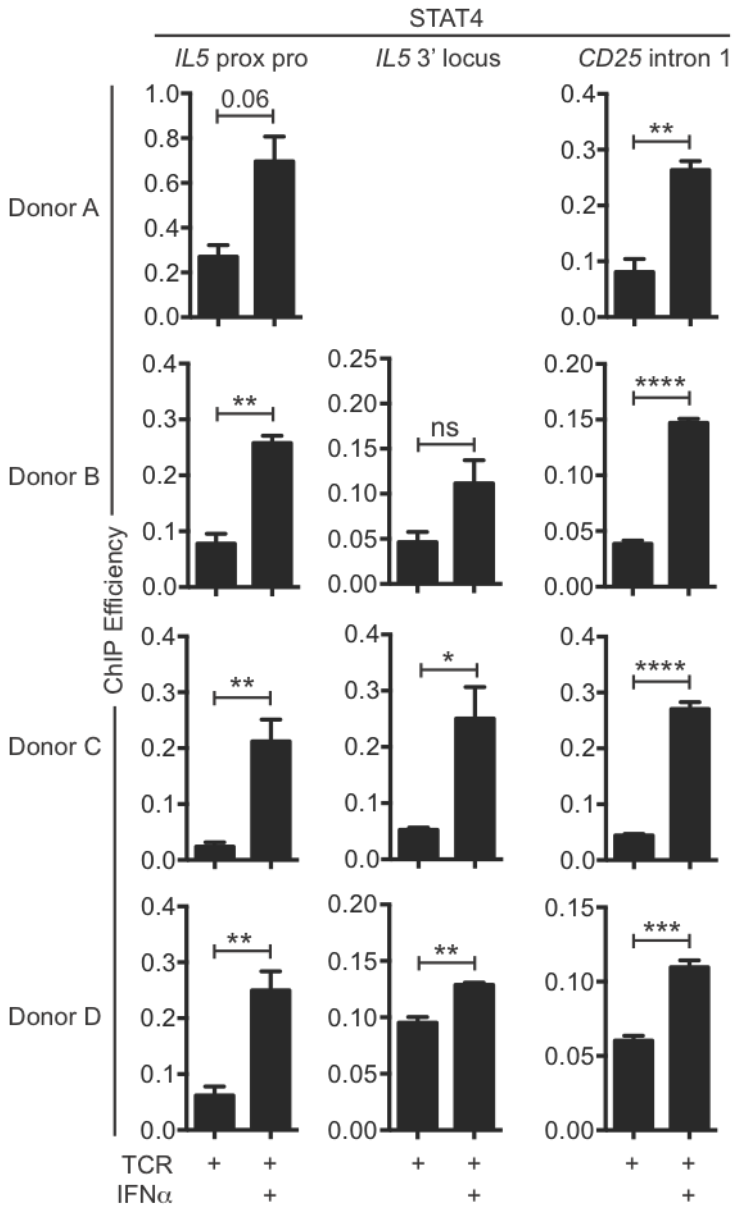


Figure 5.11: IFN-α-mediated STAT4 binding in multiple donors by ChIP-qPCR. *In vitro* polarized human Th2 cells were restimulated with plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) for 2 hrs in the presence or absence of IFN-α (1000 U/ml), and ChIP lysates were prepared and probed for STAT4 binding to DNA. qPCR was used to analyze the human *IL5* proximal and 3' locus sites, and the human *CD25* intron 1 site as a positive control. ChIP efficiencies were determined as described in the methods section (Chapter 2). Student's t-test was used to determine significance; * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

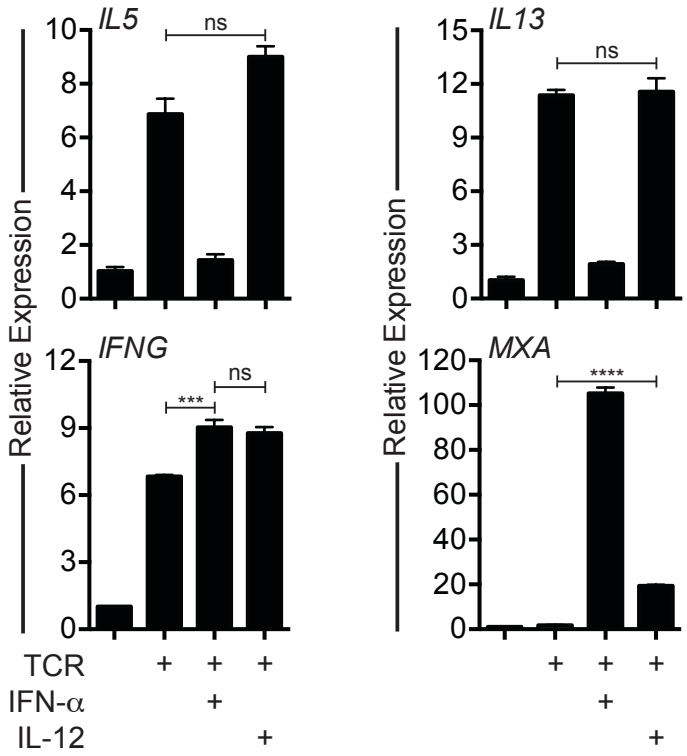


Figure 5.12: IL-12 treatment is insufficient in suppressing *IL5* and *IL13* gene expression. Human $CD4^{+}CD45RO^{+}$ T cells were stimulated using plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) for a total of 4 hrs in the presence or absence of IFN-α or IL-12. Each sample was analyzed relative to their respective 'No TCR' controls and compared to the reference gene, *PPIA*. One-way ANOVA with a Bonferroni post-hoc test was used to determine statistical significance. * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

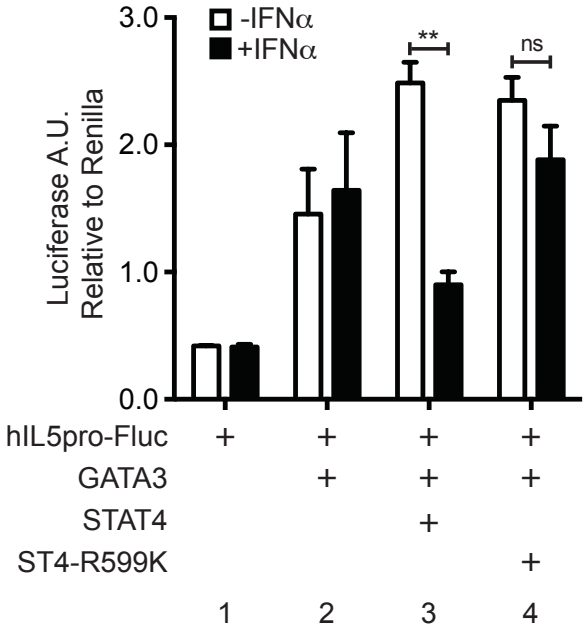


Figure 5.13: STAT4 is required for IFN-α-mediated *IL5* suppression. T-Antigen-expressing Jurkat cells were utilized for all transfections. Cells were transfected with a total of 28 ug DNA by electroporation and rested 24 hrs. Cells were stimulated with soluble anti-hCD3 (1:1000) and rhIL-2 (50 U/ml) in the presence or absence of IFN-α for 6 hrs, and luciferase and renilla were quantified. (A) GATA3-GFP, human IL5pro-fluciferase, and CMVpro-renilla were co-transfected into cells in the presence or absence of murine STAT4-GFP, murine STAT4-R599K-GFP, then stimulated as described above, and the reporter expression off the IL5 promoter was assessed. Data representative of three individual experiments, and are plotted as the ratio of luciferase to renilla signal. Two-way ANOVA with a Bonferroni post-hoc test were used to determine statistical significance. * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

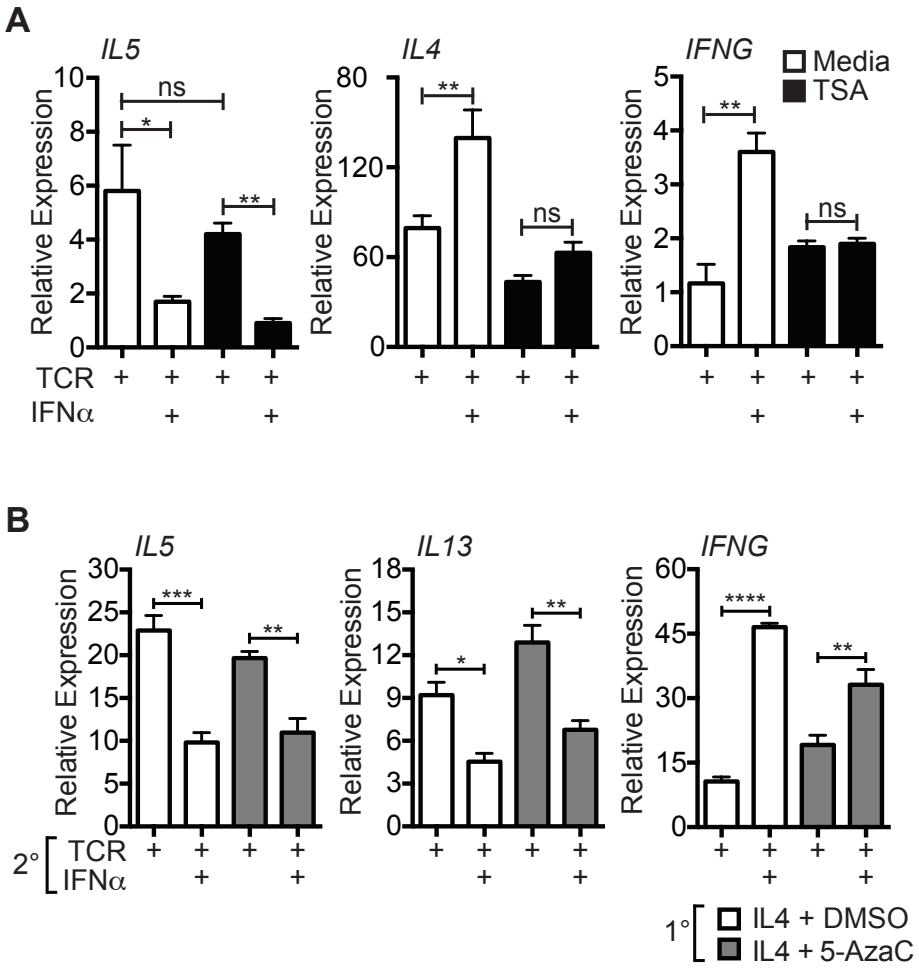


Figure 5.14: IFN-α signaling is sufficient to suppress *IL5* gene expression in the presence of a histone deacetylase inhibitor. (A) Human $CD4^{+}CD45RO^{+}$ T cells were pretreated with TSA (10 nM) for 45 min, then stimulated with plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) in the presence or absence of IFN-α (1000 U/ml) for 4 hrs. Each sample was analyzed relative to their respective 'No TCR' controls and compared to the reference gene, *PPIA*. (B) *In vitro* polarized human Th2 cells where provided DMSO or 5-AzaC daily during the 7 d polarization. Cells were restimulated with plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) for 4 hrs in the presence or absence of IFN-α (1000 U/ml). Each sample was analyzed relative to their respective 'No TCR' controls and compared to the reference gene, *PPIA*. Two-way ANOVA with a Bonferroni post-hoc test was used to determine statistical significance in (A) and (B). * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

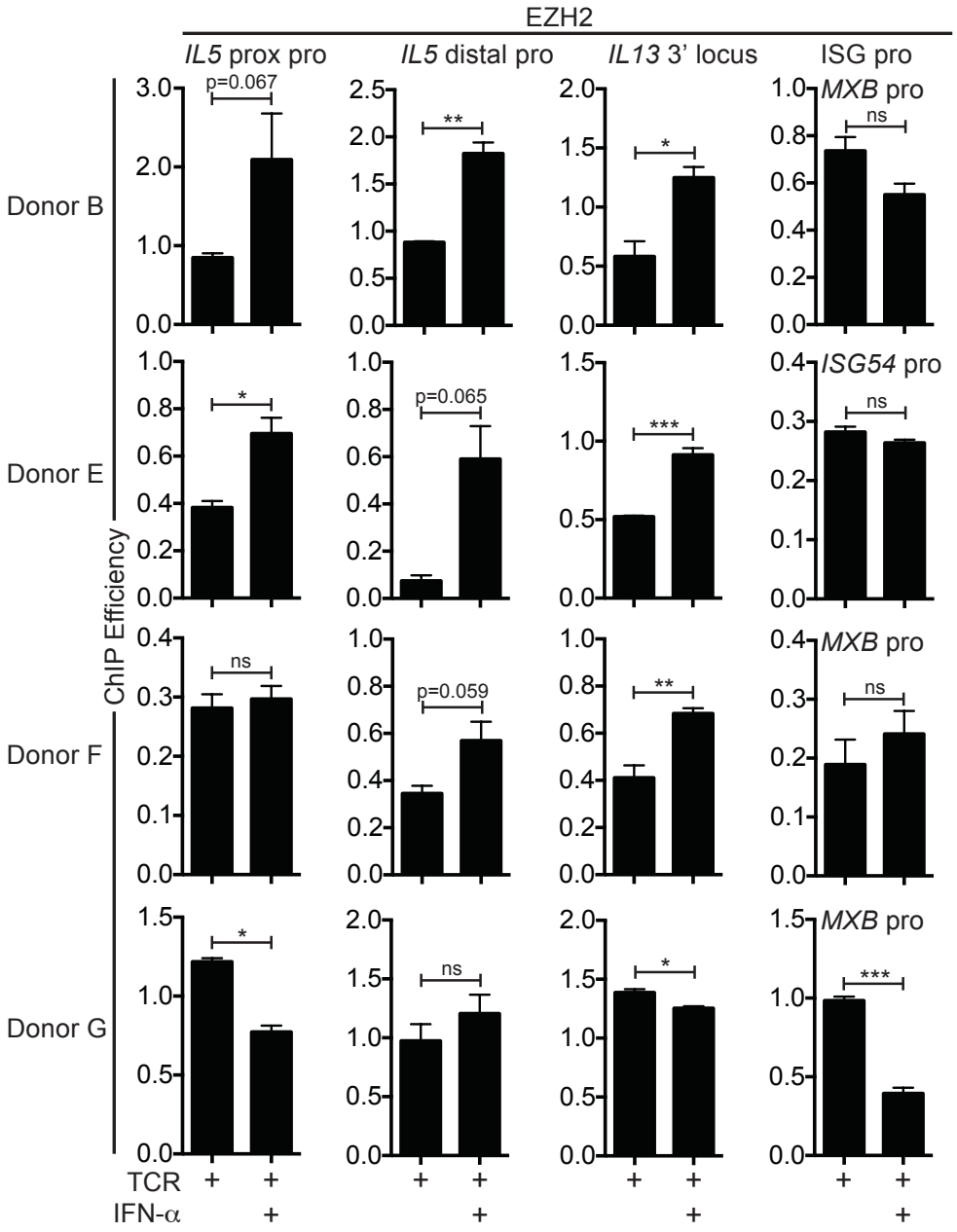
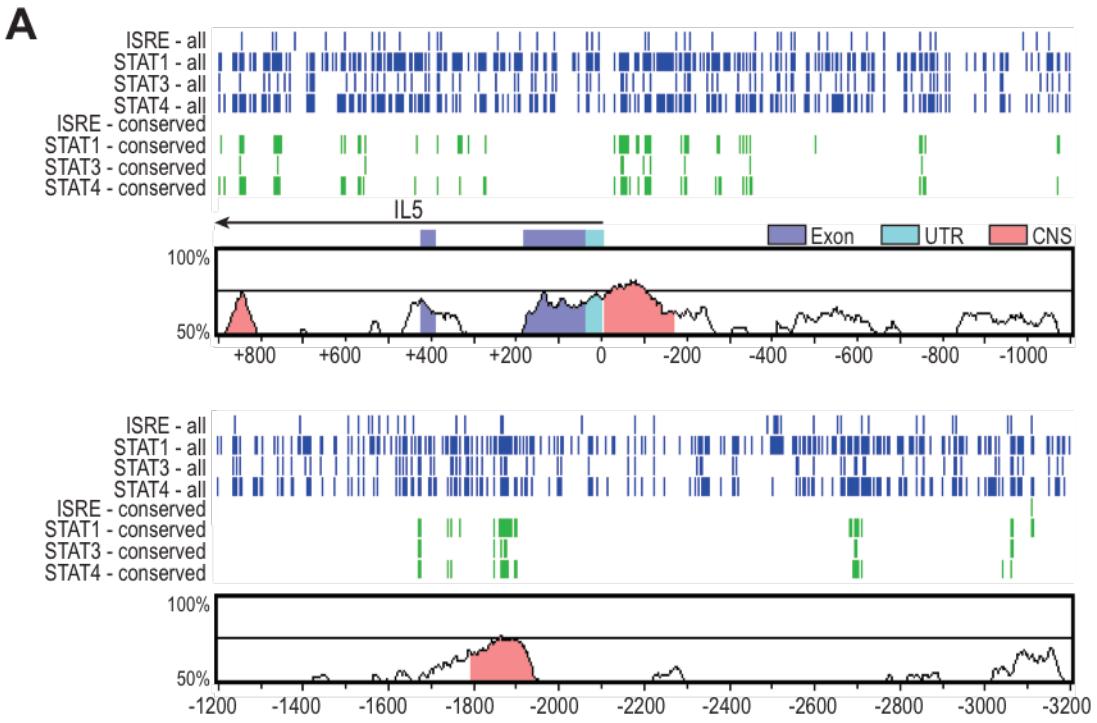
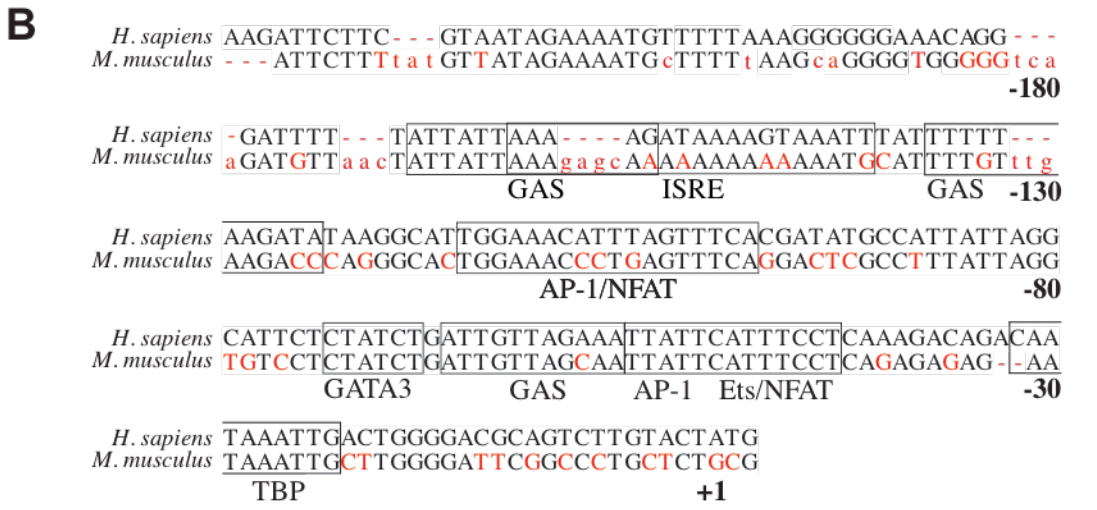


Figure 5.15: IFN-α-mediated EZH2 binding patterns within the Th2 locus and ISG promoters. *In vitro* polarized human Th2 cells were restimulated with plate-bound anti-hCD3 (3 μg/ml) and rhIL-2 (50 U/ml) for 2 hrs in the presence or absence of IFN-α (1000 U/ml), and ChIP lysates were prepared and probed for EZH2 binding to DNA. qPCR was used to analyze the human *IL5* proximal and distal sites, the *IL13* 3' locus, and the human *MXB* or *ISG54* promoter. ChIP efficiencies were determined as described in the methods section (Chapter 2). The four donors shown are representative of all the donors performed for this experiment. Student's t-test was used to determine significance; * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.


A
ISRE - all
STAT1 - all
STAT3 - all
STAT4 - all
ISRE - conserved
STAT1 - conserved
STAT3 - conserved
STAT4 - conserved
IL5
Exon
UTR
CNS
100%
50%
+800 +600 +400 +200 0 -200 -400 -600 -800 -1000
-1200 -1400 -1600 -1800 -2000 -2200 -2400 -2600 -2800 -3000 -3200


B
H. sapiens AAGATTCTTC - - - GTAATAGAAAATGTTTTTAAAGGGGGGAAACAGG - - -
M. musculus - - - ATTCTTTtatGTTATAGAAAATGcTTTTtAAGcaGGGGTGGGGGtca
-180
H. sapiens - GATTTT - - - TATTATTAAA - - - - AGATAAAAGTAAATTTATTTTTT - - -
M. musculus aGATGTTaacTATTATTAAAgagcAAAAAAAAAAAATGCATTTTGTttg
GAS ISRE GAS -130
H. sapiens AAGATATAAGGCATTGGAAACATTTAGTTTCACGATATGCCATTATTAGG
M. musculus AAGACCCAGGGCACTGGAAACCCTGAGTTTCAGGACTCGCCTTTATTAGG
AP-1/NFAT -80
H. sapiens CATTCTCTATCTGATTGTTAGAAATTATTCATTTCCTCAAAGACAGACAA
M. musculus TGTCCTCTATCTGATTGTTAGCAATTATTCATTTCCTCAGAGAGAG - - AA
GATA3 GAS AP-1 Ets/NFAT -30
H. sapiens TAAATTGACTGGGGACGCAGTCTTGTACTATG
M. musculus TAAATTGCTTGGGGATTCGGCCCTGCTCTGCG
TBP +1

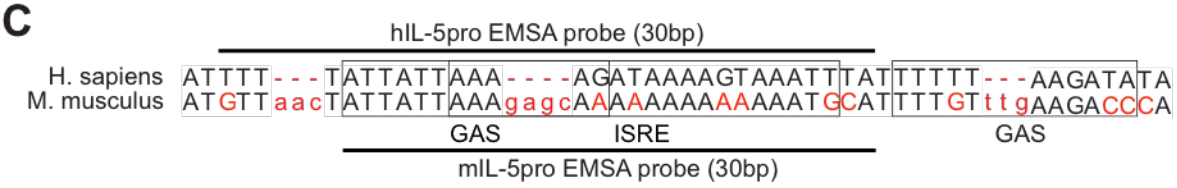

C
hIL-5pro EMSA probe (30bp)
H. sapiens ATTTT - - - TATTATTAAA - - - - AGATAAAAGTAAATTTATTTTTT - - - AAGATATA
M. musculus ATGTTaacTATTATTAAAgagcAAAAAAAAAAAATGCATTTTGTttgAAGACCCA
GAS ISRE GAS
mIL-5pro EMSA probe (30bp)

Figure 5.16: Human and murine IL5 promoter conservation and putative STAT binding sites. (A) VistaPlot analysis of the human and mouse IL-5 promoters, and their relative conservation across the region. Conserved sequences greater than 70% are denoted 'CNS' (http://genome.lbl.gov/vista). Putative ISRE and GAS motifs are in blue, while conserved motifs between human and mouse are denoted in green (B) The proximal *IL5* promoter of the human and mouse genomes were aligned using Ensembl (http://ensembl.org) and Mobyle BoxShade version 3.3.1 (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::boxshade). Conserved base pairs between the human and mouse sequences are in black, mouse-specific nucleotides are capitalized in red, and additions to either sequence are lowercase in red. Consensus motifs of various transcription factors are outlined in black. Conservation determined using Jaspar database at an 80% motif identity. (C) The same region as in (B) but with demarcated sequences used as DNA probes in the EMSA experiments. The ChIP-qPCR primers span this region, with PCR products ~170-200 bp in length.

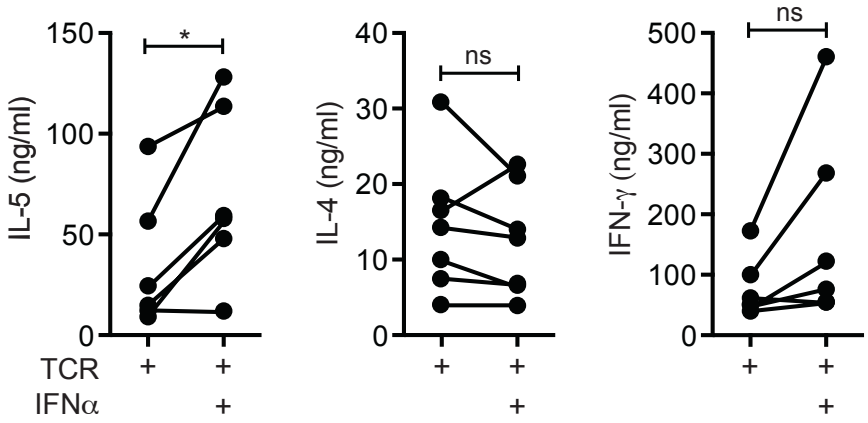


Figure 5.17: Murine IL-5 expression is not sensitive to acute IFN-α-mediated suppression. *In vitro* polarized Balb/c Th2 cells were restimulated with plate-bound anti-mCD3 (3 μg/ml) and rhIL-2 (50 U/ml) for 24 hrs in the presence or absence of IFN-α (A/D) (1000 U/ml) and supernatants were assayed for the designated cytokines by ELISA. Each line represents a unique mouse. Statistical significance was determined by a pair-wise student's t-test in; * ≥ 0.05, ns: not significant.

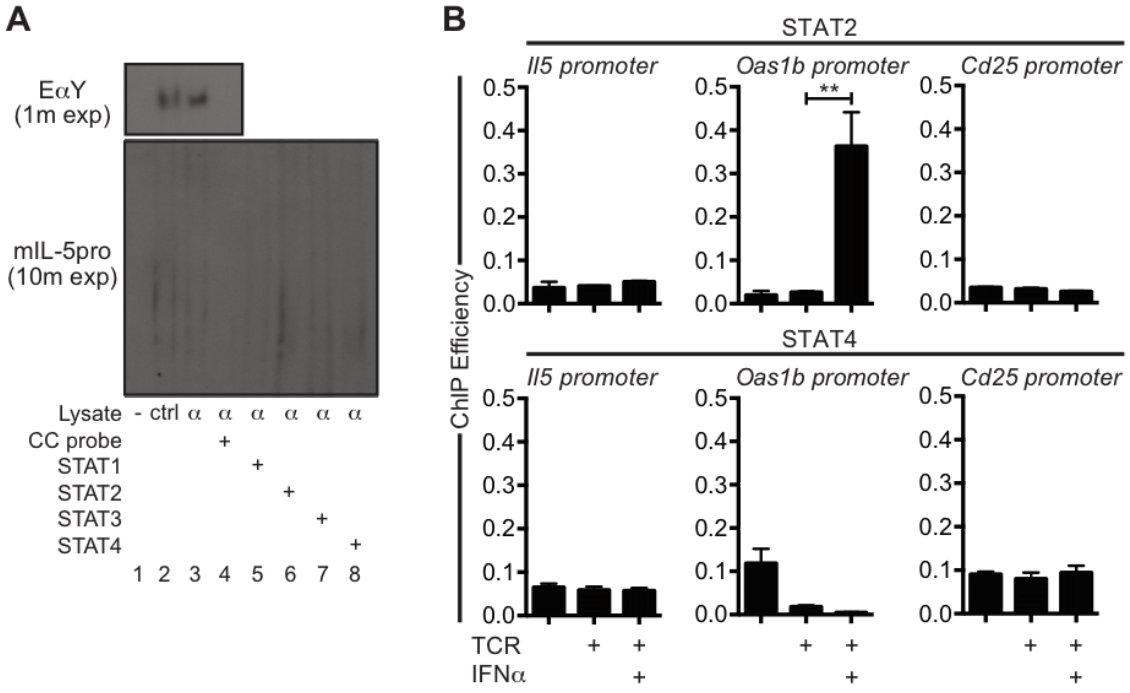


Figure 5.18: The murine Il5 promoter fails bind STAT2 or STAT4 in response to IFN-α treatment. (A) Whole murine splenocytes were stimulated with plate-bound anti-mCD3 (3μg/ml) and rhIL-2 (50 U/ml) for 2 hrs in the presence (α; lanes 3-8) or absence (ctrl; lane 2) of IFN-α (A/D) (1000 U/ml). Nuclear lysates were utilized for *in vitro* binding assays to the EαY or mIL-5pro probe in the presence or absence of STAT proteins (lanes 5-8) or a cold competitor probe (CC, lane 4). (B) *In vitro* polarized Balb/c Th2 cells were restimulated with plate-bound anti-CD3 and rh-IL-2 (50 U/ml) for 2 hrs in the presence or absence of IFN-α (1000 U/ml) and ChIP lysates were prepared and probed for STAT2 and STAT4 binding to DNA. qPCR was used to analyze specific regions of the mouse Il5 'ISRE/GAS' site, while the mouse Oas1b promoter was assessed as a positive control. ChIP efficiencies were determined as described in the methods. Data in (A) and (B) is representative of 3 separate experiments, respectively. Statistical significance in (B) was determined by one-way ANOVA with a Bonferroni post-hoc test in; ** ≥ 0.01, ns: not significant.

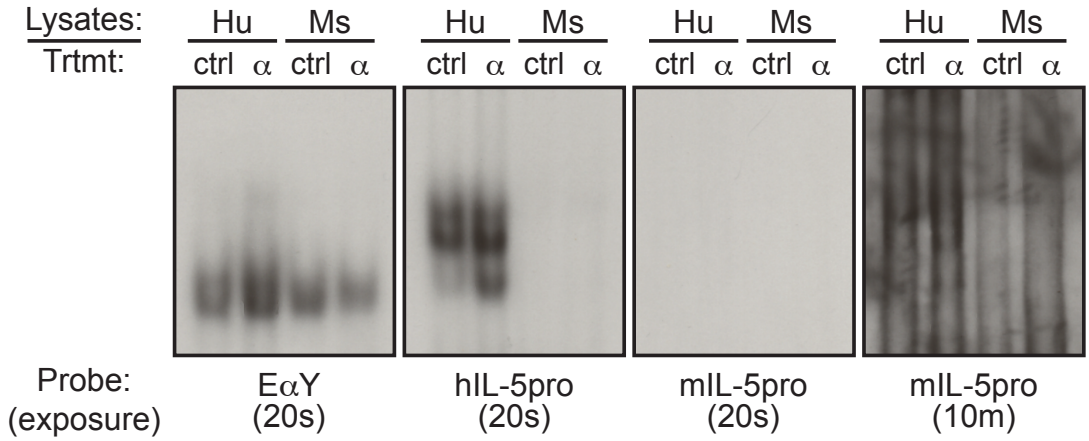


Figure 5.19: Human STAT proteins fail to interact with the murine Il5 promoter. Human and murine nuclear lysates were prepared as described in Figure 5.5B and Figure 5.18A and were utilized for *in vitro* binding assays to the EαY, hIL-5pro, or mIL-5pro probes to assess binding of nuclear proteins to each probe in response to IFN-α treatment. The human and mouse IL-5pro probe sequences (30 bp each) are shown in Figure 5.16C. Data is representative of 2 separate experiments.

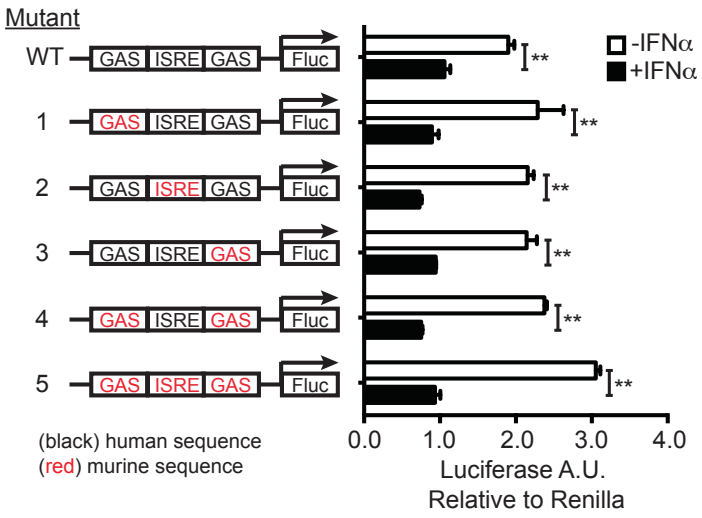


Figure 5.20: Human-to-murine mutations of the human *IL5* promoter fail to reverse IFN-α-mediated gene suppression. T-Antigen-expressing Jurkat cells were utilized for all transfections. Cells were transfected with a total of 28 ug DNA by electroporation and rested 24 hrs. Cells were stimulated with soluble anti-hCD3 (1:1000) and rhIL-2 (50 U/ml) in the presence or absence of IFN-α for 6 hrs, and luciferase and renilla were quantified. GATA3-GFP, STAT4-GFP, and CMVpro-renilla were co-transfected into cells in the presence of the human IL5pro-luciferase or five different IL5 promoter mutants, then stimulated as described above, and the reporter expression of renilla and luciferase were assessed. Data representative of two individual experiments, and are plotted as the ratio of luciferase to renilla signal. Two-way ANOVA in (A) and (B) with a Bonferroni post-hoc test were used to determine statistical significance. * ≥ 0.05, ** ≥ 0.01, *** ≥ 0.001, **** ≥ 0.0001, ns: not significant.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

Summary

IFN-α/β is a pleotropic cytokine that mediates the antiviral response through the activation of hundreds of ISGs. Although the biological implications of this antiviral pathway have been studied at length, much less is known regarding the role of this cytokine in modulating the immune response through the suppression of gene expression. Previous studies have observed the ability of IFN-α/β signaling to suppress gene expression, however, it is largely unknown how this process occurs. This body of work demonstrates a role for IFN-α/β signaling in modulating the epigenetic landscape of the GATA3 gene during Th2 development (Chapter 3) and in suppressing human *IL5* expression in a STAT4-dependent manner in memory Th2 cells (Chapter 4 and 5). Previous work conducted by Dr. Huber demonstrated that human Th2 development is suppressed in response to IFN-α treatment, even in the presence of exogenous IL-4 (Huber et al., 2010). He showed that IFN-α treatment suppressed the expression of GATA3 at the mRNA and protein level; however, the extent to which IFN-α signaling was mediating the GATA3 auto-activation loop remained unknown.

In Chapter 3, Dr. Huber and I developed a greater understanding regarding the effects of IFN-α signaling in human Th2 cells and how IFN-α/β alters the epigenetic profile of a T cell (Huber et al., 2014). The work presented demonstrates that IFN-α suppresses GATA3 as a function of cell division, which seems to be mediated through the repression of the IL-4-

sensitive, alternative exon 1 transcript of GATA3, exon 1a. Indeed, the expression of the exon 1a-mediated transcript variant is suppressed by IFN-α as a function of cell division as well, and these observations correlate with enhanced DNase hypersensitivity at the conserved non coding sequence 1 (CNS-1) approximately 2 kb from exon 1a. These findings correlate with enhanced H3K27me3 as well as increased total nucleosome content (H3), and the inability of GATA3 to bind to the CNS-1 site in the presence of IFN-α. Taken together, these results provide evidence that IFN-α can override signaling cues from IL-4, and prevent GATA3-mediated Th2 stability. These findings also showed that pre-committed Th2 cells are responsive to the repressive effects of IFN-α upon restimulation. However, I did not provide an explanation as to how IFN-α is initially suppressing GATA3 expression, and whether this regulation targets GATA3 at the transcript or protein level. As described in the introduction, once the *GATA3* gene becomes activated through STAT6 signaling, GATA3 protein induces its own expression. It could be the case that the histone modifications at this site are a byproduct of IFN-α-mediated suppression of this gene, or that the epigenetic modifications are directly induced by IFN-α signaling, which in turn prevents the GATA3 auto-activation loop from being triggered. Indeed, there is one study that demonstrates that IFN-α-mediated gene suppression required the methyltransferase activity of EZH2, which correlated with enhanced STAT2 binding at the same promoter ISRE as where EZH2 was bound (Testoni et al., 2011a). Although this study did not formally demonstrate the requirement for STAT2 in recruiting the EZH2-complex to the DNA, the enhancement of repressive mark H3K27me3 within 1 hr of IFN-α treatment was described for a number of other IFN-α-suppressed genes by the same group (Testoni et al., 2011b). If this mechanism of IFN-α-mediated EZH2 recruitment exists in our system, it could be the case that GATA3 CNS-1 site is

being quickly silenced by IFN-α treatment, which would prevent GATA3 protein from binding to CNS-1, preventing the auto-activation loop from occurring at this juncture.

In addition to suppressing Th2 development, the data presented in Chapter 3 showed that IFN-α is able to suppress fully differentiated Th2 cells during restimulation. This finding led us to wonder how acute IFN-α signaling suppresses fully differentiated Th2 cells. In Chapters 4 and 5, I showed that acute IFN-α signaling suppresses *IL5* and *IL13*, but not *IL4* at both the mRNA and protein level. This differential regulation of the Th2 cytokine genes was somewhat surprising because Dr. Huber found that pre-committed Th2 cells treated with IFN-α suppress the production of IL-4, IL-5 and IL-13 upon restimulation (Huber et al., 2010). However, these experiments are fundamentally distinct from the acute treatment with IFN-α I presented in Chapter 4. In Dr. Huber's previous work, *in vitro* polarized Th2 cells were restimulated for an additional 7 d in the presence of IFN-α, then restimulated at d 14 with PMA and ionomycin for intracellular staining (Huber et al., 2010). This is a considerably longer period of time for the cells to see IFN-α compared to the experiments I conducted in Chapters 4 and 5, in which *ex vivo* memory $CD4^+$ T cells or *in vitro* polarized Th2 cells were treated for less than 24 hrs with IFN-α in the presence of TCR stimulation. It could be the case that the acute responses I have observed in response to IFN-α preface the long-term effects, including the epigenetic modifications I observed in Chapter 3. Furthermore, I demonstrate that IFN-α suppresses *IL5* and *IL13* by suppressing the rate of nascent transcription. This finding correlates with STAT4 binding within the human *IL5* promoter, and using a reporter assay, I demonstrate that STAT4 activity is required to mediate IFN-α-induced gene suppression. Despite the requirement for activated STAT4 in this mechanism, it is still unclear what species of STAT4 dimer drives *IL5* gene suppression. STAT1/STAT4 (Collison et al., 2012), STAT4/STAT4 (Jacobson et al., 1995),

STAT3/STAT4 (Jacobson et al., 1995; Parham et al., 2002) have been shown to bind DNA in response to different cytokine-induced Jak/STAT signaling pathways. Furthermore, STAT4 dimers have been shown to interact with each other through N-terminal domain interactions (Vinkemeier et al., 1998). It has been proposed that this N-terminal interaction allows a STAT4 tetramer complex to interact with low affinity GAS elements that do not necessarily contain the complete consensus motif (Xu et al., 1996). This type of tetramer complex could potentially play a role in the findings I have presented here, because the GAS/ISRE elements within the human *IL5* promoter contain approximately 80% of the consensus motif, as determined using Jaspar. Additionally, the use of purified STAT4 and other STAT species using biochemical approaches in STAT knockout cells would be useful to deteremine which specific STAT protein is required for IFN-α-mediated gene suppression.

At this time, the exact cis regulatory element within the *IL5* promoter that is required to mediate gene suppression is unknown, however, it lies within the 1.8 kb region of the *IL5* promoter. Although the murine Il5 promoter element is disrupted and does not contain the putative GAS/ISRE element, mutating the human *IL5* promoter to the mouse sequence did not ameliorating IFN-α-mediated gene suppression. There is however, a GAS element closer to the Il5 transcriptional start site that is conserved between human and mouse that is proximal to an AP-1/Ets/NFAT site that has been shown play a role in driving Il5 expression (Figure 5.16B) (Lee et al., 1995). It could be that this element is bound by STAT6, which has been shown to recruit the enhancesome to the IL-4 promoter through interaction with CPB and p300 (Gingras et al., 1999; McDonald and Reich, 1999). The findings presented in Chapter 5 suggest that there are additional cis regulatory elements that mediate human *IL5* gene suppression, possibly acting in concert with each other in response to IFN-α signaling.

IFN-α/β regulation of non-Th2 cells

The data presented here provide an example as to how IFN-α/β acts to suppress gene expression, which directly suppresses Th2 cell function. As Th2 cells play a direct role in driving an inflammatory environment in chronic allergic diseases, it is interesting that IFN-α/β also negatively regulates other cell types that play a role in this dysregulated Th2 state. Eosinophils and basophils play a role in driving the acute hypersensitivity driven by IgE and IL-5 (Bischoff et al., 1990; Cameron et al., 2000; Yoshimoto et al., 2009). Both of these types of granulocytes have been shown to be regulated by IFN-α signaling *in vitro*. In eosinophils, IFN-α treatment inhibits both IL-5 production and the release of preformed proteins, including eosinophil cationic protein and neurotoxin (Aldebert et al., 1996). Chen *et al* observed that IFN-α suppressed IL-3-mediated priming of basophils, which led to a reduction in IL-4 and IL-13 expression from these cells (Chen et al., 2003). B cells and the production of IgE also play a central role in driving acute hypersensitivity in atopic diseases. The importance of B cell production of IgE in human disease has been demonstrated by blocking IgE therapeutically using omalizumab, an anti-IgE monoclonal antibody (Humbert et al., 2014). Adults with severe allergic asthma treated with omalizumab showed reduced expression of the high affinity FcεRI on pDCs and basophils, and improved Forced Expiratory Volume in one second (FEV_1) after 16 weeks of treatment (Garcia et al., 2013). The use of omalizumab has also been shown effective in children less than 12 yrs old by multiple studies (Busse et al., 2011; Lanier et al., 2009). Furthermore, in a recent follow-up study of less than 10 children, children who received omalizumab treatment for their moderate and severe allergic asthma benefitted from the therapy up to three years after cessation of the clinical trial, exemplified by no need for inhaled corticosteroids or rescue inhalers (Baena-

Cagnani et al., 2015). Because blocking IgE production is currently being targeted therapeutically, it is interesting that *in vitro* treatment of B cells with IFN-α suppresses IL-4-mediated IgE class switching in human and mouse (Finkelman et al., 1991; Pene et al., 1988). Taken together, these reports demonstrate that IFN-α/β signaling seems to override the Th2-mediated responses, and acts to suppress the allergic-inducing effects of Th2 cells, granulocytes and B cells. In addition to the cell types described above, ILC2s have been shown to drive a Th2-dominant environment, and in mice lacking an adaptive immune system, ILC2s were sufficient in promoting lung inflammation in response to papain challenge (Halim et al., 2014). These lineage-negative cells express the master transcription factor GATA3 and express CRTH2 on the cell surface (Mjösberg et al., 2011; 2012). Furthermore, in response to cytokine activation, ILC2s produce IL-5 and IL-13, leading to granulocyte recruitment and mucus production (Huang et al., 2015; Klein Wolterink et al., 2012). It would be interesting to determine whether ILC2s are responsive to IFN-α similar to Th2 cells. If STAT4 is expressed in these cells, then it is plausible that IFN-α signaling could suppress IL-5 and IL-13 expression from these cells, or possibly GATA3, although the role for STAT4 in IFN-α-mediated GATA3-suppression has not been established.

The therapeutic potential of IFN-α/β for the treatment of atopic diseases

IFN-α/β is widely used to treat many severe chronic diseases, including multiple sclerosis, hepatitis C virus (HCV) infection and certain types of cancers (George et al., 2012). The use of IFN-α/β therapy in atopic diseases has only recently been explored through either clinical case studies or by retrospective analyses of prior clinical trials. Schmitz and colleagues

documented that the use of low-dose IFN-α rapidly improved lung function and cellular responses, including an increased Th1 population and enhanced expression of IL-10 by PMBCs (Simon et al., 2003). In the same study, the use of prednisone to control disease symptoms in 100% of participants was significantly reduced 5-10 months after IFN-α therapy began. Within weeks of receiving therapy, all patients showed an increase in lung function and greater physical activity (Simon et al., 2003). Another case study that treated three severe glucocorticoid-dependent asthmatics demonstrated that IFN-α injections almost completely abolished daily asthma attacks (Kroegel et al., 2006). This study also reported a ≥ 60% reduction in the daily use of short-acting β2 agonists, and reduced emergency room visits per year by at least 70% in all participants (Kroegel et al., 2006). Additionally, there are a handful of reports in HCV patients demonstrating that IFN-α/β can mitigate allergic symptoms in a subset of asthma patients during treatment for chronic viral infection. One case study showed that a patient's adult-onset asthma was cured, even after cessation of IFN-α/β therapy (Yamamoto et al., 2005). Another study demonstrated that individuals with HCV infection that responded to IFN-α/β therapy had reduced serum IL-33 (Cacopardo et al., 2012). Other reports are not as clear; a study in 2003 demonstrated that IFN-α-responders being treated for HCV showed control of asthmatic symptoms when their conventional asthma therapies failed at IFN-α/β therapy onset (Kanazawa et al., 2003). How a persistent viral infection alters the immune response in an atopic individual remains unclear; however, there does seem to be a correlation between IFN-α-responsiveness and the suppression of allergic disease. Controlled clinical trials will need to be conducted in order to determine the efficacy of IFN-α/β as a treatment for severe atopic diseases, such as steroid-resistant allergic asthma.

To date, there is only one published randomized trial using IFN-β in allergic asthma patients (Djukanović et al., 2014). The study enrolled 147 asthmatics using inhaled corticosteroids to control their symptoms that had a history of virus-induced exacerbations. The participants were given either inhaled IFN-β (n = 72) or placebo (n = 75) within 24 hours of developing symptoms from influenza or rhinovirus infection. IFN-β did not significantly reduce the endpoint of viral infection, however, individuals on IFN-β treatment had enhanced morning peak expiratory flow recovery and a reduced need for additional therapy to control symptoms during the viral infection (Djukanović et al., 2014). Additionally, in smaller studies within the clinical trial, IFN-β treatment led to a reduction in virally-induced moderate asthma exacerbations, and patients used their rescue inhalers less on days 5 and 6 of the infection compared to placebo controls. The use of IFN-β in this clinical trial to control virus-induced exacerbations provides evidence that this cytokine could be useful in specific situations, and further studies regarding the efficacy of this treatment are needed.

Although suppressing the inflammatory cell types driving allergic diseases directly is a popular approach for the development of new therapeutic strategies, another possibility is through the regulation of these cell types *in vivo* by enhancing the regulatory cell population. IFN-α has been shown to enhance IL-10 production from $CD4^+$ T cells (Ramgolam et al., 2009; Schandené et al., 1996; Zhang et al., 2009) as well as LPS-stimulated macrophages (Chang et al., 2007a). Murine $CD4^+$ Treg cell transfer models have been shown to reduce eosinophil recruitment, Th2 cytokine production, and attenuate airway hyperresponsiveness in response to subsequent ovalbumin challenge (Kearley et al., 2008; Xu et al., 2012). Furthermore, Borish *et al* observed that IL-10 was undetectable in the BAL fluid of most of the adult asthmatics used in their study, whereas IL-10 was present in the BAL fluid from healthy controls (Borish et al.,

1996). In a recent study using a transgenic mouse model susceptible to developing food allergy, Rivas *et al* demonstrated that induced Treg (iTreg) cells undergo reprogramming in response to IL-4R-mediated signaling (Noval Rivas et al., 2015). Interestingly, Treg cells from children with milk allergy were fewer in number and had increased GATA3 and IRF4 expression compared to healthy controls. Upon restimulation with β-lactoglobulin, there were a greater percentage of $CD4^{+}FOXP3^{+}$ IL-4 and IL-13 producing cells compared to non-allergic or non-milk specific allergic T cells (Noval Rivas et al., 2015). Based on the data presented in this thesis, it could be the case that IFN-α treatment could prevent IL-4-mediated reprogramming of these iTreg cells by preventing the upregulation of GATA3 expression. Dr. Huber has shown that IFN-α is able to suppress Th2 development in the presence of IL-4 (Huber et al., 2010), and the data presented in Figure 4.14 demonstrate that acute IFN-α signaling suppresses *IL4RA* chain gene expression, but not STAT6 (Figure 4.14). It may be the case that these iTreg cells are sensitive to additional reprogramming that might rescue their ability to suppress the Th2-dominant environment.

CpG/allergen therapy to suppress Th2-dominant inflammation

The side effects of IFN-α/β therapy are well documented, especially in the treatment of HCV infection (Dumoulin et al., 1999; Fattovich et al., 1996; Okanoue et al., 1996), and include flu-like symptoms, nausea, and can be as severe as autoimmune disease-like symptoms. To induce long-term tolerance in an antigen-dependent manner, and mitigate the systemic side effects of injected IFN-α/β, the use of adjuvant CpG treatment with allergy injections was developed. CpG (Cytosine-Guanine Dinucleotide DNA) is unmethylated DNA that stimulates the innate immune system through TLR-9 signaling (Hemmi et al., 2000). Early studies in mice

demonstrated the therapeutic potential of CpG using a *Schistosoma mansoni* egg inflammation model (Kline et al., 1998). Intraperitoneal injection of CpG at the time of challenge resulted in the prevention of airway eosinophilia and enhanced IFN-γ and IL-12 production in BAL fluid (Kline et al., 1998). Enhanced IL-12 and IFN-γ production are not necessarily required for the suppression of Th2-mediated pathology and cytokine production, however, in IL-12-, IFN-γ-, or IL-12/IFN-γ-deficient mice, approximately 10 times the amount of CpG was required to protect from airway eosinophilia induced by *Schistosoma mansoni* eggs compared to the WT mice receiving challenge and CpG treatment (Kline et al., 1999). However, these studies utilized a helminth-mediated inflammation model instead of using purified allergen. Santeliz *et al* demonstrated that CpG conjugated to the major short ragweed allergen amb a 1 reduced airway hyperreactivity compared to animals challenged with amb a 1 only (Santeliz et al., 2002). Further, administration of amb a 1 with a control sequence failed to prevent airway hyperreactivity, demonstrating that the presence of CpG mediates this therapeutic effect (Santeliz et al., 2002). Studies that utilized an experimental challenge in humans also show promise. One study asked whether amb a 1/CpG injections of ragweed-allergic individuals altered the PBMC response upon *in vitro* challenge (Simons et al., 2004). Cells from amb a 1/CpG-treated individuals, but not placebo controls, expressed reduced IL-5, CCL17, and CCL22 production upon allergen restimulation *in vitro* (Simons et al., 2004). Although the Th1 cytokine IFN-γ was enhanced 2 weeks after injection, this effect seemed to be transient, whereas the effects of reduced IL-5 were more prolonged and maintained at 16 weeks-post injection (Simons et al., 2004). This study demonstrates the potential for the use of allergen/CpG therapy to modulate *in vivo* allergen-specific responses. This study was followed by a randomized, double-blinded, placebo-controlled phase 2 trial with the same ragweed allergen, amb a 1, conjugated to a CpG-

containing immunostimulatory DNA sequence. Twenty-five adults with ragweed allergies were recruited and were injected once a week for six weeks with amb a 1/CpG or placebo prior to ragweed season. Each individual was monitored for allergic symptoms in the two subsequent ragweed seasons based on the visual-analogue scale, peak-season daily nasal symptom diary scores, and overall quality-of life scores (Creticos et al., 2006). During both ragweed seasons, amb a 1/CpG injected-patients had a higher peak-season rhinitis score using the visual-analog scale, and peak-season daily nasal symptom diary scores compared to placebo controls. Further, individuals receiving the treatment showed a significant correlation between a reduction of IL-4-positive basophils and reduced visual-analogue scores during the first ragweed season. Although this study had limited participants, it suggests that allergen/CpG therapy induces long-term symptom relief in ragweed-specific allergic rhinitis (Creticos et al., 2006). Despite these promising results, not all atopic diseases improve in response to CpG therapy. One study found that atopic asthmatics treated with nebulized CpG for four weeks did not see attenuated FEV_1 scores post-allergen challenge compared to the placebo controls, despite enhanced ISG expression (Gauvreau et al., 2006). This study differs from the other studies in that CpG was given for four weeks prior to allergen challenge, and was not provided via injection. Another study demonstrated that seven injections of CpG were able to provide symptom control in 66% of individuals compared to placebo controls, even in the absence of inhaled steroids (Beeh et al., 2013). Based on this data, it seems that CpG therapy paired with the specific insulting allergen can potentially drive a Th1 or Treg response, or at least mitigate the Th2-dominant response. Interestingly, human pDCs treated with CpG induce $CD4^+CD25^+$ Treg cell differentiation in a antigen-nonspecific manner (Moseman et al., 2004). However, the need for specific antigen in

the context of *in vivo* T cell modulation will likely play an important role in driving prolonged tolerance.

Future Directions

Although the data presented in Chapter 3 identify that IFN-α/β signaling enhances the epigenetic silencing mark H3K27me3 as well as overall nucleosome content at the *GATA3* locus, we still do not understand how this process is mediated. It would be useful to ChIP for STAT2 and STAT4 and sequence the sites bound by these transcription factors in naïve $CD4^+$ T cells within hours of IFN-α/β treatment in the presence or absence of IL-4 and TCR stimulation. I hypothesize that the later time points that Dr. Huber and I measured *GATA3* mRNA (d 3) and epigenetic landscape profile (d 5) are the product of an upstream mechanism that occurs in response to acute IFN-α/β signaling. Furthermore, as STAT binding within the *GATA3* locus would only correlate with Th2 suppression, a dominant negative STAT protein could be utilized to determine whether it is necessary for the suppression of Th2 development. Additionally, enhanced H3K27me3 is associated with heterochromatin that is inaccessible to the enhancesome and subsequent transcription (Consortium et al., 2013; Yuan et al., 2012). It would be interesting to determine how permanent this phenotype is in the absence of IFN-α/β signaling. This could be conducted using *in vitro* polarized Th2 cells, in which cells are given IL-4, IL-4/IFN-α or IFN-α for 7 d. Subsequently, each population would be restimulated in the absence of additional cytokines, and the abundance of H3K27me3 and transcript expression could be monitored over time. Further, a fraction of the cells could be given an IL-4-neutralizing antibody during the second week to determine how TCR-mediated signals affect the negative regulatory environment

at the epigenetic level in the presence or absence of any IL-4 these cells would be making in response to TCR stimulation. This experiment would demonstrate whether IFN-α-mediated H3K27me3 is permanent or transient, and have implications for a therapeutic strategy.

A major question that has yet to be answered with these experiments and previous work conducted by Dr. Huber is at which step within the GATA3 auto-activation loop is IFN-α/β suppressing GATA3 expression. GATA3 has been shown to activate its own expression, which correlates with its ability to bind CNS-1 and its own promoter. IFN-α/β could be preventing this at a number of steps. First, it could directly suppress gene expression of GATA3. Nuclear run-on could be used to determine whether this is the case, however, this experiment would be technically difficult due to the expression of GATA3 already present in naïve $CD4^+$ T cells (Pai et al., 2003). Perhaps a system could be developed in which a GATA3 mutant was expressed that could not bind DNA. If TCR stimulation and STAT6 were present, I hypothesize that the inability of GATA3 to enhance its own expression would not effect the gene expression. Perhaps then, you could distinguish whether a reduction of nascent transcription of the GATA3 gene was due to a suppression of nascent transcription in response to IFN-α/β. If the rate of nascent transcription were maintained, it would suggest that IFN-α/β signaling acts on the GATA3 protein itself to suppress gene expression, and subsequent Th2 stability. Dr. Huber demonstrated that IFN-α/β does not enhance protein degradation of GATA3, nor does it prevent GATA3 translocation to the nucleus (Chapter 3). It could be the case that IFN-α/β mediates a post-translational modification that reduces its affinity for DNA, or prevents GATA3 from recruiting other enhancers to induce gene expression. Recently, Hosokawa *et al* identified a role for GATA3 methylation in suppression the expression of the murine Il5 gene (Hosokawa et al., 2015). GATA3 was shown to be methylated within the N-terminal zinc finger domain at arginine

256 and arginine 261. Using a GATA3-methylated mimic, the authors showed that this modification, especially at position 261, suppressed IL-5 expression, but left the expression of IL-4 and the suppression of IFN-γ intact (Hosokawa et al., 2015). Although these arginine residues are conserved between mouse and human, it is intriguing that this post-translational modification of GATA3 induces the differential regulation of the Th2 cytokine genes, which is what I have observed in response to acute IFN-α/β signaling (Chapter 4.1-4.3). However, the authors did not measure GATA3 binding to its own locus. It could be the case that a post-translational modification is induced in response to IFN-α/β signaling, which prevents GATA3 from binding DNA, or recruiting the transcriptional machinery and other enhancers required to drive GATA3 gene expression.

There are apparent difference between the IFN-α/β-mediated pathway that suppresses Th2 development and that of acute cytokine suppression. The two most obvious examples are that 1) the presence of IFN-α/β during Th2 development prevents IL-4, IL-5 and IL-13 expression upon restimulation, and 2) the expression of GATA3 is suppressed in the presence of IFN-α/β during Th2 development, while GATA3 expression is unaffected in *ex vivo* total memory $CD4^+$ T cells. It is likely that IFN-α/β signaling effects these two populations of cells differently, as the epigenetic landscape of these naïve versus committed Th2 cells has been shown to be considerably distinct, for example, within the Th2 cytokine locus and Ifng locus (Agarwal and Rao, 1998; Ansel et al., 2003; Lee et al., 2006). Thus, STAT proteins activated in response to IFN-α/β treatment likely bind to different sites within the genome. However, the signaling intermediates utilized by IFN-α/β to induce this suppression could be similar. It could be the case that STAT4 binds distinct sites within the GATA3 locus that prevents Th2 stability during lineage commitment. Alternatively, continuous IFN-α/β treatment of memory Th2 cells

could possibly lead to a similar enhanced H3K27me3 at specific genes, including GATA3. Despite IL-4 being insensitive to acute IFN-α/β treatment, I have not measured this cytokine at later time points. It could be the case that the kinetics of IL-4 regulation are distinct from that of IL-5 and IL-13, and a kinetic analysis of this cytokine in TCR-stimulated memory Th2 cells would easily address this question.

All donors utilized for these experiments were considered 'healthy' individuals that did not suffer from chronic atopic diseases, although some donors reported mild seasonal allergies, but their cells were not utilized for experiments during an immune response to seasonal allergens. I did not assess allergen sensitivity by skin prick test in these donors. It has been demonstrated that PBMCs, pDCs and BAL cells individuals with difficult-to-control allergic asthma secrete less IFN-α/β and IFN-λ in response to viral or TLR challenge *in vitro* and *in vivo* (Contoli et al., 2006; Durrani et al., 2012; Gill et al., 2010; Sykes et al., 2012; Wark et al., 2005). However, there is a population of individuals who are able to control their disease with conventional therapies, and cells from these individuals make IFN-α/β and IFN-λ similar to levels of non-asthmatic controls (Bochkov et al., 2009; Lopez-Souza et al., 2009; Sykes et al., 2012; 2013). Despite a clear indication that there is a defect in the production of these antiviral cytokines in severe asthmatics, there are far fewer studies that looked specifically at the IFN-α/β-responsiveness of $CD4^+$ T cells from allergic individuals. One such study used PBMCs from house dust mite allergic individuals and asked whether treatment with IFN-α or IFN-λ suppressed the Th2 cytokine genes (Pritchard et al., 2012a). Both IFN-α or IFN-λ treatment were able to suppress allergen-specific expression of IL-13, while only IFN-α was able to suppress IL-5 expression (Pritchard et al., 2012b). Based on this study, it seems that PBMCs from allergen-sensitive individuals are regulated in a somewhat similar manner compared to PBMCs from

healthy controls. It would be interesting to measure the T cell response to *in vitro* IFN-α/β treatment from individuals from different atopic groups. For example, healthy T cell responses would be compared to well-controlled and non-controlled allergic asthma. Naïve $CD4^+$ T cells, total memory $CD4^+$T cells, and pDCs could be isolated from the same patient, and the effects of IFN-α/β could be measured from the T cells, while the production of IFN-α/β and IFN-λ from pDCs would be determined in response to viral infection or TLR stimulation. This would provide insight into how the T cells from individuals whose pDCs do not make IFN-α/β or IFN-λ correlate to their T cells' ability to be regulated by this cytokine.

In addition to looking at the cellular functions of each of these populations of cells, it would be interesting to determine whether single nucleotide polymorphisms (SNPs) exist within genes of interest that might affect an asthmatic individuals T cells' ability to respond to IFN-α/β. This sort of study could be correlated to serum IgE, eosinophilia, and severity of symptoms. I would be interested in whether there are SNPs within the STAT2, STAT4, IFNAR1, and IFNAR2 genes to determine whether a positive correlation exists between a particular SNP and the risk of developing asthma. Park *et al* found that although there was no association between STAT4 SNPs and the risk of asthma in South Korean participants, there was a positive correlation between one intronic SNP and the production of IgE specific to mite allergens (Park et al., 2005). Li *et al* found that another specific SNP within the STAT4 gene that did correlate with the risk of asthma in a cohort in China (Li et al., 2007). Finally, Hsieh *et al* utilizing Taiwanese children found that a specific STAT2 SNP was also associated with increased risk of asthma (Hsieh et al., 2009). Although the association with STAT2 seems to be an indication of the role of the IFN-α/β in this disease, there are a number of reasons for the correlation between STAT4 and atopic disease. It could be the case that these specific SNPs induce differences in the

Th1 development or function, thereby skewing Th2 responses. Alternatively, these SNPs could play a role in IFN-α/β-mediated signaling, which corresponds to a more direct route within the Th2 cell that causes an increased risk in developing asthma. It would also be interesting to correlate these SNPs with IFN-α/β-mediated suppression of granulocytes and B cell-IgE production. To my knowledge, no SNP analysis has been reported on the correlation between IFNAR1 and IFNAR2 and atopic diseases.

Implications and Concluding Remarks

This body of work has provided insight into the way differentiating $CD4^+$ T cells and fully committed Th2 cells respond to IFN-α/β signaling. These populations of cells distinctly respond to exogenous cues differently due to the lack of epigenetic programming in the former population. This likely provides unique STAT binding sites in the two populations, in addition to the classical ISGs that are likely sensitive to IFN-α/β signaling in both populations. Despite IFN-α/β being expressed in response to viral infections in all individuals, respiratory viral infections are the leading cause of asthma exacerbations (Gern, 2015). Although the exact mechanism for this is unknown, it is apparent that cytokines produced in response to viral infections seem to be enhanced in some instances, including the production of IL-25, TSLP, IL-33, and IL-13 (Beale et al., 2014; Jackson et al., 2014; Message et al., 2008). Perhaps the exaggerated inflammatory response, paired with a defect in the ability to produce IFN-α/β contributes to this observation. It is clear that FcεR crosslinking inhibits IFN-α/β production, and enhanced SOCS1 expression in asthmatics was recently shown to suppress IFN-λ production in response to rhinovirus infection (Gielen et al., 2015). Finally, inflammatory mediators, such as histamine, have also been shown

to suppress the expression of CpG-mediated IFN-α/β production (Mazzoni et al., 2003). It is apparent that there are a number of ways in which the Th2 inflammatory response is able to override the production of IFN-α/β and IFN-λ. There are likely a number of consequences resulting from reduced cytokine production, one of which is the possible inability to balance the Th2 adaptive immune response. The data presented here, paired with the studies I discussed demonstrating the role of IFN-α/β in inducing an IL-10 response *in vitro*, and CpG/allergen therapy demonstrating a transient Th1 response *in vivo*, indicate the potential in balancing the dominant nature of Th2 inflammation in atopic individuals. Pairing such therapies with the widely successful omalizumab therapy has the potential to induce long-term tolerance in individuals that suffer from uncontrolled atopic disease.

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