COUNTER-REGULATION OF HUMAN $CD4^+$ T HELPER 2 LYMPHOCYTE DEVELOPMENT AND STABILITY BY TYPE I INTERFERON

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DEDICATION

To Sarah, Karsten, and Lukas.

ACKNOWLEDGEMENTS

Graduate school is a long journey, and many people have helped me along the way. I cannot begin describing my work before acknowledging them for their personal and professional support, so I here offer my thanks:

To Dr. David Farrar, the best mentor I could have asked for. You were brilliant, insightful, gentle when possible, but firm when I needed a kick in the pants. Thank you for everything you have taught me both scientifically and personally, for being the keel that kept me steady through the natural ups and downs of research, and for the listening ear that cared about more than just data in the notebook. I'm honored to have you as both a mentor and a friend.

To my thesis committee – Dr. Becky Gruchalla, Dr. James Chen, Dr. Ivan D'Orso, Dr. Michelle Gill, and Dr. Jerry Niederkorn – for your enthusiasm, focus, guidance, and tidbits of wisdom. It's been a pleasure working with you.

To all of my lab buddies – Ann, Lalo, Kristan, Navin, Sarah, Didem, Leo, Loderick, Annalisa, Dyan, and Ann-Marie – who sharpened me with science chats, made the Farrar lab an awesome place to work, and shared many happy times I'll still remember when my research career is done. I can't imagine being in a lab without you guys.

To Angie, Christina, Ann-Marie, Julia, Jennifer, Laura, Elizabeth, and Bonnie, for coaxing the Moflo and Arias to do magical things with lasers, water and electricity.

To Mariana Ibarra, who tirelessly handled our glass cleaning and autoclaving and always had a friendly smile.

To Kole Roybal and Mark Borromeo for assistance with microscopy and ChIP, techniques I would not have managed otherwise.

To Karen Kazemzadeh, Renee Gugino, and Harmony Hilton, who cheerfully kept the Immunology program running smoothly.

To Karen, Bettye, Cathy, Natalia, Andrea, and Naomi, who have handled countless details to keep the research environment operating.

To Dad, Mom, Kelly, Jay, Alexia, Mommy, Daddy, Stacy, and Matt, for their constant love and support.

To Sarah, Karsten, and Lukas – my family and my best friends. How could I possibly describe the undeserved joy you bring to my life. I love you all very much.

COUNTER-REGULATION OF HUMAN $CD4^+$ T HELPER 2 LYMPHOCYTE DEVELOPMENT AND STABILITY BY TYPE I INTERFERON

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

September, 2012

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Innate cytokines shape the differentiation of $CD4^+$ T cells from naïve precursors into multiple functional subsets in order carry out effective adaptive immune responses to diverse immunological stimuli. Interleukin-12 controls the development of T helper 1 (Th1) cells, which fight infection by intracellular pathogens such as bacteria and viruses. Helminth parasites and allergens induce the production of IL-4, which drives differentiation to Th2. The IL-12 and IL-4 signaling pathways also counter-regulate each other, though this balance favors Th2 as IL-4 signaling overrides the effects of IL-12. In addition, IL-4 induces expression of the GATA3 transcription factor that stabilizes the Th2 phenotype through auto-regulatory maintenance of

its own expression. A role for type I interferon (IFN-α/β), a major antiviral cytokine, has been described more recently in Th1 cells, but the potential role of IFN-α/β in counter-regulating Th2 development is poorly understood.

My work reveals that IFN-α/β blocks IL-4-induced Th2 differentiation of human $CD4^+$ T cells by inhibiting expression of GATA3. The loss of GATA3 in developing Th2 cells leads to reduced secretion of the Th2 cytokines, IL-4, IL-5, and IL-13, and reduced expression of chemoattractant receptor expressed in Th2 cells (CRTh2). IFN-α/β also suppresses GATA3 in committed Th2 cells, leading to a loss of the Th2 phenotype. GATA3 transcription utilizes two distinct first exons, exon 1A and 1B, which are controlled by separate promoters, but IL-4 signaling in Th2 cells leads to preferential utilization of the upstream exon 1A transcript. IFN-α specifically blocks expression of the 1A but not 1B transcript, indicating a specific regulation of Th2 cells. Furthermore, IFN-α appears to induce epigenetic silencing of an upstream conserved non-coding sequence I, which may be required for optimal exon 1A transcription in Th2 cells. This work reveals an unexpected role for IFN-α in selectively inhibiting Th2 but not Th1 differentiation, which may be important for ensuring appropriate development of antiviral immunity. In addition, the ability of IFN-α to suppress both developing Th2 cells and previously committed Th2 cells suggests that IFN-α may be useful as a novel therapeutic for atopic diseases.

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Huber, J.P., Ramos, H.J., Gill, M.A., and Farrar, J.D. (2010). Cutting edge: Type I IFN reverses human Th2 commitment and stability by suppressing GATA3. The Journal of Immunology 185, 813-817.

Huber, J.P., and Farrar, J.D. (2011). Regulation of effector and memory T-cell functions by type I interferon. Immunology 132, 466-474.

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3C – chromosomal conformation capture

Ab – antibody

AP-1 – activation protein-1

APC – allophycocyanin

APC – antigen-presenting cell

AHR – airway hyperresponsiveness

BSA – bovine serum albumin

Bt – biotin

CBA – cytometric bead array

cDMEM – complete Dulbecco's modified Eagle medium

cDNA – complementary DNA

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

CTCF – CCCTC-binding factor

DC – dendritic cell

DTT – dithiothreitol

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

EMSA – electrophoretic mobility shift assay

FAIRE – formaldehyde-assisted isolation of regulatory elements

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

FluA – influenza A virus

GAS – γ-IFN activation site

GFP – green fluorescent protein

GM-CSF – granulocyte-macrophage colony stimulating factor

h – human

HCV – hepatitis C virus

HRP – horseradish peroxidase

IFN – interferon

IFNAR – interferon-α/β receptor

Ig - immunoglobulin

IL – interleukin

IP – immunoprecipitate

IRF – interferon regulatory factor

IRS – insulin receptor substrate

ISG – interferon stimulated gene

ISGF3 – interferon stimulated gene factor 3

ISRE – interferon stimulated response element

JAK – Janus kinase

LCR – locus control region

MAPK – mitogen-activated protein kinase

MFI – mean fluorescence intensity

MHC – major histocompatibility complex

NF-κB – nuclear factor κB

NFAT – nuclear factor in activated T cells

NK – natural killer cell

NOD – nucleotide-binding oligomerization domain

OVA – ovalbumin

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

PI-3 kinase – phosphatidylinositol 3-kinase

PHA – phytohemagglutinin

PMA – phorbol 12-myristate 13-acetate

PMSF – phenylmethylsulfonyl fluoride

PRR – pattern recognition receptor

qPCR – quantitative real-time PCR

rh – recombinant human

RIG-I – retinoic acid-inducible gene I

RIPA – radioimmune precipitation assay

RNA – ribonucleic acid

RSV – respiratory syncytial virus

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

Tc2 – T cytotoxic type 2

TCF-1 – T cell factor 1

TCR – T cell receptor

Th1 – T helper type 1

Th2 – T helper type 2

Th17 – T helper type 17

TLR – Toll-like receptor

TSLP – thymic stromal lymphopoietin

U – units

WT – wild type

Y – tyrosine

YAC – yeast artificial construct

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Initiation of $CD4^+$ T cell responses

The immune system is a highly complex network of cells, proteins, and molecules that protect us from a diverse array of dangers. Effective protection entails the distinguishing of both self from non-self as well as dangerous from innocuous. Detection of a pathogen (dangerous and non-self) leads to an inflammatory response that is tailored to the specific characteristics of the pathogen in order to effectively clear the infection. This immunity can be generally divided into two branches. The first branch is the antigen-nonspecific innate immune system, which specializes in the initial recognition of the infection. The second branch is adaptive immunity, which depends on instruction from innate cells but provides much more specific protection as well as immunological memory.

An important link between these two branches is the function of professional antigen presenting cells (APCs) that reside in the host periphery to detect invasion by a pathogen. APCs recognize pathogens via pattern recognition receptors (PRRs), which are germline-encoded and respond to conserved pathogen-associated microbial patterns (PAMPs) derived from various classes of microbes (Janeway and Medzhitov, 2002; Kapsenberg, 2003). Important examples of

PRRs include Toll-like receptors (TLRs) on the cell surface and in endosomal compartments, as well as cytoplasmic sensors such as nucleotide-binding oligomerization domain (NOD) receptors and retinoic acid-inducible gene I (RIG-I). Binding of these receptors by their cognate microbial ligand alerts innate immune cells to the presence of the pathogen and initiates innate responses such as phagocytosis of the pathogen. In addition, APCs enable adaptive T cell immunity.

One particular class of APCs, dendritic cells (DCs), plays a critical role in activating $CD4^+$ T cells. Pathogen recognition by DCs leads to DC maturation, wherein they upregulate major histocompatibility complex class II (MHC II) and the co-stimulatory molecules CD80 and CD86. Mature DCs also express chemokine receptors like CCR7 that allow migration from the periphery to secondary lymphoid organs where DCs present microbial antigens to $CD4^+$ T cells. In addition to providing stimulation to the T cell receptor (TCR) and costimulation to receptors like CD28, DCs also secrete cytokines that guide $CD4^+$ T cell differentiation into various effector subsets (Kapsenberg, 2003).

As DCs produce different cytokines depending on the PRRs that are activated, cytokine signaling provides a way to program $CD4^+$ T cells with unique functions appropriate for responding to different classes of pathogens. Signaling from these cytokines through their cognate receptors on the $CD4^+$ T cells induces differentiation of the T cells into various subsets. Each of these subsets is defined by the expression of particular transcription factors, as well as the secretion of unique sets of effector cytokines that regulate the behavior of other immune cells, ultimately leading to productive clearance of the infection (Glimcher and Murphy, 2000; Murphy and Reiner, 2002). Figure 1.1 shows the major $CD4^+$ T cell subsets, their "master regulator" transcription factors, and the main cytokines they secrete.

Th1 development and type I interferon

Since the discovery of $CD4^+$ T cell subsets, a major quest in T cell biology has been to understand the signals that control the differentiation of these subpopulations. One of the first signals identified was found to control T helper type 1 (Th1) differentiation, with interleukin-12 (IL-12) being the key cytokine governing this pathway (Hsieh et al., 1993; Macatonia et al., 1993; Macatonia et al., 1995). IL-12 is produced during bacterial, viral, and protozoan infections (Trinchieri, 2003). Binding of IL-12 to its receptor (IL-12R) on $CD4^+$ cells triggers the activation of the Janus kinases JAK2 and Tyk2 (Bacon et al., 1995a), leading to the phosphorylation and activation of signal transducer and activator of transcription 4 (STAT4) (Bacon et al., 1995b; Jacobson et al., 1995). Phosphorylated STAT4 plays a critical role during Th1 commitment by promoting expression of T-bet (Szabo et al., 2000; Szabo et al., 2002; Ramos et al., 2007; Placek et al., 2009), and recent studies have defined unique roles for both STAT4 and T-bet in regulating IFN-γ gene expression within committed Th1 cells (Thieu et al., 2008). Finally, IFN-γ enhances both T-bet and IL-12Rβ2 expression, reinforcing IL-12-mediated Th1 commitment (Szabo et al., 1997; Afkarian et al., 2002). Thus, in both mice and humans, IL-12 signaling through STAT4 and T-bet was established as a key pathway to IFN-γ production and the Th1 phenotype.

Another major cytokine associated with Th1 development is type I interferon (IFN-α/β). IFN-α/β was first reported in 1957 by Isaacs and Lindenmann as an activity that "interfered" with influenza A infection (Isaacs and Lindenmann, 1957; Isaacs et al., 1957), and has proven to be a critical regulator of innate immunity via its pleiotropic actions on virtually all somatic cell types. IFN-α/β programs a state of resistance to intracellular pathogens and serves to alarm cells

of both innate and adaptive immunity to the threat of infections. IFN-α/β is primarily induced by viral infection but can also be secreted in response to a variety of biological stresses including bacterial infection, UV irradiation, inflammation, and heat shock (De Maeyer and De Maeyer-Guignard, 1998; Doly et al., 1998).

Type I interferon is a family of highly related monomeric secreted proteins (Stark et al., 1998). In humans, there are approximately 20 IFN-α subtype genes in addition to individual genes encoding IFN-β, κ, ε, and ω. In the mouse, there exists an additional region of gene duplications resulting in approximately 20 genes encoding IFN-ζ isoforms (also known as "limitin") (Hardy et al., 2004).

Despite the large number of IFN-α/β subtype genes, only one IFN-α/β receptor (IFNAR) has been identified, which is ubiquitously and constitutively expressed (Stark et al., 1998). All IFN-α/β isoforms tested can bind the IFNAR, albeit with varying affinities. The IFNAR is a heterodimeric complex composed of two type I transmembrane subunits designated R1 and R2. Both the human and mouse IFNARs are constitutively associated with the JAK kinases Jak1 and Tyk2 (Stark et al., 1998). Before cytokine activation, the N-terminus of STAT2 mediates an interaction with the cytoplasmic tail of the IFNAR2 (Li et al., 1997). Upon receptor activation by IFN-α/β, the two receptor subunits co-ligate and promote activation of the JAK kinases that phosphorylate tyrosine (Y) residues within the cytoplasmic domains of the IFNAR1/2 chains (Qureshi et al., 1996; Yan et al., 1996). STAT2 becomes phosphorylated on Y-690 located just distal to the SH2 domain. Unlike STAT2, STAT1 is recruited to the receptor complex indirectly by docking to phosphorylated Y-690 on STAT2 (Qureshi et al., 1996). The STAT1-STAT2 heterodimer then associates with IRF-9 to form the interferon-sensitive gene factor-3 (ISGF3). ISGF3 regulates expression of the majority of interferon-sensitive genes (ISGs) by directly

transactivating interferon-sensitive response elements (ISREs) found within their promoters. Gene expression is also induced by the binding of STAT1-STAT2 heterodimers to γ-interferon activation sites (GAS) (Ghislain et al., 2001). Thus, STAT2 plays a critical role in IFN-α-induced gene expression (Figure 1.2).

Virtually all cells have the inherent capacity to secrete some level of IFN-α/β in response to certain viral infections. However, professional APCs, particularly plasmacytoid DCs (pDCs), are a key source of IFN-α/β. pDCs are a specialized subset of dendritic cells whose maturation is guided by innate cytokines (IL-3, Flt2 ligand, GM-CSF, and IL-4) and signaling through pattern recognition receptors during infections (Liu et al., 2005; Naik et al., 2007). These signals promote the secretion of a variety of innate cytokines, notably IL-12, IL-18, and importantly, IFN-α/β (Ito et al., 2002; Iho, 2003; Naik et al., 2007). While these cells are not as efficient at activating $CD4^+$ T cells as monocyte-derived dendritic cells due to their lower expression of MHC-II, pDCs play a significant role in promoting Th priming through cytokine secretion (Cella et al., 2000; Krug et al., 2003).

In addition to the role of IFN-α/β in the innate response, it has been reported to drive Th1 development much like IL-12, though not without controversy. In mice, STAT4 activation was not detected in response to IFN-α/β compared to IL-12 (Jacobson et al., 1995), yet studies with human cells reported just the opposite, suggesting a species difference in IFN-α/β-mediated STAT4 phosphorylation (Cho et al., 1996; Rogge et al., 1998; Farrar et al., 2000b). However, as new and more specific reagents became available, low levels of phosphorylated STAT4 could be detected in mouse cells in response to IFN-α/β (Berenson et al., 2004). Given the pronounced role of IL-12 signaling through STAT4 to drive Th1 commitment, these early studies assumed that any signaling pathway that activated STAT4 would promote Th1

development. Recent studies have challenged this assumption. Virtually all receptors that signal via the JAK/STAT pathway promote STAT tyrosine phosphorylation within minutes following receptor engagement. However, the duration of signaling varies between receptors and among STAT family members. Hilkens and colleagues first demonstrated a clear difference in the duration of STAT4 tyrosine phosphorylation between IL-12 and IFN-α/β signaling in human $CD4^+$ T cells, with IL-12 promoting sustained STAT4 activation compared to IFN-α/β signaling (Athie-Morales et al., 2004). The inability of IFN-α/β to maintain STAT4 activation was correlated with a marked deficit in IFN-α/β -dependent Th1 development. Further kinetic comparisons of IL-12 and IFN-α/β clearly demonstrated that while IL-12 promoted STAT4 phosphorylation up to 24 hrs, STAT4 was rapidly dephosphorylated within 6 hrs of IFN-α/β stimulation (Ramos et al., 2007). As a result, only cells treated with IL-12 expressed sustained levels of T-bet sufficient for IFN-γ secretion and Th1 commitment. Thus, while IFN-α/β may be more efficient at promoting acute STAT4 phosphorylation in human cells than in mice, IFN-α/β cannot sustain STAT4 phosphorylation and is therefore not sufficient to drive Th1 commitment in either species (Berenson et al., 2004; Persky et al., 2005; Berenson et al., 2006; Davis et al., 2008).

Th2 development

The second major subset of $CD4^+$ T cells is Th2. Multiple pathways have been reported to contribute to Th2 differentiation, but the first signal identified was IL-4 (Murphy et al., 2000; Paul and Zhu, 2010). Th2 responses develop during infections with helminths and some fungi, though the mechanism by which they induce IL-4 production is uncertain. Nevertheless, soon

after the discovery of $CD4^+$ T cell heterogeneity (Mosmann et al., 1986), IL-4 was shown to enhance the production of the Th2-associated cytokines IL-4, IL-5, and IL-13 from murine cells in vitro (Betz and Fox, 1990; Le Gros et al., 1990; Swain et al., 1990; Seder et al., 1992b). Likewise, loss of IL-4 impaired Th2 development (Kopf et al., 1993). Of particular interest was the fact that IL-4 induced Th2 cytokine production from purified T cell cultures activated with anti-CD3-coated plates, indicating that IL-4 can act directly on the T cells rather than working through an intermediate cell type (Le Gros et al., 1990). Furthermore, wild type (WT) mice challenged with the helminth parasite *Nippostrongylus brasiliensis* mount a Th2 response characterized by increased IL-5 production and eosinophilia, but IL-4-/- mice are unable to do so (Kopf et al., 1993). Cells from WT and IL-4-/- mice are both able to produce IL-5 when differentiated with IL-4 in vitro, indicating that disruption of IL-4 does not inherently block a cell's ability to produce other Th2 cytokines. Thus, IL-4 is important for Th2 development both in vitro and in vivo.

While IL-4 is needed for optimal Th2 development, the initial source of IL-4 that starts the differentiation process has not been clearly identified. Basophils (Seder et al., 1991; Sokol et al., 2008), DCs (d'Ostiani et al., 2000; Maroof et al., 2006), eosinophils (Moqbel et al., 1995), mast cells (Bradding et al., 1992), and NKT cells (Yoshimoto and Paul, 1994; Wang et al., 2006) can all produce IL-4. However, IL-4 production by these cells types is not required for Th2 responses (Schmitz et al., 1994; Brown et al., 1996; Kim et al., 2010). Instead, the IL-4 may come from the newly activated naïve T cells themselves. Naïve $CD4^+$ T cells from mice deficient in the IL-4 receptor (IL-4R) retain the ability to produce IL-4 at levels sufficient to promote Th2 differentiation of WT cells (Noben-Trauth et al., 2000). Th2 development is also affected by the strength of the TCR stimulation (Tao et al., 1997a), but this effect can be blocked by addition of anti-IL-4 neutralizing antibodies to the culture (Tao et al., 1997b), suggesting that the IL-4

induced by TCR signaling may be sufficient to drive differentiation of those cells to Th2. Therefore, multiple cell types may provide IL-4 during Th2-inducing immune responses.

In both mice and humans, IL-4 signals through two different heterodimeric receptors (Nelms et al., 1999). The first, type I, is comprised of the IL-4 receptor alpha chain (IL-4Rα) and the common gamma chain (γc). IL-4 can also signal through a second receptor formed by the pairing of IL-4Rα with IL-13Rα, but naïve $CD4^+$ T cells lack expression of this second chain and thus bind IL-4 via the type I receptor (Chomarat and Banchereau, 1998). Ligand binding triggers the phosphorylation of multiple tyrosine residues in the cytoplasmic tail of the IL-4Rα via JAK1 and JAK3, associated with the IL-4Rα and γc chains respectively. These phosphorylated tyrosines act as docking sites for STAT6 as well as Insulin receptor substrate (IRS) 1 and 2. IRS-1/2, after binding, are phosphorylated by JAK1 and signal through PI3K kinase, though this pathway is primarily responsible for IL-4's effects on T cell proliferation and survival (Wang et al., 1993a; Wang et al., 1993b; Sun et al., 1995). On the other hand, the Th2-inducing effects of IL-4 are mediated by STAT6 (Kaplan et al., 1996; Shimoda et al., 1996; Nelms et al., 1999). After binding to phosphorylated IL-4Rα, STAT6 is phosphorylated on tyrosine 641 (Y641), and mutation of this residue abolishes STAT6 function (Mikita et al., 1996). Following this phosphorylation event, STAT6 molecules homodimerize via binding of their SH2 domains to phospho-Y641, and the STAT6 dimer migrates to the nucleus where it binds DNA and promotes gene transcription. Loss of STAT6 results in a loss of Th2 cytokine expression, but no change in IFN-γ expression (Kaplan et al., 1996; Shimoda et al., 1996). Furthermore, STAT6-/- mice fail to mount a normal Th2 response to *Nippostrongylus brasiliensis* infection (Takeda et al., 1996) and are unable to clear the parasite (Urban et al., 1998). Thus, STAT6 is a critical link between IL-4 and the Th2 differentiation program.

STAT6 activation promotes expression of a key transcription factor, GATA3, which is both necessary and sufficient for Th2 development and promotes transcription at the Th2 cytokine locus containing the IL-4, IL-5, and IL-13 genes (Siegel et al., 1995; Zhang et al., 1997; Zheng and Flavell, 1997; Ouyang et al., 1998; Scheinman and Avni, 2009; Onodera et al., 2010). Deletion of GATA3 in differentiated Th2 cells via a Cre-expressing retrovirus leads to reduced secretion of the Th2 cytokines but increased IFN-γ secretion (Pai et al., 2004). Deletion of GATA3 in Th2 cells in vivo using OX40-Cre, which deletes GATA3 mainly in activated peripheral $CD4^+$ T cells, results in reduced IL-4 expression, complete loss of IL-13, and inability to clear a *Nippostrongylus brasiliensis* infection (Zhu et al., 2004). Similarly, people with only one functional GATA3 allele show reduced IgE levels and a reduced Th2-Th1 cell ratio (Skapenko et al., 2004). Further evidence indicates that GATA3 functions downstream of STAT6 in Th2 cells. Ectopic expression of GATA3 in STAT6-/- mice restores Th2 cytokine production (Ouyang et al., 2000). Additionally, the appearance of DNase hypersensitivity sites associated with chromatin accessibility at the IL-4 gene is lost in STAT6-deficient mice (Agarwal and Rao, 1998), but this requirement can be overcome by ectopic expression of GATA3 (Lee et al., 2000; Ouyang et al., 2000). GATA3 also enables Th2 cytokine expression in cells previously differentiated to Th1, indicating both that GATA3 is instructive and not just permissive, and that the function of GATA3 is dominant over the effects of Th1-inducing signals like IL-12 (Lee et al., 2000; Farrar et al., 2001). Finally, GATA3 acts acutely to inhibit expression of the IL-12Rβ2 subunit (Ouyang et al., 1998). Consequently, induction of GATA3 serves to block Th1 development while positively regulating Th2 commitment. Moreover, GATA3 is also critical for the long-term phenotypic stability of Th2 cells. Ectopic expression of GATA3 enables expression of the endogenous GATA3 gene even in IL-4-/- and STAT6-/- mice (Lee et al., 2000; Ouyang et al., 2000; Ranganath and Murphy, 2001). Thus, GATA3 is involved in an auto-regulatory

feedback loop that maintains Th2 commitment even in the absence of further IL-4 signaling, though it's not yet understood exactly how GATA3 works to promote its own expression. A model of IL-4 signaling through GATA3 is shown in Figure 1.3.

Other cytokine signals linked to Th2 development in addition to IL-4 include IL-2, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (Wang and Liu, 2009; Paul and Zhu, 2010; Ziegler, 2012). IL-2 signaling through STAT5 is required for Th2 development (Cote-Sierra et al., 2004), but IL-2 is insufficient to drive maximal expression of the Th2 cytokines (Swain et al., 1990; Cote-Sierra et al., 2004), and IL-2 does not abrogate the requirement of GATA3 (Zhu et al., 2004). Furthermore, induction of Th2 development by direct treatment with IL-25 or TSLP is dependent on IL-4 signaling (Angkasekwinai et al., 2007; Omori and Ziegler, 2007). Finally, though IL-33 can act directly on differentiated Th2 cells to enhance cytokine production, it does not appear to drive differentiation from naïve precursors (Schmitz et al., 2005). Thus, IL-4 remains the primary cytokine signal driving Th2 development.

There is also evidence of non-cytokine signals that promote IL-4-independent Th2 differentiation. Stimulation of purified IL-4Rα-/- $CD4^+$ T cells through the TCR is sufficient to induce some IL-4 production, although the expression is lower than that of cells from WT mice (Noben-Trauth et al., 1997). Similarly, while IL-4Rα-/- mice have an impaired Th2 response to a *Nippostrongylus brasiliensis* infection, the response is not completely abolished. However, though TCR stimulation can act independently of IL-4 signaling through STAT6, it induces GATA3 expression (Rodriguez-Palmero et al., 1999), perhaps through activation of the transcription factor T cell factor 1 (TCF-1) (Yu et al., 2009). Interactions between OX-40 and OX-40L on $CD4^+$ T cells and DCs respectively are important for TSLP-induced Th2 responses, but this pathway has a much more significant role in recall responses than primary induction of Th2 development (Seshasayee et al., 2007). Finally, Notch signaling contributes to T helper

differentiation, with the Delta1 and Jagged ligands favoring Th1 and Th2 development respectively (Maekawa et al., 2003; Amsen et al., 2004; Okamoto et al., 2009). However, the effects of Notch signaling can be overridden by Th1- or Th2-inducing cytokine signals (Ong et al., 2008), and Notch-induced Th2 development of $CD4^+$ T cells STAT6-/- mice still proceeds through GATA3 (Amsen et al., 2007). These data, combined with the previous discussion of the requirement for GATA3, emphasize the critical role of this transcription factor in Th2 differentiation.

Following induction of GATA3 expression, it promotes transcription of IL-4, IL-5, and IL-13. These three cytokines are clustered together into a 160kb sequence termed the IL-4 locus. The genes encoding IL-4 and IL-13 neighbor each other but are separated from the IL-5 gene by an intervening gene, RAD50. Many conserved non-coding sequences (CNS) in this region harbor DNase I hypersensitivity sites that appear during Th2 development (Agarwal and Rao, 1998; Takemoto et al., 1998; Loots et al., 2000; Ansel et al., 2006), reflecting chromatin remodeling that renders the DNA accessible to transcription factors, like NFAT and AP-1, that are acutely activated by TCR signaling (Szabo et al., 1993; Rooney et al., 1995). The appearance of these hypersensitivity sites is mediated by GATA3 (Lee et al., 2000; Ouyang et al., 2000), which binds at multiple locations in the IL-4 locus. In mice, GATA3 has been shown to bind directly to the IL-5 and IL-13 promoters (Zhang et al., 1998; Kishikawa et al., 2001). Another binding site in a conserved intronic regulatory element (CIRE) in the IL-4 gene is conserved in both mice and humans, and deletion of that binding site impairs IL-4 production by memory Th2 cells (Tykocinski et al., 2005). At each of these individual cytokine loci, deletion of the GATA3 binding site results in reduced expression of that cytokine but not the others. Nevertheless, these three cytokines are coordinately expressed (Mosmann et al., 1986; Bucy et al., 1995), being under the control of additional cis-regulatory elements including multiple

enhancer regions (Ranganath et al., 1998; Takemoto et al., 1998; Loots et al., 2000; Lee et al., 2001; Mohrs et al., 2001; Solymar et al., 2002; Yamashita et al., 2002; Tanaka et al., 2011). GATA3 also binds in a number of these regulatory sequences, thereby contributing to the coordinated cytokine expression. In addition, a locus control region (LCR) located at the 3' end of the RAD50 gene is required for Th2 cytokine production (Lee et al., 2003; Spilianakis and Flavell, 2004; Lee et al., 2005; Koh et al., 2010). The promoters of all three cytokine genes are brought into close proximity both to each other and to the LCR (Spilianakis and Flavell, 2004), suggesting that the RAD50 gene is looped out of the way so that the cytokine genes may be coordinately regulated. Interestingly, the LCR has been reported to be remodeled by STAT6 but not by GATA3 (Lee and Rao, 2004), suggesting cooperation of these two factors for optimum Th2 cytokine production and further underlining the importance of IL-4 signaling for Th2 development.

Tissue-specific regulation of GATA3

The central role of GATA3 in driving Th2 development is intriguing since it is also critical for many other biological processes. During embryonic development, GATA3 is expressed in many different tissues, including the central and peripheral nervous systems, kidney, urogenital tract, endocardium, mammary glands, and thymus (Yamamoto et al., 1990; Ko et al., 1991; George et al., 1994; Lakshmanan et al., 1999; Asselin-Labat et al., 2007). As a consequence of GATA3's role in the nervous system, whole-body deletion of GATA3 is embryonically lethal due to lack of sympathetic neurons (Lim et al., 2000). In adult mice, GATA3 expression persists in a number of these tissues including the nervous system, thymus, and Th2 cells.

In the thymus, GATA3 supports positive selection and commitment to the $CD4^+$ lineage. GATA3 is expressed from the earliest stages of thymocyte development and is required for thymocyte survival and proliferation (Hendriks et al., 1999; Zhang et al., 2012). Later in the thymic development, GATA3 is required during selection due to its role in promoting expression of the TCR-β chain (Henderson et al., 1994; Pai et al., 2003). Finally, GATA3 promotes commitment to the CD4 lineage (Hernandez-Hoyos et al., 2003). Proper GATA3 function in the thymus requires a limited range of expression. Too much GATA3 is detrimental, as ectopic expression of GATA3 in thymocytes skews them toward a mast cell progenitor phenotype (Taghon et al., 2007), while cells expressing too little GATA3 will fail to pass thymic selection.

The need for GATA3 in multiple tissues, and the necessity of tightly controlling GATA3 levels in the thymus while also allowing further induction in Th2 cells, raises an important question: how can the GATA3 gene be selectively regulated to accommodate these different functions? Two solutions have been described so far. The first solution is the presence of multiple tissue-specific enhancers (Lakshmanan et al., 1999; Hosoya-Ohmura et al., 2011). Several of these enhancers are located somewhat distantly from the GATA3 proximal promoter. One particular enhancer required for normal expression of GATA3 in T cells is located 280kb downstream of the GATA3 gene (Hosoya-Ohmura et al., 2011). A YAC construct that contains 650kb around the GATA3 gene but lacks this distant downstream enhancer is insufficient to replicate normal GATA3 expression in murine T cells (Lakshmanan et al., 1999), indicating a complex requirement of multiple cis-regulatory elements for proper GATA3 expression.

A second mechanism by which GATA3 expression is controlled is the expression of two different transcripts. GATA3 mRNA, like that of some other GATA family members, comprises two different transcripts distinguished from each other by the use of distinct first exons (1A and 1B) spliced onto a shared exon 2 (Asnagli et al., 2002; Amsen et al., 2007; Fang et al., 2007;

Scheinman and Avni, 2009; Yu et al., 2009; Nakata et al., 2010; Onodera et al., 2010). Exon 1A and 1B are controlled by distinct promoters that are separated by approximately 10kb of DNA. Since the protein coding sequence begins in the middle of exon 2, these two different transcripts would not be expected to yield different versions of the GATA3 protein. Instead, the existence of the two promoters may provide a mechanism that enables increased GATA3 transcription specifically in certain cell types. Asnagli *et al.* showed that both transcripts were expressed on day 3 in developing murine Th2 cells, but exon 1B expression was transient and decreased by day 4 while exon 1A remained highly expressed out to day 7. Thus, the GATA3 exon 1A appears to play a key role in GATA3 expression in Th2 cells.

In contrast to exon 1A utilization in Th2 cells, thymic T cells primarily express the GATA3 exon 1B transcript (Asnagli et al., 2002). Notch signaling occurs in developing thymocytes prior to GATA3 expression (Rothenberg, 2012), though it is not clear that Notch directly initiates GATA3 expression in that context. Induction of GATA3 by Notch in Th2 cells seems to depend on mobilization of the Notch effector protein RBPJ to the GATA3 exon 1A promoter (Amsen et al., 2007) and preferential induction of the exon 1A transcript (Fang et al., 2007). Consequently, the absence of exon 1A mRNA in thymocytes suggests that other mechanisms control expression of GATA3 in the thymus. Since GATA3 is so critical for successful generation of naive $CD4^+$ T cells, the immune system must have a mechanism for stabilizing GATA3 levels within the optimal range for thymocytes while also allowing for increased expression during Th2 commitment. The dual transcripts of GATA3 may be part of this mechanism, allowing IL-4 to raise GATA3 levels during Th2 development via the exon 1A transcript. However, though there are multiple reports of the exon 1A and exon 1B transcripts in mice, the role of the exon 1A transcript in human Th2 cells remains to be evaluated.

Th2 immunity

Th2 cells provide protection against helminth parasite infections largely through the production of IL-4, IL-5, and IL-13. IL-5 is a potent activating and chemotactic factor for eosinophils (Sanderson, 1992; Warringa et al., 1992), leading to hypereosinophilia. IL-13 stimulates mucous hypersecretion from in the mucosal epithelia, as well as subepithelial airway fibrosis (Zhu et al., 1999). IL-4 and IL-13 induce antibody isotype switching in B cells to IgE (Del Prete et al., 1988; Geha, 1992), while CD40-CD40L interactions on the T and B cells respectively result in IgE production that can be further enhanced by IL-5 (Pene et al., 1988b). IgE then arms mast cells, basophils, and eosinophils to respond in an antigen-dependent manner. When stimulated by an antigen, these cells secrete an array of inflammatory mediators including histamine, proteases, cytokines, prostaglandins, and leukotrienes. These molecules increase vascular permeability and cellular infiltration, directly attack helminth tissues, propagate the inflammation, and trigger peristalsis of the muscles lining the intestinal tract which, along with IL-13-induced mucous production from goblet cells, aids expulsion of the worms (Robinson, 2000; Finkelman et al., 2004; Bloemen et al., 2007). Th2 cells also participate in the effector response, and their chemotaxis is aided by receptors like chemoattractant receptor expressed on Th2 cells (CRTh2) (Nagata et al., 1999; Cosmi et al., 2000), which is controlled by GATA3 (De Fanis et al., 2007) and is sensitive to prostaglandin D2 (PGD2) (Quapp et al., 2007). PGD2 is secreted by mast cells and recruits Th2 cells into sites of active inflammation where they participate in the effector response through the production of cytokines (Xue et al., 2005).

These processes are beneficial when targeted against helminths, but they cause unwelcome damage when they are directed against foreign but innocuous antigens in the form of allergens. During an allergic response, $CD4^+$ T cells are inappropriately activated against

allergen peptides and coordinate inflammation in the upper and lower respiratory tract that can result in devastating pathology (Holgate and Polosa, 2008; Holgate, 2012). The exact set of symptoms varies between patients, but several common features shared with anti-helminth responses include elevated serum IgE antibody titers, hypereosinophilia, mucous hypersecretion, and constriction of the smooth muscle lining the airways. Long-term remodeling of the lung tissue results from fibrosis and thickening of the airways. The aggregate result of these symptoms is reduced airspace in the lungs and shortness of breath (asthma) and a predisposition to constriction of the airspace in response to allergenic exposure (airway hyperresponsiveness/AHR).

Given this basic definition, not all asthma is Th2-mediated allergic asthma, but allergic asthma accounts for over 50% of the more than 20 million people in the USA with asthma, including 2.5 million children under 18 years of age (AAFA, 2012). Worldwide, the World Health Organization estimated in 2007 that 300 million people suffered from asthma, and ~70% of these people also had allergies (WHO, 2007). Additionally, allergic diseases also extend beyond just asthma to include a variety of other conditions such as atopic dermatitis, allergic rhinitis, and serious anaphylactic reactions to peanuts and penicillin. The prevalence of asthma and allergic diseases continues to rise, increasing both the health burden of these conditions and the importance of improving our understanding and treatment of these conditions.

Balancing T helper cell responses

Ensuring appropriate development of Th1 and Th2 immune responses is critical for clearance of an infection. One example of this requirement is infection with the intracellular parasite *Leishmania major* (Reiner and Locksley, 1995). Two different murine strains, Balb/c

and C57BL/6, respond to *Leishmania major* infection very differently and with different outcomes. C57BL/6 mice develop a Th1 response characterized by IFN-γ production, which is effective for clearance of intracellular pathogens, and these mice are consequently able to eradicate the parasite. In contrast, Balb/c mice develop a Th2 response, characterized by production of IL-4 and IgE but poor production of IFN-γ. Consequently, these mice fail to clear the infection. In a reverse example, studies of people infected with the helminth *Schistosoma haematobium* showed that elevated IgE against the egg antigens was protective, while low IgE was associated with an increased susceptibility to reinfection (Hagan et al., 1991; Caldas et al., 2000).

In order to ensure appropriate T helper differentiation, cytokine signaling promotes certain subsets while inhibiting others. Early studies of T helper development showed that cells committed to either Th1 or Th2 and produced either IFN-γ or IL-4, IL-5, and IL-13 (Mosmann et al., 1986; Swain et al., 1990; Kopf et al., 1993). This suggested that differentiating cells not only turned on one set of cytokine genes, they also turned the others off. Indeed, later studies showed that chromatin remodeling of the IL-4 locus, as evidenced by the appearance of DNase I hypersensitivity sites, occurred in Th2 cells but not Th1 cells (Agarwal and Rao, 1998). Likewise, only Th1 cells remodeled the IFN-γ gene. Furthermore, Th1- and Th2-specific transcription factors regulate each other. For example, in murine cells, IL-12 signaling reduces GATA3 expression to levels below that of an undifferentiated cell (Ouyang et al., 1998). On the other hand, ectopic expression of GATA3 in developing Th1 cells blocks IL-12 signaling by inhibiting expression of the IL-12Rβ2 through an IL-4-independent mechanism. Ectopic expression of GATA3 into previously differentiated Th1 cells also enables IL-4 production while suppressing secretion of IFN-γ (Farrar et al., 2001). Subsequent studies showed that GATA3 blocks

expression of STAT4 (Usui et al., 2003). Ectopic expression of STAT4 was able to restore IL-12Rβ2 expression, even the cells were co-transduced with a GATA3-containing retrovirus. However, co-transduction of T-bet could not overcome the activity of GATA3 (Usui et al., 2003), even though ectopically expressed T-bet suppresses expression of endogenous GATA3 (Szabo et al., 2000; Usui et al., 2006). This suggests that T-bet contributes to Th1 development indirectly by inhibiting an inhibitor of STAT4 activation, indicating an intricate network of counter-regulation. However, though these studies help to clarify the hierarchy of these different factors, they fail to clarify the relative priority of different cytokine signals since many of these experiments were performed with retroviral constructs that lack normal cis-regulatory elements. Indeed, murine cells that are differentiated to Th1 for one week and are then reactivated for a second week with IL-4 lose their ability to secrete IFN-γ upon a tertiary activation and instead begin secreting IL-4 (Szabo et al., 1995). In contrast, cells differentiated to Th2 produce IL-4 and no IFN-γ even when re-cultured with IL-12 or anti-IL-4 neutralizing antibodies. Thus, IL-4 appears to be a dominant signal in mice that induces a stable Th2 phenotype and also redirects Th1 cells to Th2.

Relationship between respiratory infections and allergies

The ability of the immune system to properly direct T helper differentiation becomes particularly important when encountering both Th1- and Th2-inducing challenges simultaneously. The tissues of the upper and lower respiratory tract serve as a major barrier to both allergens and respiratory pathogens encountered daily in the air we breathe. This makes the lung environment vulnerable to complex immune reactions involving a mix of both Th1- and Th2-mediated inflammatory responses. It might be surmised that a Th1-mediated response

would dampen allergic inflammation. For example, Balb/c mice sensitized to ovalbumin (OVA) develop a Th2-mediated allergic response that leads to AHR upon aerosol challenge, but this response is significantly reduced if the mice are infected with *Mycobacterium bovis* (Gouveia et al., 2012). On the other hand, adoptive transfer of OVA-specific Th1 cells into OVA-sensitized mice exacerbated the allergic inflammation instead of suppressing it (Hansen et al., 1999). Similarly, though the greatest identifiable risk for development of asthma is sensitization to aeroallergens early in life (Simpson et al., 2010), respiratory viral infections are both a significant cause of asthma exacerbation and a significant risk factor for later wheezing and asthma development (Lemanske et al., 2005; Jackson et al., 2008). Not surprisingly, deficient production of IFN-β (Wark et al., 2005) and IFN-λ (Contoli et al., 2006) are associated with increased viral-induced asthma exacerbation.

The Role of IFN-α/β in Th2 Development

The association between allergies and a deficiency in type I interferon raises several interesting questions. What is the relationship between IL-4 signaling and IFN-α signaling? Given the dominance of IL-4 over Th1 development, does IL-4 override any effects of IFN-α in Th1 development? Alternatively, given the strong connection between type I interferon and Th1 development and the fact that IL-4 signaling through GATA3 represses Th1 development, it is plausible that IFN-α/β might cross-regulate Th2 development. Previous studies in both mice and humans do not offer much clarity on this point. Injection of IFN-α into mice resulted in reduced IL-4 mRNA and increased IFN-γ mRNA in total splenocytes compared to untreated mice (Finkelman et al., 1991). However, the cells were not fractionated prior to analysis, so it is not

possible to say which cell populations contributed this change. Injection of IFN-α into mice has also been shown to impair protective Th2 immunity to *Nippostrongylus brasiliensis* (Urban et al., 1993). However, both of these results could potentially be explained by the additional activity of other cytokines like IL-18 (Matikainen et al., 2001; Freudenberg et al., 2002; Berenson et al., 2006), so it is difficult to infer whether these results reflect a specific effect of IFN-α on Th2 differentiation. Treatment of murine $CD4^+$ T cells with IFN-α in vitro showed a small reduction compared to untreated cells, but no cells were treated with both IFN-α and IL-4 (Wenner et al., 1996). Similarly, IFN-α/β treatment of bulk human $CD4^+$ T cells during acute stimulation seemed to inhibit IL-5, but not IL-4 or IL-13 (Schandene et al., 1996). There are reports that treatment with IFN-α/β can improve the condition of patients with hypereosinophilia (Zielinski and Lawrence, 1990; Butterfield and Gleich, 1994), and this is paralleled by reduced eosinophilia in mice treated with IFN-α (Meritet et al., 2001). Yet despite these results, the effect of IFN-α on IL-4-induced Th2 differentiation was still not established. Understanding the role that IFN-α plays in this process is important both for our understanding of Th2 development (Figure 1.4), and also for our understanding of how allergic asthma is affected by the cytokines induced during respiratory virus infections (Figure 1.5). Thus, I have sought to more carefully define the role of IFN-α in the development and function of human Th2 cells.

Hypothesis: IFN-α/β plays a counter-regulatory role in human IL-4-driven Th2 cells.

Aim 1: To determine the role of IFN-α/β during Th2 differentiation

Aim 2: To determine the role of IFN-α/β in differentiated Th2 cells

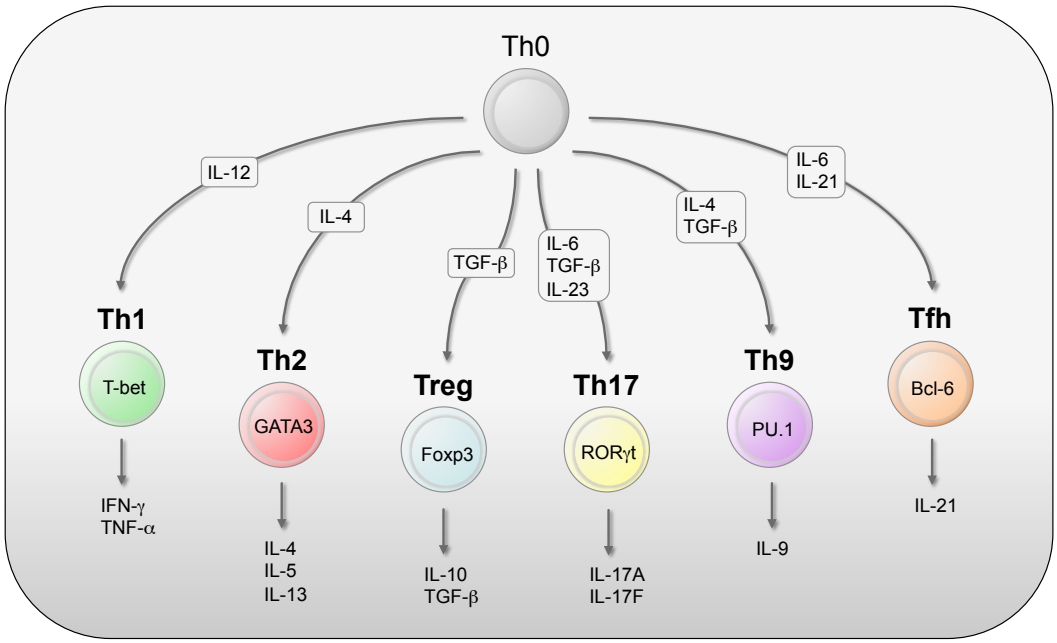

Figure 1.1. Innate cytokines control CD4⁺ T cell differentiation. Various combinations of innate cytokines promote differentiation of newly activated T helper cells into distinct functional subsets that express unique transcription factors and cytokines in order to appropriately respond to diverse immunological challenges.

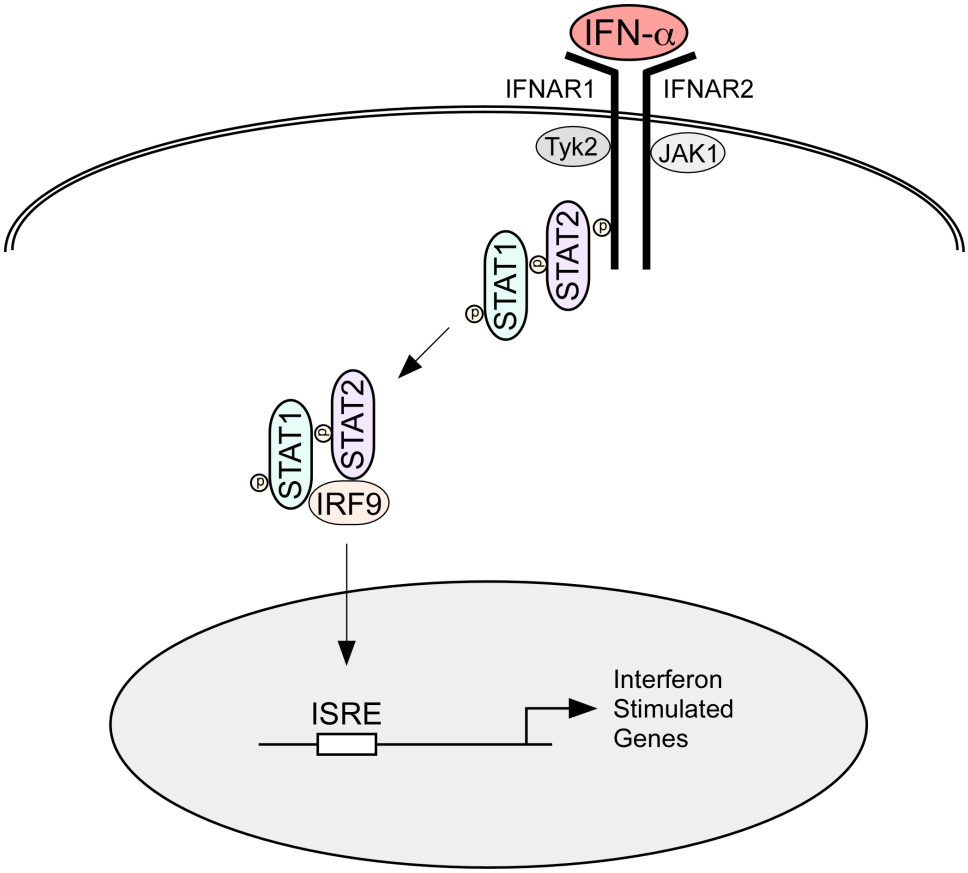

Figure 1.2. A model of type I interferon signaling. Binding by IFN-α to its receptor activates STAT2 and formation of the ISGF3 transcription factor complex. This complex then binds to interferon-sensitive response elements (ISRE) to activate expression of interferon stimulated genes. Although not pictured here, activated STAT2 can also dimerize with STAT1 and bind to GAS elements.

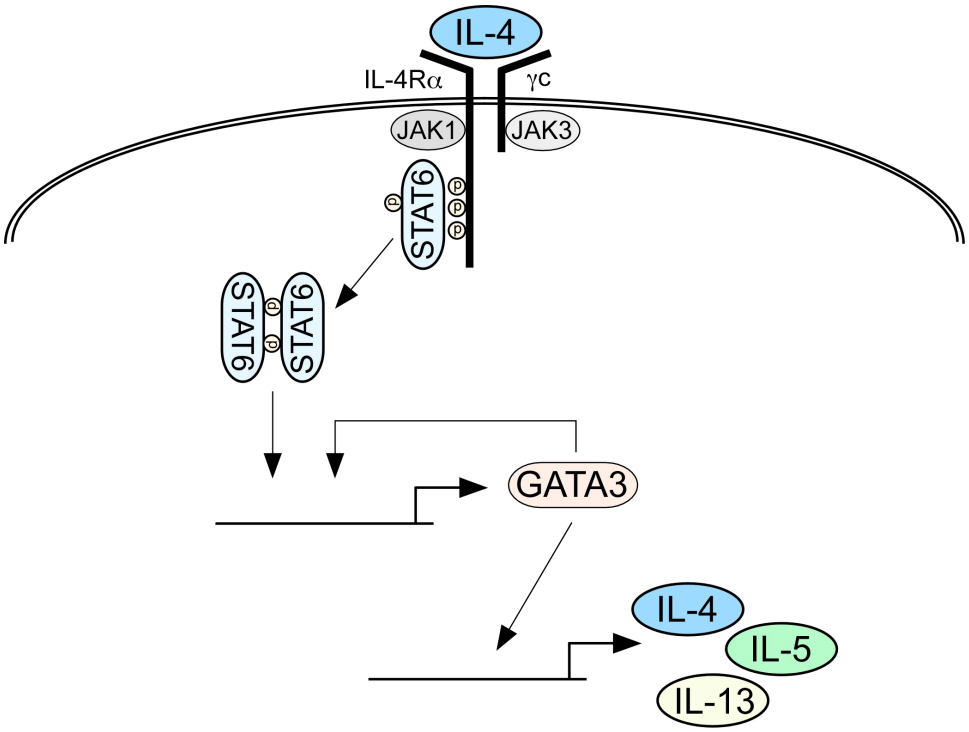

Figure 1.3. A model of IL-4 signaling. Binding by IL-4 to its receptor triggers phosphorylation and dimerization of STAT6, which then upregulates the transcription factor GATA3. GATA3 stabilizes the Th2 phenotype by promoting its own expression, and also enables expression of the Th2 effector cytokines IL-4, IL-5, and IL-13.

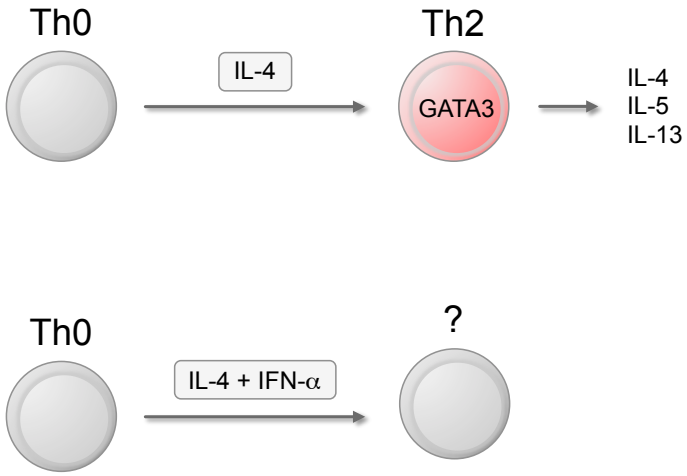

Figure 1.4. Potential regulation of Th2 development by IFN-α. IL-4 signaling in undifferentiated $CD4^+$ T cells leads to expression of GATA3 and the Th2 cytokines. IFN-α produced during viral infections, though insufficient to drive Th1 differentiation, may counter-regulate the development of Th2 cells in order to promote appropriate antiviral immunity.

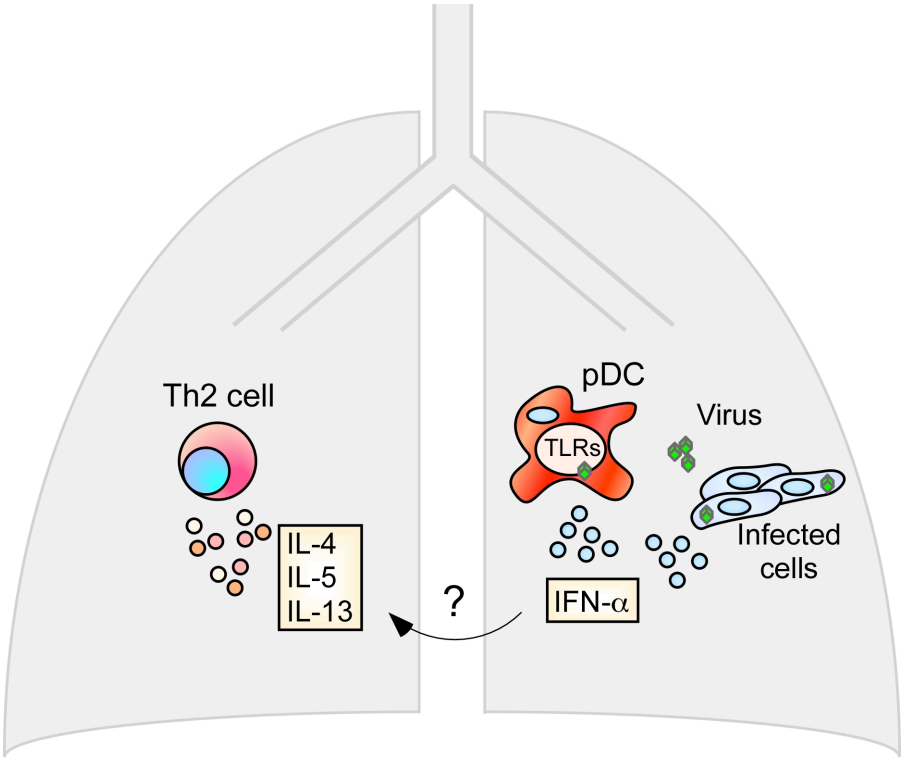

Figure 1.5. Potential regulation of Th2 cells in the lung. Cells from patients with allergic asthma produce less IFN-α when encountering respiratory viruses. Infection with these viruses also exacerbates the asthma. Understanding how normal virus-induced IFN-α production affects Th2 development and function may provide insight into the complex relationship between antiviral responses and allergic asthma.

CHAPTER II

MATERIALS AND METHODS

Human subjects

100-180 ml of peripheral blood was obtained from healthy adult volunteers by venipuncture. Informed consent was obtained from each donor according to guidelines established by the Internal Review Board (University of Texas Southwestern Medical Center). Alternatively, buffy coats were purchased from Carter Blood Care (Dallas, TX).

Mice

OT-II TCR transgenic mice were housed in specific pathogen-free conditions in accordance with guidelines established by the Institutional Animal Care and Use Committee (UT Southwestern Medical Center).

Cytokines, antibodies, and reagents

Recombinant human IL-4 (rhIL-4), rhIL-12, rhIFN-γ, rhIFN-αB2, rhIFN-αD, and the anti-human IL-4 (anti-hIL-4) antibody were purchased from R&D Systems (Minneapolis, MN). rhIFN-αA, the anti-hIFN-α/β receptor (IFNAR2) antibody, and polyclonal antisera against hIFN-α and hIFN-β were purchased from PBL Laboratories (Piscataway, NJ). rhIFN-β1a was a generous gift of M. Racke (University of Ohio). rhIL-29 (IFN-λ1) was purchased from Peprotech

(Rocky Hill, NJ). rhIL-2 was obtained from the NIAID Resources for Researchers (NIH, Bethesda, MD). The anti-hCD3 and anti-hCD28 antibodies were purchased from BioLegend (San Diego, CA). Additional anti-hCD3 (OKT3) antibody was purified from ascites fluid, and anti-hIL-12 (20c2) and anti-hIFN-γ (4S.B3) antibodies were purified from hybridomas.

The AlexaFluor 700 (Alexa700)-conjugated anti-hIL-2, phycoerythrin (PE)-conjugated anti-hIL-5, allophycocyanin (APC)-conjugated anti-hIL-13 antibodies, and rabbit polyclonal antisera against GATA3 (Poly6071) was purchased from BioLegend (San Diego, CA). The PE-conjugated anti-hCD4 and APC-conjugated anti-hCD4 antibodies were purchased from Invitrogen (Carlsbad, CA). The fluorescein isothiocyanate (FITC)-conjugated anti-hCD45RA, Alexa647-conjugated anti-hGATA3, Alexa647-conjugated anti-hCD294 (CRTH2), Alexa647-conjugated isotype, PE-conjugated anti-hIL-4, FITC-conjugated anti-hIL-4, PE-Cy7-conjugated anti-hIFN-γ, anti-hCD124 (IL-4Rα)-biotin, anti-hSTAT6 antibodies, and rabbit polyclonal antisera against phospho-Y641-hSTAT6 were purchased from BD Biosciences (San Jose, CA). The anti-hGATA3 (HG3-31) antibody and rabbit polyclonal antisera against GATA3 (H-48) and STAT2 (C-20 and L-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antiserum against tri-methyl-H3K27 and acetyl-H4 were purchased from EMD Millipore (Billerica, MA). The anti-tri-methyl-H3K4 antibody and rabbit polyclonal antiserum against H3 were purchased from Abcam (Cambridge, MA). The unconjugated murine IgG1 isotype control and the unconjugated anti-human Lamin antibodies were purchased from Cell Signaling Technology (Danvers, MA). The goat anti-hIgG/anti-hIgA/anti-hIgM antibody cocktail, rabbit control IgG, biotin-conjugated goat anti-rabbit Fab fragments, and biotin-conjugated goat anti-mouse Fab fragments were purchased from Jackson ImmunoResearch (West Grove, PA).

Phorbol-12-myristate-13-acetate (PMA) and MG132 were purchased from A.G. Scientific, Inc. (San Diego, CA). Streptavidin (SA)-PerCP was purchased from BD Biosciences. Phytohemagglutinin (PHA) was purchased from Calbiochem (La Jolla, CA). Brefeldin A was purchased from Epicentre (Madison, WI). SA-conjugated Qdot655 was purchased from Invitrogen. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin antiserum was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Carboxyfluorescein diacetate succinimidyl ester (CFSE), ionomycin, and 5-azacytidine were from Sigma-Aldrich (St. Louis, MO).

Human $CD4^+$ T cell cultures

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy adult volunteers. Heparinized whole blood was subjected to ficoll density centrifugation using Lymphocyte Separation Media (Cellgro, Manassas, VA). Buffy coats were further purified by incubating 45 min at 37°C on plates coated with goat anti-hIgG/anti-hIgM/anti-hIgA to obtain a total lymphocyte population. To obtain naïve human $CD4^+$ T cells, hPBMCs were stained with FITC-conjugated anti-hCD45RA and PE- or APC-conjugated anti-hCD4 antibodies, and $CD4^+$ $CD45RA^+$ cells were sorted on a MoFlo cell sorter (DakoCytomation, Fort Collins, CO) or a FACSAria cell sorter (BD, Franklin Lakes, NJ) at greater than 90% purity. Alternately, untouched $CD4^+$ $CD45RA^+$ cells were isolated using the Human Naive $CD4^+$ T Cell Enrichment Set (BD Biosciences) according to the manufacturer's instructions with purities greater than 90%.

Cells were activated at 2-2.5 x 10^6 cells/ml for three days on culture plates coated with 1-5 μg/ml anti-hCD3 + 1-5 μg/ml anti-hCD28 in the presence of 50 units (U)/ml rhIL-2, and

cultured in complete Iscove's Modified Dubelcco's Medium (HyClone, Logan, UT) supplemented with 1 mM sodium pyruvate (Hyclone), 2 mM L-glutamine (Hyclone), 10 U/ml penicillin (Hyclone), 10 μg/ml streptomycin (Hyclone), 50 μM β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA), non-essential amino acids (Hyclone), and 10% fetal bovine serum (FBS) (Valley Biomedical, Inc., Winchester, VA), (cIMDM). Alternately, cells were activated with 1 μg/ml PHA. Cells were cultured at 37°C in 5% CO_2.

Cytokine activation conditions were as follows unless otherwise indicated: Neutralized – anti-hIFN-γ, anti-hIL-12, anti-hIL-4, and anti-hIFNAR2; IL-4 – anti-hIFN-γ, anti-hIL-12, rhIL-4, and anti-hIFNAR2; IFN-α – anti-hIFN-γ, anti-hIL-12, anti-hIL-4, and rhIFN-αA; IL-4+IFN-α – anti-hIFN-γ, anti-hIL-12, rhIL-4, and rhIFN-αA. Unless otherwise indicated, IFN-α is always IFN-αA. Cytokines and neutralizing antibodies were added at the following concentrations: anti-hIFN-γ (4S.B3), 5 μg/ml; anti-hIL-12 (20C2), 5 μg/ml; anti-hIL-4, 2 μg/ml; anti-hIFNAR2; 2 μg/ml; rhIL-12, 10 ng/ml; rhIL-4, 20 ng/ml; rhIFN-αA, 1000 U/ml; rhIFN-αB2, 1000 U/ml; rhIFN-αD, 1000 U/ml; rhIFN-β, 1000 U/ml; rhIFN-λ, 100 ng/ml. On day three, cells were split into fresh media containing 50U/mL IL-2 and were rested to day 5 or day 7. For some experiments, cells were restimulated on day 7 for a further 2, 5, or 7 days as described.

Isolation and activation of murine splenocytes

Whole spleens from WT OT-II mice mice were harvested and leukocytes were isolated from the spleen through gentle grinding, followed by lysis of red blood cells. Splenic cells were activated with OVA peptide and cultured in cIMDM in the presence of polarizing cytokines as follows: Neutralized – anti-mIL-4 (clone 11B11), anti-IL-12 (TOSH), and anti-IFN-γ (R46A2),

IL-4 – rmIL-4 (10 ng/ml), anti-mIL-12, and anti-mIFN-γ, IFN-α – rm/hIFN-α(A/D) (1000 U/ml), anti-mIL-4, anti-mIL-12, and anti-mIFN-γ, or IL-4+IFN-α – (rmIL-4, rm/hIFN-αA/D, anti-mIL-12, and anti-mIFN-γ. On day 3, cells were split into cIMDM containing 50 U/ml rhIL-2 and rested to day 5.

Flow cytometric analysis

Naïve human $CD4^+$ T cells were differentiated for 7 days as described above. On day 7, cells were washed and rested overnight at 2 x 10^6 cells/ml in cIMDM without IL-2. Cells were left unstimulated or were restimulated for 6 hours at 37°C, 5% CO_2 with 50 ng/ml PMA + 1 μM ionomycin in the presence of 1 μg/ml Brefeldin A.

For flow cytometric detection of CRTh2 (CD294), cells were washed with PBS containing 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and labeled for 20 minutes at room temperature with anti-hCD294-Alexa647.

For intracellular cytokine staining, cells were washed in phosphate buffered saline (PBS) (Hyclone) and fixed for 20 minutes at room temperature in PBS containing 5% formalin (Mallinckrodt, Phillipsburg, NJ). Cells were then washed and permeabilized for 10 minutes at room temperature in PBS containing 0.5% BSA and 0.1% saponin (Sigma-Aldrich). Cells were labeled for 20 minutes at room temperature with fluorescently conjugated antibodies as noted in the figures. Cells were analyzed on a FACSCalibur or LSRII cytometer (BD), and the data were processed using FlowJo software (Tree Star, Ashland, OR).

For flow cytometric analysis of GATA3, human $CD4^+$ T cells were cultured as above to day 5. Cells were then harvested and stained with the Foxp3 Staining Kit (eBioscience, San Diego, CA) according to the manufacturer's instructions using anti-hGATA3-Alexa647 and a

corresponding isotype control. For co-staining of GATA3 and cytokines, cells were harvested on day 5 and left unstimulated or were immediately restimulated for 6 hours at 37°C, 5% CO_2 with 50 ng/ml PMA + 1 μM ionomycin in the presence of 1 μg/ml Brefeldin A prior to staining.

For assessment of proliferation, freshly isolated naïve human $CD4^+$ T cells were washed and resuspended in PBS at 1 x 10^7 cells/ml. Cells were labeled with 1.25 mM CFSE for 10 minutes at room temperature, and labeling was stopped by addition of cIMDM containing 20% FBS. Cells were washed extensively in cIMDM and differentiated for 5 days as described above. Dilution of CFSE was analyzed on an LSRII cytometer (BD), and the data were processed using FlowJo.

Analysis of GATA3 protein stability

Naïve human $CD4^+$ T cells were differentiated for 5 days as described above. On day 5, cells were washed, resuspended at 2 x 10^6 cells/ml in cIMDM, and incubated for 2 hours at 37°C, 5% CO_2 with 50μM MG132 in DMSO or with DMSO alone (vehicle). Cells were then harvested and stained for GATA3 as described above.

Intracellular staining for STAT6 phosphorylation

Whole STAT6 and phospho-tyrosine-641-STAT6 were detected by intracellular staining as follows: Freshly isolated naïve $CD4^+$ T were treated with cytokines for 10 min or 30 min as indicated in the text, then harvested and split into two parallel pools. Cells were incubated in 5% formalin/PBS followed by fixation in cold 100% methanol. Following fixation, cells were washed extensively in staining buffer (PBS, 0.5% BSA, and 1 mM sodium pervanadate ($NaVO_4$)),

permeabilized in staining buffer containing 0.1% saponin, and stained in three layers. Cells were first stained with unconjugated mouse anti-STAT6 or with rabbit anti-P-Y641 STAT6 antibodies (BD Biosciences), followed by biotinylated goat anti-mouse Fab or biotinylated goat anti-rabbit Fab (Jackson), then followed by SA-PerCP. Cells were collected on a FACSCalibur (BD), and the data were analyzed using FlowJo.

Quantitation of cytokine secretion

Naïve human $CD4^+$ T cells were activated for 7 or 14 days as described above, and were then restimulated at 1-2 x 10^5 cells/ml for 24-48 hours with 50 ng/ml PMA + 1 μM ionomycin or by activation on anti-CD3-coated plates (1-5 μg/ml). Cell-free supernatants were analyzed for IL-4, IL-5, IL-13 or IFN-γ cytokine concentrations by enzyme-linked immunosorbent assay (ELISA) using ELISA MAX kits (Biolegend, San Diego, CA) according to the manufacturer's protocols. Alternatively, multiple cytokine concentrations were quantified by Cytometic Bead Array (BD Biosciences) or the Meso Scale Discovery platform (Gaithersburg, MD) according to the manufacturer's instructions.

Co-culture of $CD4^+$ T cells and plasmacytoid dendritic cells

Naïve human $CD4^+$ T cells were differentiated for 7 days under Neutralized or IL-4 conditions as described above. At day 7, cells were reactivated either by culture on plates coated with 3 μg/ml anti-hCD3 and anti-hCD28 or by co-culturing with purified pDCs that had been previously activated for 18 hours at 2 x 10^4 per well in a round-bottom 96-well plate with rhIL-3 +/- Flu A (pDCs were purified and activated by Dr. Michelle Gill). Prior to co-culture, a small

aliquot of the supernatant from the activated pDCs was saved for an IFN-α ELISA (performed by Dr. Michelle Gill). During the reactivation, cells were cultured for 2 days in Neutralized or IL-4 conditions that were supplemented with polyclonal antisera against hIFN-α and hIFN-β. On day 2, cell-free culture supernatants were collected for cytokine quantification and cells were split into fresh media containing 50U/mL IL-2 and were rested to day 7. At day 7, cells were rested overnight and then either activated for intracellular cytokine staining as described above, or activated for 48 hours on a plate coated with 5 μg/ml anti-hCD3 to generate cell-free supernatants. All cell-free supernatants were analyzed by the Meso Scale Discovery platform per manufacturer's instructions.

Assessment of gene expression by quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from cultured cells using an RNeasy Mini Kit (QIAgen, Valencia, CA) or an Arcturus Pico Pure kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Optionally, following isolation, RNase-free DNase I digestion was performed for 15 minutes at 37°C. RNA was reverse transcribed for 1 hour at 42°C. The resultant cDNA was subjected to qPCR analysis with Brilliant II SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA) or Maxima SYBR Green Master Mix (Thermo Scientific) on an ABI7300 cycler (Applied Biosystems) using primers directed against human mRNA transcripts as listed in Table 1. Primers directed against human GAPDH or PPIA were used as a reference. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All primers were synthesized by Integrated DNA Technologies (Coralville, IA) or Sigma Aldrich. Primer sequences are found in Table 1.

Live cell sorting of CFSE-labeled cells for mRNA analysis

Naïve human $CD4^+$ T cells were labeled with CFSE and differentiated for 5 days under Neutralized, IL-4, IFN-α, or IL-4+IFN-α conditions as described above. On day 5, cells were washed sorted based on CFSE dilution on a MoFlo cell sorter (DakoCytomation) or a FACSAria cell sorter (BD Biosciences). mRNA was harvested from CFSE sorted cells as described above and used for qPCR analysis.

Retroviral transduction of human T cells

The GFPRV (Ranganath et al., 1998) and GATA3-GFPRV (Ranganath et al., 1998; Farrar et al., 2001) retroviral expression constructs have been described. Retroviral supernatants for transduction were generated by calcium chloride transfection of the Phoenix amphotropic packaging cell line (Orbigen, San Diego, CA). Retroviral supernatants for transduction were generated by calcium chloride transfection of the Phoenix amphotropic (PhA) packaging cell line (Orbigen, San Diego, CA). PhA cells were maintained in Dubelcco's Modified Eagle Medium (Hyclone) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 0.015% sodium bicarbonate ($NaHCO_3$), non-essential amino acids, and 10% FBS (cDMEM). Immediately prior to transfection, cells were given fresh media supplemented with 25 μM chloroquine. The transfection mixture was prepared as follows: 25-30 μg plasmid DNA was suspended in a solution of 0.244 M calcium chloride ($CaCl_2$). The DNA/$CaCl_2$ mixture was combined 1:1 with 2 X HBS (50 mM HEPES, pH 7.05, 10 mM KCl, 280 mM NaCl, 12 mM dextrose, 1.5 mM $Na2HPO_4$) and mixed vigorously for 10-15 seconds. This

mixture was added drop-wise to PhA cells, and the cells were incubated at 37°C, 5% CO_2 for 8 hours. The transfection mixture was then exchanged for fresh cDMEM, and the cells were rested for 16 hours. Media was then exchanged for fresh cDMEM, cells were incubated at 32°C, and retroviral supernatants were harvested at 24 hour intervals and used immediately for transduction of T cells.

Naïve human $CD4^+$ T cells isolated as described above were activated at 2 x 10^6 cells/ml on culture plates coated with 3 μg/ml anti-hCD3 + 3 μg/ml anti-hCD28 in cIMDM under IL-4 conditions as described above until d3, then split into fresh media containing IL-2 and rested to day 7. Cells were then reactivated with 3 mg/ml anti-hCD3 + 3 mg/ml anti-hCD28 in IL-4 conditions and 600 U/ml rhIL-2. Cells were incubated at 37°C, 5% CO_2 for 24 hours and then transduced with retroviral supernatants in the presence of 5 μg/ml polybrene and IL-4 conditions and rhIL-2 as described above for 60 minutes at 1000 x *g*. Transduction was repeated for two consecutive days. On day 3, cells were split 1:10 into fresh cIMDM containing 50 U/ml rhIL-2 and rested to day 14. At day 14, GFP^+ cells were sorted and reactivated with anti-CD3/anti-CD28 plus 50U/ml rhIL-2 in the presence or absence of rhIFN-αA. On day 2 following the GFP sort, cell-free supernatants were collected for ELISA analysis and cells were split 1:10 into fresh cIMDM containing 50 U/ml rhIL-2 and rested for a further 5 days (for intracellular staining of GATA3 as described above) or 7 days. At day 7 following the GFP sort, cells were reactivated for 48 h with anti-hCD3 + anti-hCD28 to generate cell-free supernatants for ELISA analysis. Alternatively, cells were harvested on day 3 following the GFP sort and analyzed for endogenous and ectopic GATA3 mRNA expression.

Assessment of GATA3 cellular localization by immunofluorescence

Naïve $CD4^+$ T cells were isolated from human PBMCs, activated for 5 days under the polarizing conditions described above, and then incubated 2 hours +/- rhIFN-αA, +/- reactivation with 3 μg/ml OKT3 and anti-hCD28. The cells were then adhered to coverslips coated with 0.1% poly-L-lysine (Sigma-Aldrich) and fixed with 4% paraformaldehyde in PBS. Cells were then stained by placing the coverslips facedown in PBS + 1% BSA containing anti-hCD4-PE and incubating overnight at 4°C. Cells were then washed with PBS, permeabilized with the FoxP3 Staining Kit (eBioscience) as described for flow cytometry, and stained overnight with anti-hGATA3-Alexa647. Cells were washed with PBS, the excess buffer was drained, and the coverslips were mounted onto slides with Prolong Gold + DAPI (Invitrogen). Antibody specificity was confirmed using an Alexa647-labeled isotype control. Fluorescence microscopy was performed on a Deltavision Deconvolution microscope and images were prepared using ImageJ software (NIH, Bethesda, MD).

Assessment of GATA3 by Western blotting

Naïve human $CD4^+$ T cells isolated as described above were activated on culture plates coated with anti-hCD3 + anti-hCD28 in cIMDM under Neutralized, IL-4, or IL-4+IFN-α conditions as described above until d3, then split into fresh media containing IL-2 and were rested to day 7. Lysis was performed at 5 x 10^7 cells/ml for 1 hour at 4°C 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 (PMSF), 1 mM dithiothreitol (DTT), 10 μg/ml

leupeptin, 1 mM benzamidine, 1 μM pepstatin, and 1 mM $NaVO_4$). Samples were resolved by SDS-PAGE on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Western blotting was performed using rabbit polyclonal antisera against GATA3 (Poly6071, Biolegend) 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 Lamin as a loading control.

Electrophoretic Mobility Shift Assay (EMSA)

Naïve $CD4^+$ T cells were isolated from human PBMCs, activated for 5 days under the polarizing conditions described above, and then reactivated on day 5 for 2-4 hours with 3 μg/ml OKT3 and anti-hCD28. Cells (5-10 x 10^6 per condition) were washed in cold PBS and resuspended for 10 min on ice in 200 μl HB buffer (25 mM Tris-HCl pH 7.4, 1 mM MgCl2, 5 mM KCl) plus proteinase and phosphatase inhibitors. 20 μl NP-40 was added to 1% final concentration and incubated an additional 5 min on ice. Nuclei were pelleted at 1600 x g, the cytoplasmic fraction was saved, and nuclei were washed with 300 μl HB buffer. Nuclei were then resuspended in 25 μl NEBHS buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 1.5 mM MgCl2, 0.1% NP-40) plus proteinase and phosphatase inhibitors and incubated 30 min on ice, vortexing every 5 min. Final nuclear and cytoplasmic fractions were clarified by centrifugation at max speed and quantified by Bradford assay (Bio-Rad). 5 μg of each nuclear lysate was then incubated for 30 minutes at room temperature with 3'-biotin-labeled double-stranded DNA

probes (listed in table 2) along with poly-dI/dC in 1X EMSA buffer (5X = 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM DTT, 5 mM EDTA, 25% glycerol). All oligos were synthesized and end-labeled by Integrated DNA Technologies (Coralville, IA) and then annealed together. Specificity was confirmed using unlabeled probes and a GATA3-specific antibody (H-48, Santa Cruz Biotechnology). The complexes were then run through a 4.5% non-denaturing polyacrylamide gel at 150 V for about 2 hours and transferred to a Hybond-N^+ membrane (Amersham Biosciences). Detection of the biotinylated complexes was performed using a Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) according to the manufacturer's instructions. Densitometry analysis was performed using ImageJ software.

Chromatin Immunoprecipitation (ChIP)

For GATA3 and Histone ChIP, naïve $CD4^+$ T cells were isolated from human PBMCs, activated for 5 days under the polarizing conditions described above, and then reactivated on day 5 for 2-4 hours with 3 ug/ml OKT3 and anti-hCD28. For STAT2 ChIP, total T cells were isolated from human PBMCs and activated for 2.5 hours +/- 1000 U/mL rhIFN-α. ChIP was then performed with a Chromatin Immunoprecipitation Assay Kit (Millipore) according to a modified protocol. Briefly, 8-12 x 10^6 cells per condition were resuspended in PBS and the chromatin was then crosslinked 10 min with 1% formaldehyde (EMS, Hatfield, PA) in PBS for 10 min, then neutralized with 0.125 M glycine for 5 min. Cells were washed with PBS and resuspended in cell lysis buffer (5 mM Pipes pH 8, 85 mM KCl, 0.5% NP-40) plus proteinase and phosphatase inhibitors for 10 min on ice. Nuclei were pelleted isolated and resuspended in an SDS lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS) plus proteinase and phosphatase inhibitors for 10 min on ice. DNA was fragmented by sonication for 4X 6 min in a

Bioruptor (Diagenode, Denville, NJ). The chromatin lysates were then quantified and aliquoted for ChIP samples and input controls. Lysates were diluted ~10-fold in ChIP dilution buffer (16.7 mM Tris-HCl pH 8, 167 mM NaCl, 1.2 mM EDTA, 1.1% TritonX-100, 0.01% SDS) plus proteinase and phosphatase inhibitors, pre-cleared with Protein A/G beads (Thermo Scientific, Rockford, IL) and then incubated overnight at 4°C with 1-4 ug of primary antibodies or control antibodies, followed by incubation with Protein A/G beads for 1.5 hours. Beads were washed for 5 min with low salt buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, 0.1% SDS), high salt buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 2 mM EDTA, 1% TritonX-100, 0.1% SDS), LiCl wash buffer (10 mM Tris-HCl pH 8, 250 mM LiCl, 1 mM EDTA, 1% deoxycholate, 1% NP-40), and 1X TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). The protein/DNA complexes were eluted from the beads with elution buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 2% SDS), crosslinks were reversed by the addition of NaCl (final concentration 200 mM) and incubation for 4 hours or overnight at 65°C, during which the remaining protein was degraded with Proteinase K. The DNA was then purified with a minElute DNA purification kit (QIAgen) according to manufacturer's instructions. The purified DNA was used for qPCR analysis as described above, and ChIP efficiency was calculated according to the following formula:

$$2^{(\text{Input Ct - ChIP Ct})} \text{ x dilution factor x } 100.$$

All primers were synthesized by Integrated DNA Technologies or Sigma Aldrich. Primer sequences are found in Table 2. Primer sequences for the ISG54 promoter (hISG54pro) were taken from (Paulson et al., 2002).

Statistical analysis

All graphs are shown as mean +/- SEM. Statistical analysis was performed by one-way and two-way ANOVA using Prism software (GraphPad, San Diego, CA). Values of $p < 0.05$ were considered significant.

Table 2.1 Primers used in qPCR analysis of gene expression.

Gene	Dir.	Sequence
hGATA3	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'
hT-bet	F	5'-CCTCTCCTACCCAACCAGTATCCT-3'
	R	5'- ATGCAGGCTTCATGCTGACTG-3'
Endogenous GATA3	F	5'-CAGACACATGTCCTCCCTGAGC-3'
	R	5'-GGACTGCAGGGACTCTCGCTGGGG -3'
Retroviral GATA3	F	5'- CAGACACATGTCCTCCCTGAGC -3'
	R	5'-TAACATATAGACAAACGCACAC-3'
hRunx3	F	5'-CTTTGGGGACCTGGAACGGC-3'
	R	5'-AAGGAGCGGTCAAACTGGCG-3'
hFog1	F	5'-TGTCCAGGCGGAAACAGAGC-3'
	R	5'-GGTGGGGGTGAGTTAACATCTGC-3'
hGAPDH	F	5'-CTGCACCACCAACTGCTTAGCA-3'
	R	5'-TGATGTTCTGGAGAGCCCCG-3'
hPPIA	F	5'-GCGTCTCCTTTGAGCTGTTTGC-3'
	R	5'-ATGGACTTGCCACCAGTGCC-3'

Table 2.2. Oligos used for EMSA analysis.

Gene	Dir.	Sequence
IL-4 CIRE probe	F	TGAGTACCTATCTGGCACCATCTCTCCA- 3'BioTEG
	R	TGGAGAGATGGTGCCAGATAGGTACTCA-3'BioTEG
GATAcons competitor	F	CACTTGATAACAGAAAGTGATAACTCT
	R	AGAGTTATCACTTTCTGTTATCAAGTG
EαY probe	F	AAATATTTTTCTGATTGGCCAAAGAGTAAT-3'BioTEG
	R	ATTACTCCTTGGCCAATCAGAAAAATATTT-3'BioTEG
EαY competitor	F	AAATATTTTTCTGATTGGCCAAAGAGTAAT
	R	AATACTCTTTGGCCAATCAGAAAAATATTT

Table 2.3. Primers used in qPCR analysis of ChIP.

Gene	Dir.	Sequence
hGATA3 Primer #1	F	5'-AACATTGGGAGCTGAAAGG-3'
	R	5'-ACGACTCTTTCCTTATCTGTGC-3'
hGATA3 Primer #2	F	5'-AAACACTGGTGAGCAGGC-3'
	R	5'-CAGACCTATTCCACCCAGG-3'
hGATA3 Primer #3	F	5'-GGACTCTGGCCTTTCTACCC-3'
	R	5'-CCTGCAAAACCACCTCTCTC-3'
hGATA3 Primer #4	F	5'-CTCCTTCGACCTGCTAATGG-3'
	R	5'-TGGCAGGGTTGGGATTAAC-3'
hGATA3 Primer #5	F	5'-AACCCTGCCAGAAGACAGCG-3'
	R	5'-CAGCGAGGGAATGAATTTCACC-3'
hGATA3 Primer #6	F	5'-GGGTTTCTGTGGTGACCTTG-3'
	R	5'-TTGTTTTCCCCAATCCTCTG-3'
hGATA3 Primer #7	F	5'-CTTTCTCTCGGACACCAACAG-3'
	R	5'-TTAATCTCCCATCTGCGACC-3'
hIL-4 CIRE	F	5'-GCAGAAGGTGAGTACCTATCTGGC-3'
	R	5'-CTGCCACCAACCACCAGTTC-3'
hISG54pro	F	5'-CTCCGGAGGAAAAAGAGTCCTC-3'
	R	5'-AAATCTTCCTCTTCTGCCACC-3'

CHAPTER III

TYPE I INTERFERON REVERSES HUMAN TH2 COMMITMENT AND STABILITY BY SUPPRESSING GATA3

Most of the work in this chapter has been published in the Journal of Immunology, volume 185, pages 813-817 (Huber et al., 2010). This work is reproduced with the permission of the Journal of Immunology. Copyright 2010 The American Association of Immunologists, Inc. Experiments were performed by Jonathan P. Huber unless otherwise indicated in the text or figure legends.

Introduction

$CD4^+$ Th2 cells regulate both humoral and cell-mediated inflammatory responses to helminth pathogens as well as toxins and other foreign soluble molecules (Holgate and Polosa, 2008). In genetically predisposed individuals, Th2 cells also mediate atopic responses to normally innocuous materials, such as animal dander, pollens, and pollutants. Th2 cells mediate these processes through the selective secretion of a subset of cytokines that include IL-4, IL-5, IL-13, and, in some cases, IL-9 and IL-10 (Larche et al., 2003). Together, these cytokines lead to a cascade of events that culminate in driving inflammation at sites of allergen contact, such as the lungs in cases of allergic asthma.

Various signals contribute to a Th2 environment, such as thymic stromal lymphopoietin, OX40 ligand, Notch (Ong et al., 2008), and IL-25 (Wang and Liu, 2009); however, IL-4 remains the key signal that directly drives Th2 commitment. IL-4R signaling promotes STAT6

recruitment and phosphorylation (Kaplan et al., 1996), which ultimately regulates induction of GATA3 transcription (Kurata et al., 1999). GATA3 is a member of the 2-zinc finger GATA-binding family of transcription factors, and its expression is critical for both T cell thymic development and Th2 commitment (Pai et al., 2004). Once induced by IL-4, the GATA3 protein positively regulates Th2 cytokine gene expression and negatively regulates various aspects of Th1 commitment, such as inhibiting the expression of the IL-12Rβ2 subunit (Ouyang et al., 1998). Further, GATA3 positively regulates its own expression through an autoactivation loop, thereby maintaining Th2 stability in the absence of further IL-4 signaling (Ouyang et al., 2000; Farrar et al., 2001). The inherent stability of Th2 cells poses a significant challenge to treating allergic diseases. However, based on the emerging role of type I IFN (IFN-α/β) in the development of adaptive T cell immunity, especially in promoting Th1 responses, I hypothesized that IFN-α/β may play a counter-regulatory role in Th2 cells. Indeed, in this study, I have found that IFN-α/β potently inhibits IL-4–driven Th2 commitment in human $CD4^+$ T cells and destabilizes the Th2 phenotype by inhibiting GATA3 expression. Considering that IFN-α/β is used routinely to treat various diseases including multiple sclerosis and hepatitis C, IFN-α/β may represent a novel and readily available therapy for atopic conditions, such as allergic asthma.

Results and Discussion

Selective inhibition of human Th2 development by IFN-α/β

IL-4 is a critical regulator of Th2 development, and the downstream signaling events in this pathway dominate over the effects of other Th1-inducing signals, such as IFN-γ and IL-12. Although early reports suggested that IFN-α/β could decrease baseline Th2 cytokine secretion in naive human $CD4^+$ T cells (Schandene et al., 1996; Shibuya and Hirohata, 2005), the role of IFN-α during Th2 priming in response to IL-4 has not been investigated in human cells. Previous studies with mouse DO11.10 T cells ruled out IFN-α/β as a negative regulator of Th2 commitment (Wenner et al., 1996; Szabo et al., 1997). Indeed, Dr. J. David Farrar confirmed these previous observations by demonstrating that IFN-α does not inhibit IL-4–driven Th2 development in murine OT-II transgenic T cells (Figure 3.1). However, in this study, I tested the role of IFN-α/β in regulating human Th2 development by activating purified naive human $CD4^+$/$CD45RA^+$ T cells with plate-bound anti-CD3/anti-CD28 in the presence of cytokines or neutralizing anti-cytokine antibodies for 7 d. Cells were then restimulated and assessed for cytokine production. IL-4 potently induced Th2 development by promoting high levels of IL-4 expression (Figure 3.2A, lower panel), as well as IL-4 and IL-5 secretion (Figure 3.2B). Moreover, IL-12, but not IFN-α, promoted Th1 commitment as revealed by high levels of IFN-γ expression (Figure 3.2A, upper panel). Neither IL-12 nor IFN-γ inhibited IL-4 expression (Figure 3.2A, lower panel; 3.2B, upper panel), whereas IL-12 did inhibit IL-5 secretion by ~50% (Figure 3.2B, lower panel). In contrast, I found that IFN-α markedly blocked IL-4–driven Th2

development in human $CD4^+$ T cells (Figure 3.2A, lower panel, 3.2B). This inhibition was specific to Th2 cells, as IFN-α did not block IL-12–mediated Th1 development (Figure 3.2A, upper panel), and IFN-α consistently inhibited Th2 development in cells isolated from each of 10 healthy adult donors (Figure 3.3). IFN-α did not attenuate proliferation during priming, suggesting that IFN-α/β did not interfere with Th2 commitment by inhibiting Th2 cell expansion (Figure 3.4). Finally, multiple subtypes of IFN-α inhibited Th2 cytokine production (Figure 3.5A), while both IFN-α and IFN-β blocked Th2 development in a dose-dependent manner (Figure 3.5B), suggesting that Th2 cross regulation in human $CD4^+$ T cells is a general property of IFN-α/β signaling.

In humans, the PGD_2 receptor, CRTh2, is selectively expressed on Th2 cells (Cosmi et al., 2000) and is induced by IL-4 during Th2 development (Langenkamp et al., 2003). I assessed the expression of CRTh2 in response to cytokine activation during Th2 commitment in human $CD4^+$ T cells. IL-4 promoted the development of cells expressing CRTh2; however, IFN-α markedly blocked IL-4-driven CRTh2 expression (Figure 3.6C). A recent report suggested that IL-28/29 (IFN-λ, type III IFN) could inhibit human Th2 cells (Dai et al., 2009). I compared IL-29 (IFN-λ1) with IFN-α, and while both IFNs were able to reduce Th2 cytokine production (Figure 3.6A, B), I observed only a modest decrease in IL-4–driven CRTh2 expression in response to IL-29 that was not significantly different compared with treatment with IL-4 alone (Figure 3.6C, compare conditions 2 and 6). However, I found that IFN-α significantly blocked IL-4–mediated CRTh2 expression compared with IL-29 (Figure 3.6C). Therefore, IFN-α/β inhibits both Th2 cytokine secretion and expression of the inflammatory receptor CRTH2 that mediates recruitment and cytokine secretion from committed Th2 cells (Xue et al., 2005). Collectively, these data demonstrate a unique and species-specific inhibition of human Th2

development by IFN-α/β.

IFN-α/β destabilizes committed human Th2 cells by repressing GATA3

IL-4 regulates Th2 commitment via activation of STAT6, which drives expression of the Th2-specific transcription factor GATA3 (Kaplan et al., 1996; Kurata et al., 1999; Zhu et al., 2001). GATA3 stabilizes the Th2 phenotype by a feedback loop that uncouples the requirement for further IL-4 signaling (Ouyang et al., 1998; Farrar et al., 2001). I tested the ability of IFN-α to disrupt the stability of committed Th2 cells by first activating naive T cells for 1 week with IL-4 (Figure 3.7). These cells were then reactivated for an additional 7 d with either IL-4, anti–IL-4, IFN-α, or IL-4 + IFN-α followed by analysis of Th2 cytokine production. IL-4 efficiently promoted the development of Th2 cells, and, as expected, their stability was maintained during secondary culture regardless if the cells were activated with additional IL-4 or with anti–IL-4 (Figure 3.7A, conditions 1–3). However, IFN-α markedly inhibited Th2 cytokine expression during secondary activation even in the presence of additional IL-4. Further, IFN-α inhibited IL-4 secretion to levels observed in non-Th2 cells cultured under neutralizing conditions for 2 consecutive weeks (Figure 3.7B). Cytokine production from committed Th2 cells was also inhibited by co-culture with FluA-activated pDCs (Figure 3.8A). This inhibition was not reversed by addition of a blocking anti-hIFNAR antibody (Figure 3.8A, condition 4), likely due to overwhelming quantities of type I interferon produced by the pDCs in response to FluA (Figure 3.8B) or due to secretion of IFN-λ (Yin et al., 2012), though I cannot rule out the possibility of additional suppressive mechanisms.

A recent study by Löhning and colleagues (Hegazy et al., 2010) demonstrated that a

combination of IL-12 plus IFN-γ and IFN-α could promote IFN-γ secretion from fully committed murine Th2 cells and give rise to IL-4/IFN-γ dual cytokine-secreting cells. However, I found that in human cells, IFN-α alone suppressed IL-4 secretion both during priming (Figures 3.2, 3.3) and after secondary activation (Figure 3.7) even in the absence of IL-12. Moreover, I found that in human Th2 cells, the combination of IFN-γ plus IFN-α plus IL-12 only marginally increased IFN-γ production and secretion during secondary priming (Figure 3.9). Thus, in human $CD4^+$ T cells, IFN-α suppresses Th2 commitment and stability and is dominant over the Th2-promoting signal of IL-4, but does accomplishes this without promoting significant IFN-γ secretion either during priming or during secondary redirection.

GATA3 is required for Th2 cell stability and cytokine production. $CD4^+$ cells from mice with a T cell-specific deletion of GATA3 produce increased levels of IFN-γ but greatly reduced levels of IL-4, IL-5, and IL-13 (Pai et al., 2004). Additionally, the deletion of GATA3 specifically in Th2 cells significantly impairs their ability to produce these cytokines (Pai et al., 2004; Zhu et al., 2004). Based on these observations, I tested the ability of IFN-α to modulate the induction of GATA3 by IL-4 (Figure 3.10). IL-4 induced GATA3 mRNA in naïve $hCD4^+$ T cells (Figure 3.10A, compare conditions 1 and 2). However, cells cultured with IFN-α showed significantly reduced GATA3 transcript levels (Figure 3.10A, conditions 3 and 4). The block in GATA3 mRNA also resulted in a significant inhibition of IL-4–induced GATA3 protein by IFN-α (Figure 3.10B, C). As previously reported (Lantelme et al., 2001; Hernandez-Hoyos et al., 2003), naive $CD4^+$ T cells expressed low levels of GATA3 that were induced by TCR activation and further elevated in response to IL-4 (Figure 3.10C, conditions 1–3). In line with the observed decrease in GATA3 mRNA (Figure 3.10A), IFN-α significantly inhibited IL-4–induced GATA3 protein expression, but this was not observed in response to either IL-12 or IFN-γ (Figure 3.10C). Because I have

demonstrated that IFN-α inhibits the development of CRTh2$^+$ cells (Figure 3.6C), it is not surprising that IFN-α controls GATA3 as well, considering that GATA3 has been shown to control the expression of CRTh2 (Quapp et al., 2007). Thus, the ability of IFN-α to regulate a key Th2 transcription factor correlates with its ability to regulate CRTh2 and Th2 cytokine expression.

A variety of GATA-binding proteins, such as Runx, Fog, and T-bet, have been shown to inhibit the transcriptional regulatory activity of GATA family members, including GATA3 (Zhou et al., 2001; Kurata et al., 2002; Hwang et al., 2005; Kohu et al., 2009). I tested whether any of the select members of these GATA-binding factors were induced by IFN-α during priming. However, I found that neither Runx3 nor Fog1 (data not shown), nor T-bet (Figure 3.11) were significantly induced by IFN-α during Th2 development. T-bet expression did increase in naïve CD4$^+$ T cells cultured to day 3 in the presence of IFN-α alone, but this was abrogated by the addition of IL-4 to the culture (Figure 3.11A, compare conditions 3 and 4). Furthermore, in accordance with previous findings (Ramos et al., 2007), IFN-α was not able to sustain T-bet expression in cells cultured to day 7 (Figure 3.11B). While protein levels of these inhibitory factors have not been examined, the inability of IFN-α to increase expression at the transcriptional level suggests a novel negative regulatory mechanism for GATA3 inhibition.

IFN-α/β overrides GATA3-mediated Th2 stability

Because IFN-α limits cytokine production from committed Th2 cells, I considered whether IFN-α bypassed IL-4 signaling and inhibited GATA3 expression directly. Although induction of GATA3 by IL-4 depends upon the activation of STAT6 (Zhu et al., 2001), Dr.

Hilario J. Ramos tested this early signaling event and found that IFN-α did not significantly alter acute IL-4–driven STAT6 phosphorylation (Figure 3.12). Thus, I determined if IFN-α could inhibit GATA3 protein levels in committed Th2 cells that have already upregulated the protein (Figure 3.13). Polarization of naive T cells with IL-4 for 7 d led to a stable increase in GATA3 protein levels (Figure 3.13, conditions 1–3). However, IFN-α markedly inhibited GATA3 protein expression (Figure 3.13, conditions 4 and 5), and these data strongly suggest that IFN-α destabilizes human Th2 cells by directly interfering with GATA3 expression.

GATA3 is considered a master regulator of Th2 development based on its ability to promote Th2 commitment when ectopically expressed in cells under various counter-regulatory situations. The GATA3 feedback loop was originally discovered when cells transduced with GATA3RV subsequently upregulated the endogenous GATA3 mRNA (Ouyang et al., 2000). I hypothesized that ectopic expression of GATA3 in human Th2 cells would elevate GATA3 protein levels and thereby reverse the inhibition normally mediated by IFN-α. To test this hypothesis, I expressed GATA3 in human Th2 cells via retroviral transduction (Figure 14). GFP^+ cells were reactivated for 3 d, and the culture supernatants were analyzed for IL-5 and IL-13 (Figure 3.14A). As expected, the levels of both cytokines increased significantly in cells transduced with GATA3-GFP compared with the control retrovirus (Figure 3.14A). The addition of IFN-α to the culture inhibited the secretion of both IL-5 and IL-13 from cells transduced with empty retrovirus as well as in cells transduced with GATA3-GFP. Purified GFP^+ cells were cultured for 7 d, at which time the cells were washed, reactivated, and analyzed for cytokine secretion (Figure 3.14B). Again, addition of IFN-α to the culture inhibited the secretion of Th2 cytokines even in cells ectopically expressing GATA3. Thus, IFN-α is able to overcome ectopic expression of GATA3 and suppress the secretion of Th2 cytokines.

To confirm that GATA3 was actually expressed in the transduced cells, I analyzed GATA3 protein levels by intracellular staining at day 5 following sorting (Figure 3.14C). Indeed, GATA3 protein levels increased in cells transduced with GATA3-GFP compared with the GFPRV control. However, IFN-α treatment reduced GATA3 protein levels in cells transduced with either the empty retrovirus or with GATA3-expressing retrovirus. IFN-α treatment did not affect the retroviral mRNA levels (data not shown), further supporting the conclusion that IFN-α inhibits GATA3 protein levels.

In summary, I demonstrate a novel function for type I IFN to block Th2 development and destabilize committed Th2 cells via inhibition of GATA3. This function is lacking in mice but may be important for ensuring proper differentiation of $CD4^+$ T cells activated during viral infections in humans. As IFN-α/β has already been demonstrated to inhibit the development of Th17 but not Th1 cells (Ramos et al., 2007; Moschen et al., 2008; Ramgolam et al., 2009), IFN-α/β may restrict alternate differentiation pathways for newly activated $CD4^+$ T cells. Further, both Th2 and Th17 responses have been proposed to contribute to the pathology of allergic diseases (Pene et al., 2008; Ciprandi et al., 2010). Thus, because IFN-α/β inhibits both Th17 and Th2 pathways but does not promote Th1 cytokine expression, IFN-α/β may play a key role in controlling allergic responses.

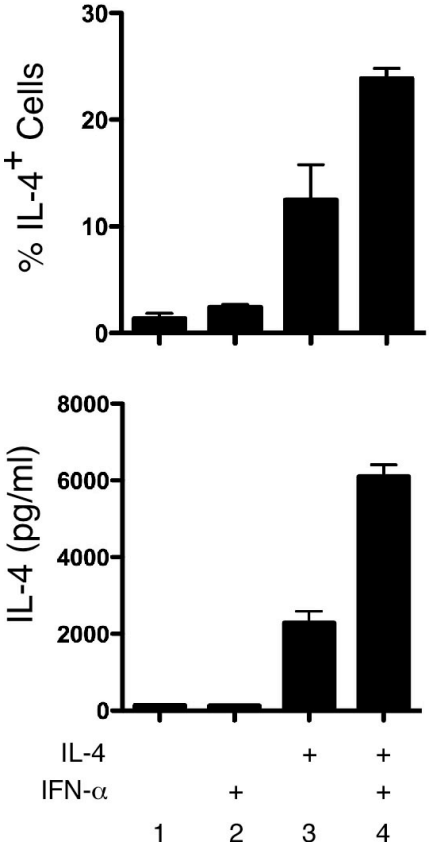

Figure 3.1. IFN-α does not inhibit murine Th2 cells. Splenic cells from OT-II transgenic mice were activated with OVA peptide and cultured for 5 days with anti-IL-4, IFN-α, IL-4, or IL-4 + IFN-α. Upper panel, cells were stimulated with PMA + ionomycin for 4 h and IL-4 was measured by intracellular staining. Lower panel, cells were restimulated with PMA + ionomycin for 36 h and IL-4 was quantified by ELISA from the culture supernatants. (This work was done by Dr. J. David Farrar.)

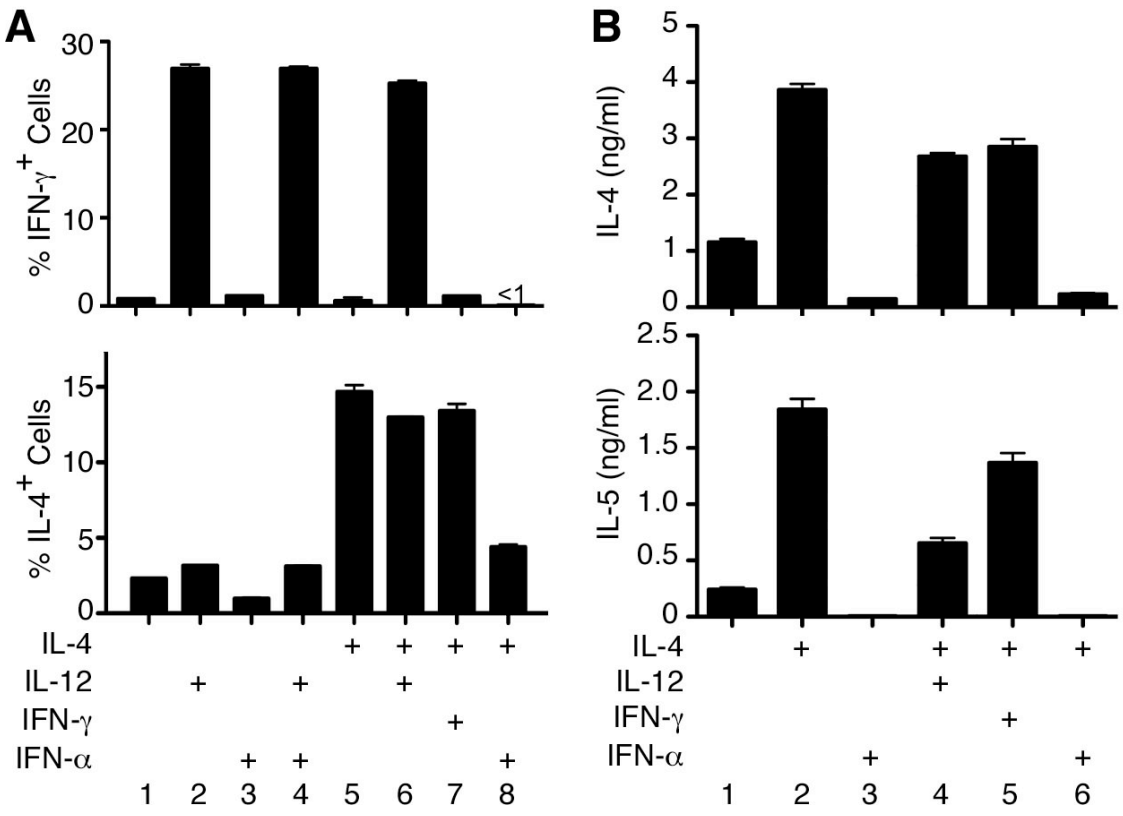

Figure 3.2. IFN-α/β inhibits human CD4⁺ Th2 development but not Th1 development. Purified human CD4⁺/CD45RA⁺ cells were activated with plate-bound anti-CD3/anti-CD28 under defined cytokine conditions. Cytokines were either neutralized with anti-cytokine antibodies (no symbol), or cytokines were added as indicated by the "+" symbol. *A*, Cells were restimulated with PMA + ionomycin, and IFN-γ and IL-4 expression were measured by intracellular staining. Data are gated on live events. *B*, Cells were restimulated with PMA + ionomycin for 24 h, and IL-4 and IL-5 were quantified from the culture supernatants by cytometric bead array.

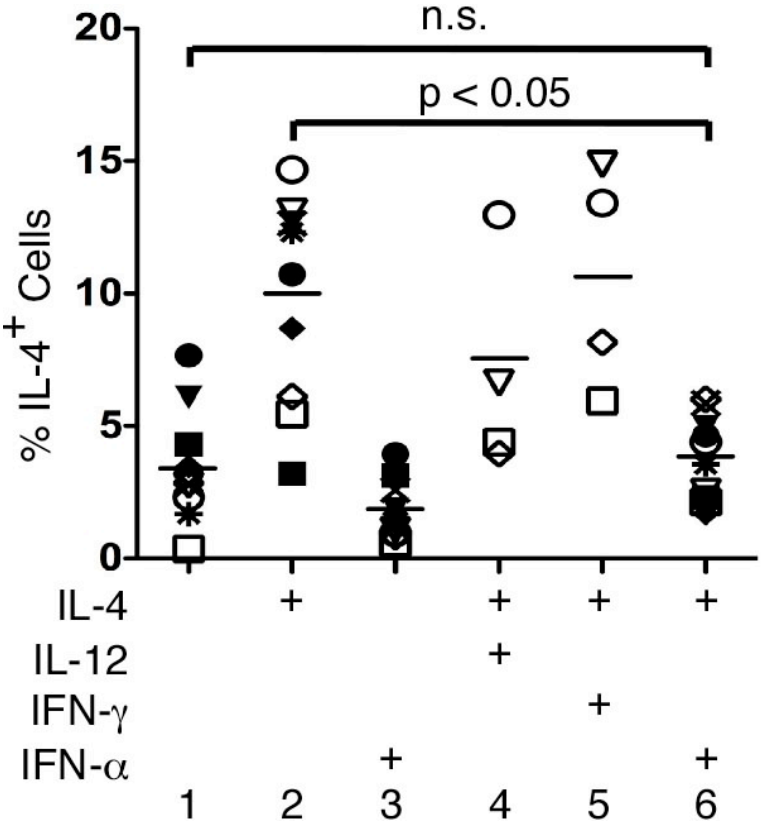

Figure 3.3. IFN-α inhibits Th2 cytokine production from multiple donors. Purified human $CD4^+/CD45RA^+$ cells were activated for 7 days with plate-bound anti-CD3/anti-CD28 under defined cytokine conditions. Cytokines were either neutralized with anti-cytokine antibodies (no symbol), or cytokines were added as indicated by the "+" symbol. IL-4 expression was measured by intracellular staining of samples from multiple donors. 10 donors were assessed with conditions 1-3 and 6, while 4 of the 10 donors were also assessed with conditions 4-5. Each symbol represents a separate donor.

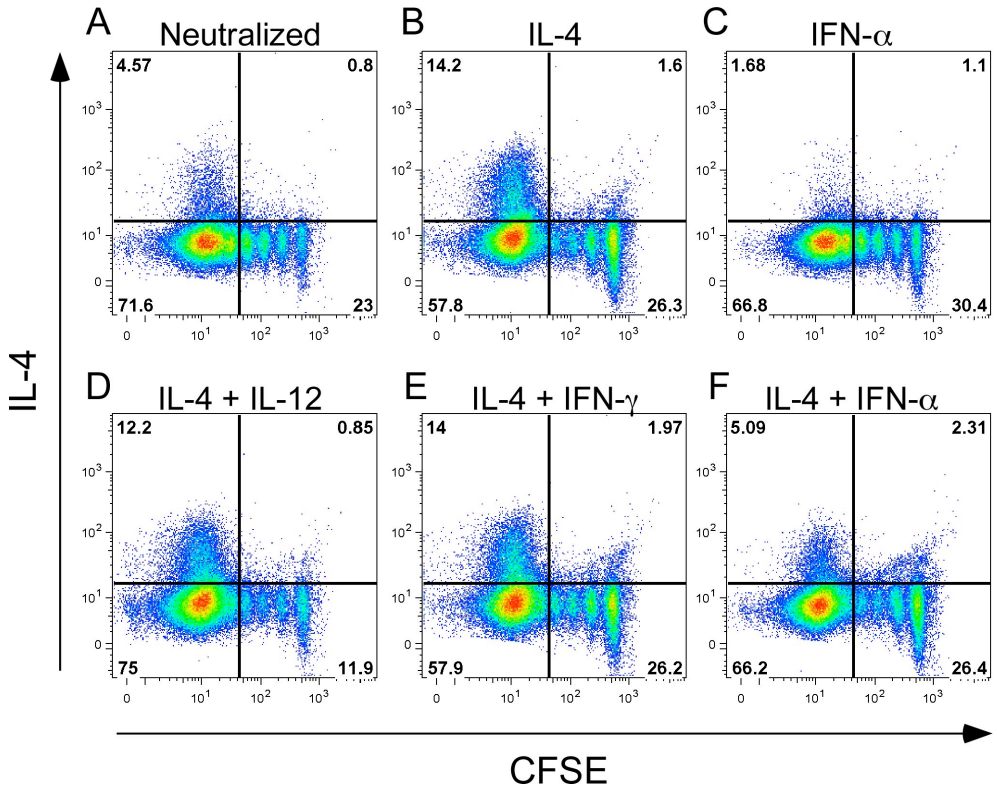

Figure 3.4. IFN-α does not block Th2 cell proliferation. Purified human $CD4^+/CD45RA^+$ cells were labeled with CFSE and activated with plate-bound anti-CD3/anti-CD28 for 5 days in the presence of the indicated cytokines. Cells were restimulated with PMA + ionomycin for 6 h, and IL-4 and IL-13 (not shown) were measured by intracellular staining.

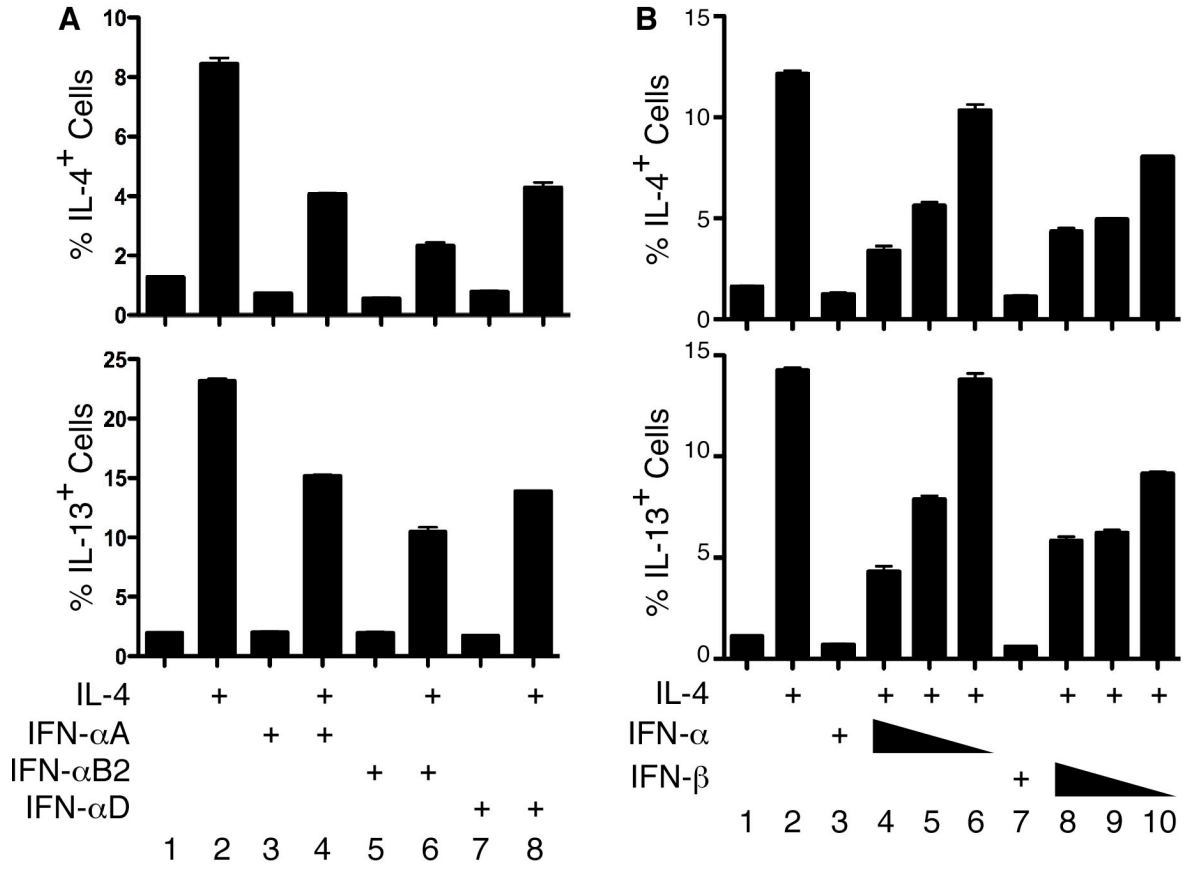

Figure 3.5. Human Th2 development is inhibited by multiple forms of type I interferon, and in a dose-dependent manner. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 for 7 days in the presence or absence of IL-4 (20ng/ml) and several subtypes of IFN-α (1000 U/ml) (*A*) or varying doses (1000, 100, and 10 U/ml) of IFN-αA or IFN-β1 (*B*). Cells were restimulated with PMA + ionomycin for 6 h, and IL-4 and IL-13 were measured by intracellular staining.

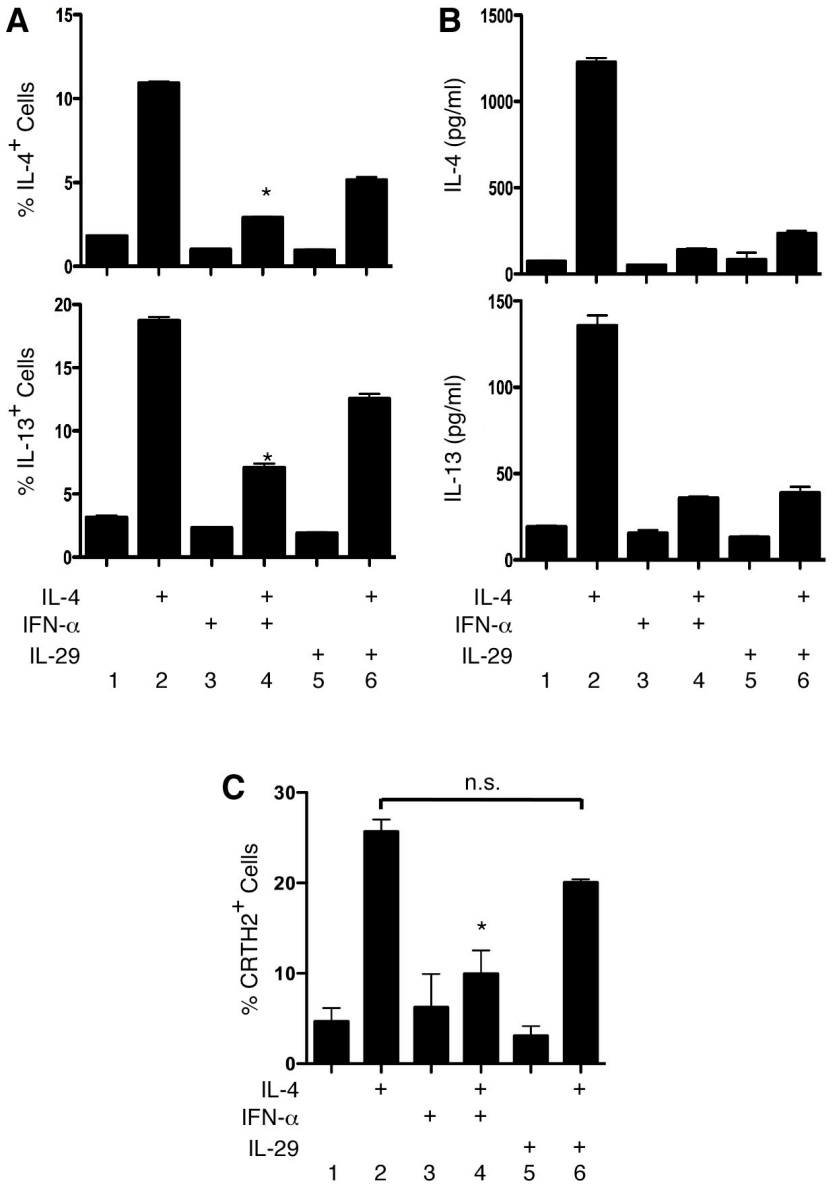

Figure 3.6 IFN-α and IL-29 (IFN-λ) regulate Th2 cytokine production, but only IFN-α blocks CRTh2. IFN-α/β inhibits human CD4+ Th2 development. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 under defined cytokine conditions. Cytokines were either neutralized with anti-cytokine antibodies (no symbol), or cytokines were added as indicated by the "+" symbol. *A*, Cells were restimulated with PMA + ionomycin, and IL-4 and IL-13 expression were measured by intracellular staining. Data are gated on live events. *B*, Cells were restimulated with anti-CD3 for 24 h, and IL-4 and IL-13 were quantified by ELISA from the culture supernatants. *C*, Induction of CRTH2 expression was assessed by flow cytometric analysis. *, $p < 0.05$ compared to IL-4 and to IL-4 + IL-29 (conditions 2 and 6).

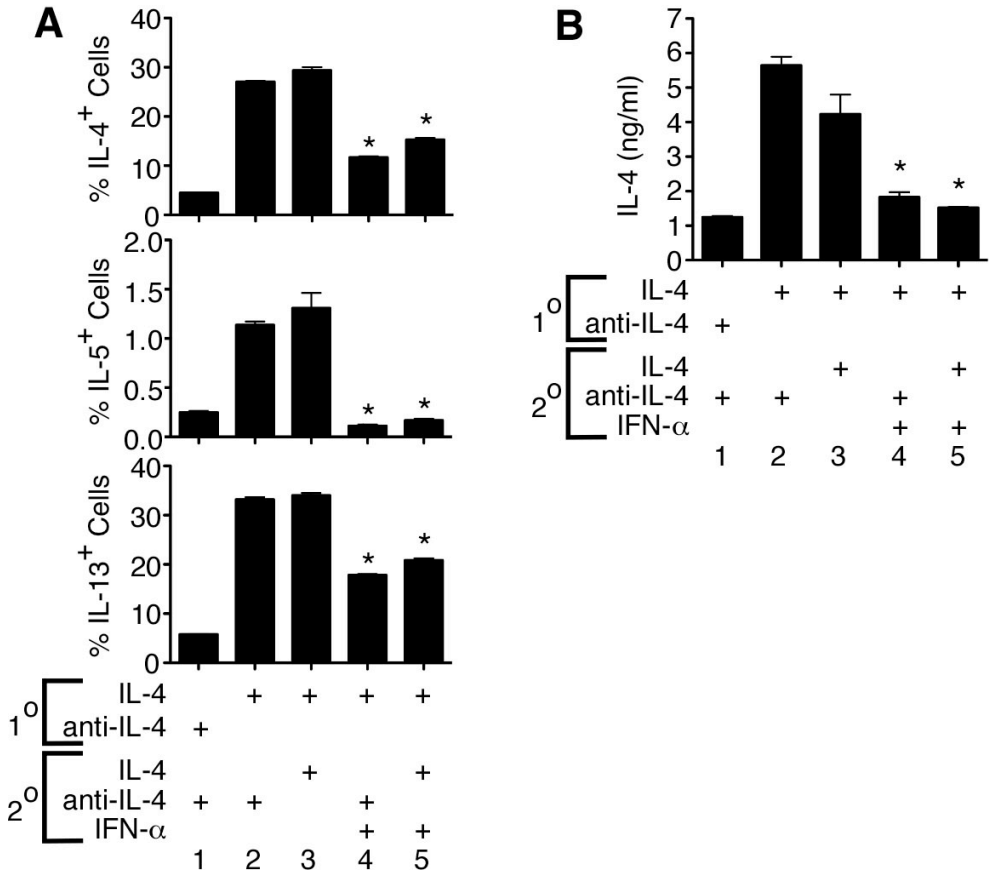

Figure 3.7. IFN-α/β suppresses Th2 cytokine expression in committed Th2 cells. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 for 7 days under neutralizing conditions or with IL-4 to promote Th2 development (1^o condition). Cells were then washed and restimulated for an additional 7 days with anti-IL-4, IL-4, IFN-α, or IL-4 + IFN-α (2^o conditions). *A*, Cells were stimulated with PMA + ionomycin for 6 h, and IL-4, IL-5, and IL-13 were measured by intracellular staining. *B*, Cells were stimulated with PMA + ionomycin for 48 h, and IL-4 was quantified by ELISA from the culture supernatants. *, $p < 0.05$ compared to IL-4 (*A* and *B*, condition 3).

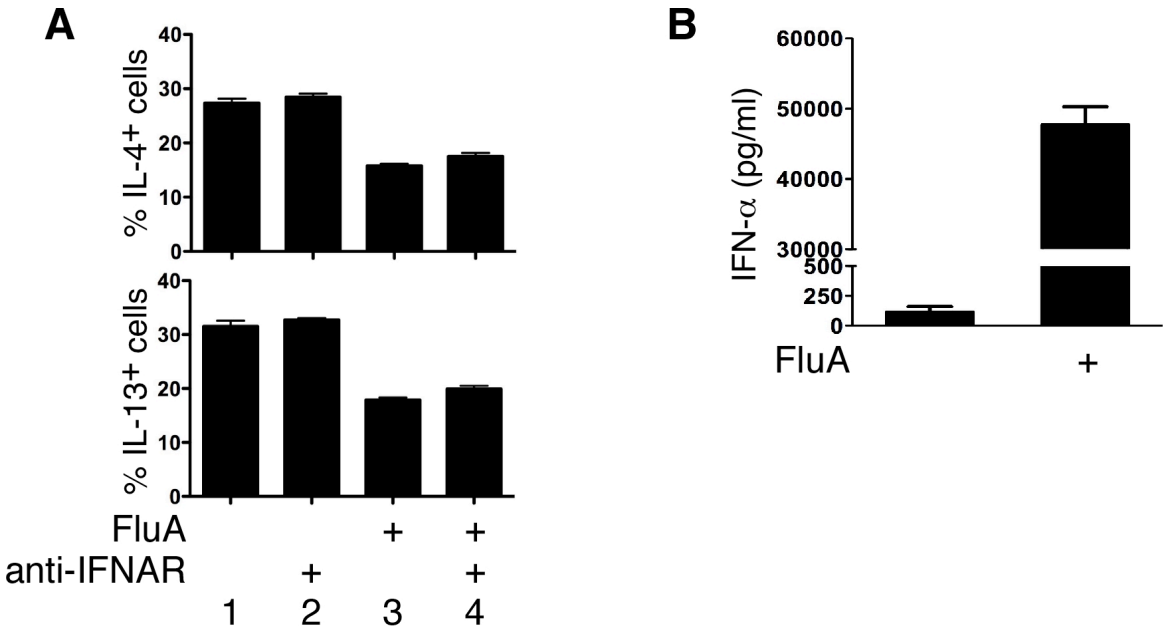

Figure 3.8. IFN-α produced by FluA-activated pDCs inhibits production of IL-4 and IL-13 by committed human Th2 cells. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 for 7 days under neutralizing conditions or with IL-4 to promote Th2 development (1^o conditions). Cells were then washed and restimulated in the presence or absence of anti-hIFNAR2 by co-culturing with pDCs activated 18 hours prior with FluA. *A*, Cells were stimulated at day 14 with PMA + ionomycin for 6 h, and IL-4 and IL-13 were measured by intracellular staining. Cells activated during the second week on coated plates showed similar results to Figure 3.7. Data shown is from cells reactivated during the second week by co-culture with pDCs. *B*, IFN-α secretion was quantified by ELISA from supernatants collected from the pDCs after 18 h of FluA treatment and prior to addition of T cells. (The pDC isolation, FluA treatment, and IFN-α ELISA were performed by Dr. Michelle Gill.)

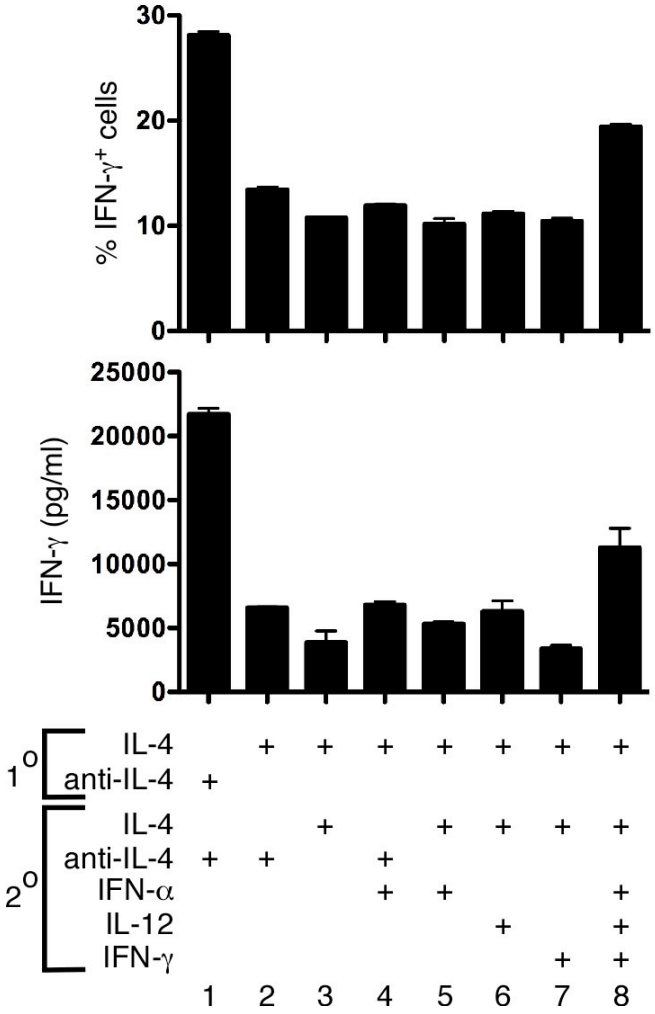

Figure 3.9. IFN-α/β does not enhance IFN-γ expression in committed Th2 cells. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 for 7 days under neutralizing conditions or with IL-4 to promote Th2 development (1^o condition). Cells were then washed and restimulated for an additional 7 days with anti-IL-4, IL-4, IFN-α, IL-12, and/or IFN-γ indicated (2^o conditions). Cells were stimulated with PMA + ionomycin for 6 h, and IFN-γ was measured by intracellular staining (upper panel). Alternatively, cells were stimulated with anti-CD3 for 48 h, and IFN-γ was quantified by ELISA from the culture supernatants (lower panel).

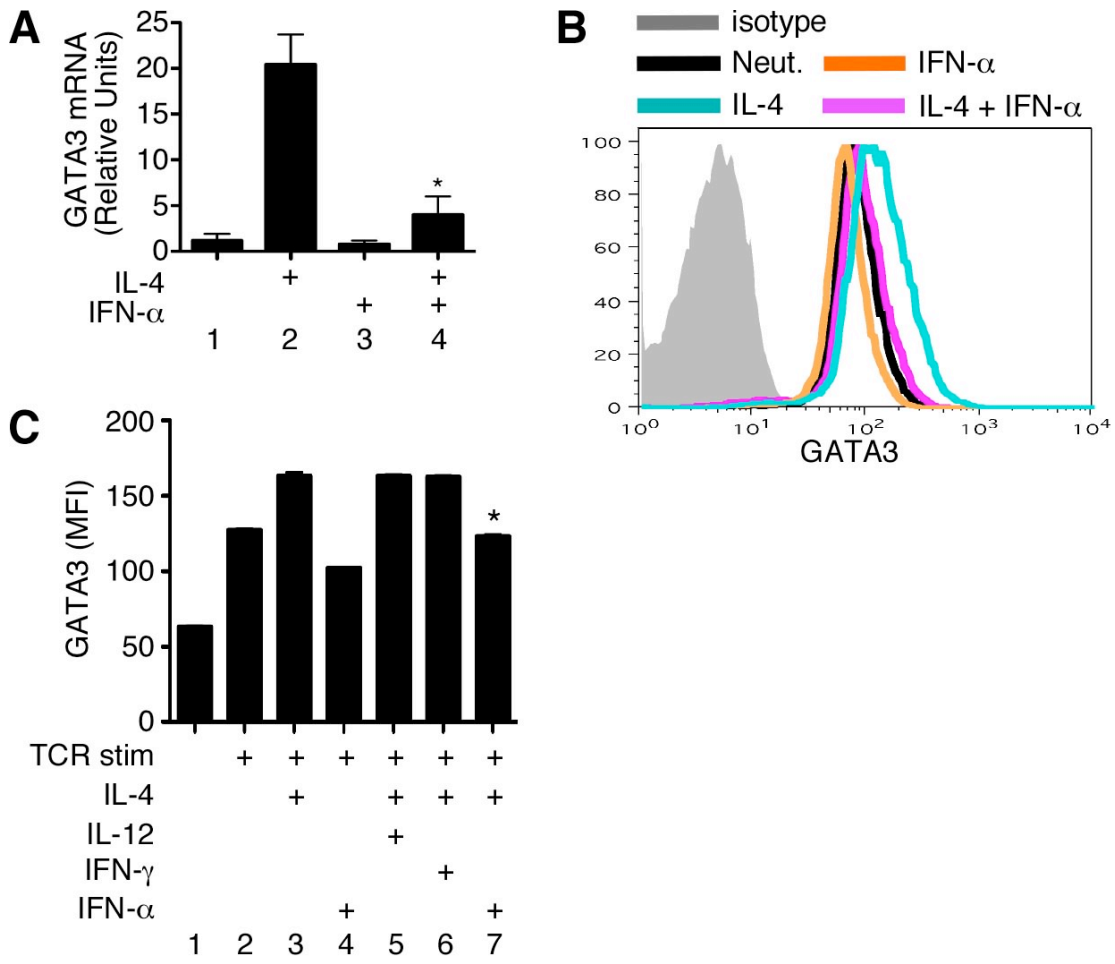

Figure 3.10. IFN-α/β specifically inhibits GATA3 expression. *A*, Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 for 72 h with IL-4, IFN-α, or IL-4 + IFN-α. mRNA was isolated from cells, and relative GATA3 mRNA was quantified by real-time PCR. Data were normalized to GAPDH expression and are shown relative to the neutralized condition. *, $p < 0.05$ compared to IL-4 (condition 2). *B*, Cells were activated as in A for 6 days, and GATA3 protein was assessed by intracellular staining and flow cytometric detection. *C*, Purified human $CD4^+/CD45RA^+$ cells were left unstimulated or were activated with plate-bound anti-CD3/anti-CD28 for 6 days in the presence of the indicated cytokines. GATA3 protein was assessed by intracellular staining and flow cytometric detection, and the data are gated on live cells and expressed as the mean fluorescence intensity of the population. *, $p < 0.05$ compared to IL-4 (condition 3).

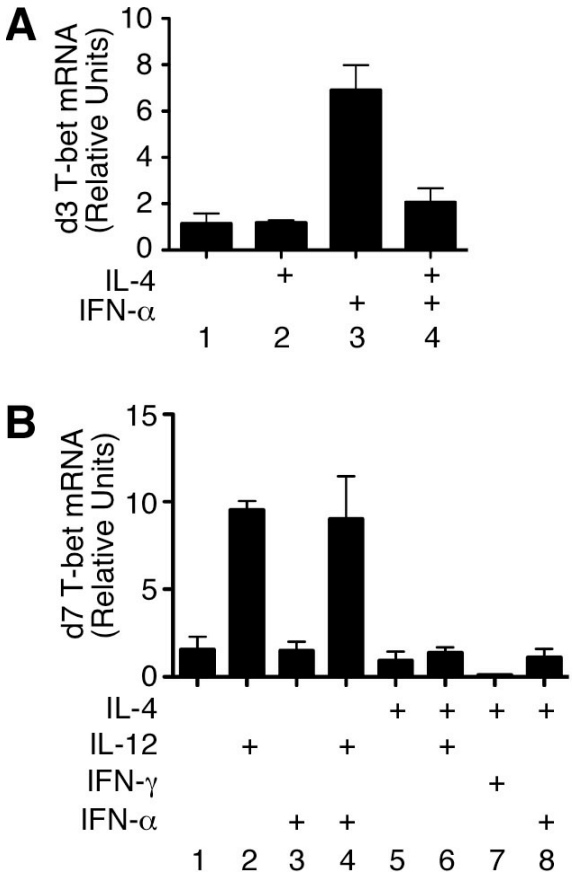

Figure 3.11. IFN-α does not upregulate T-bet in Th2 cells. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 for 3 days (*A*) or 7 days (*B*) in the presence of the indicated cytokines. mRNA was isolated from cells, and relative T-bet mRNA was quantified by real-time PCR. Data were normalized to GAPDH expression and are shown relative to the neutralized condition.

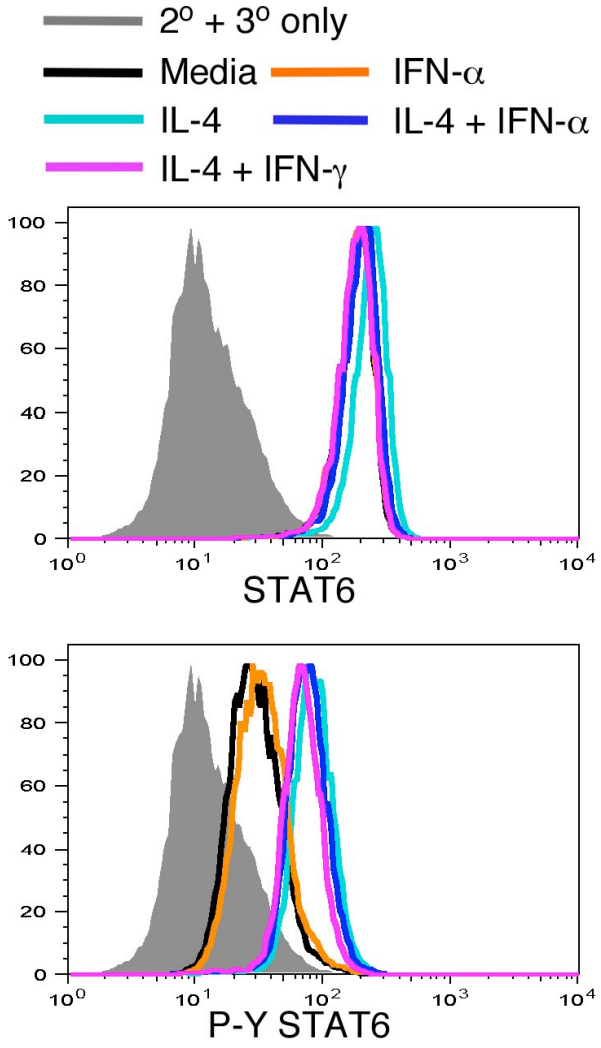

Figure 3.12. IFN-α does not affect acute STAT6 phosphorylation. Purified human $CD4^+/CD45RA^+$ cells were treated with the indicated cytokines for 10 min or 30 min (not shown) in the presence of sodium pervanadate. Total STAT6 and phosphorylated STAT6 protein was assessed by intracellular staining and flow cytometric detection. (This work was done by Dr. Hilario J. Ramos.)

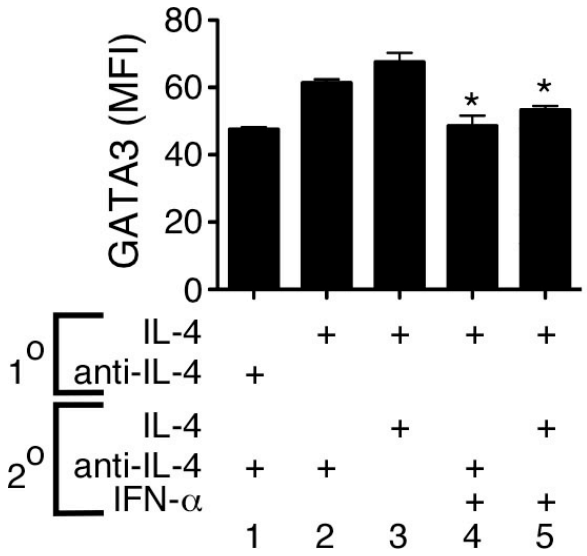

Figure 3.13. IFN-α suppresses GATA3 protein levels in committed Th2 cells. Purified human $CD4^+/CD45RA^+$ cells were activated for two consecutive weeks under 1^o and 2^o conditions as described in Fig. 2. GATA3 protein was measured on day 14 by intracellular staining. *, $p < 0.05$ compared to IL-4 (condition 3).

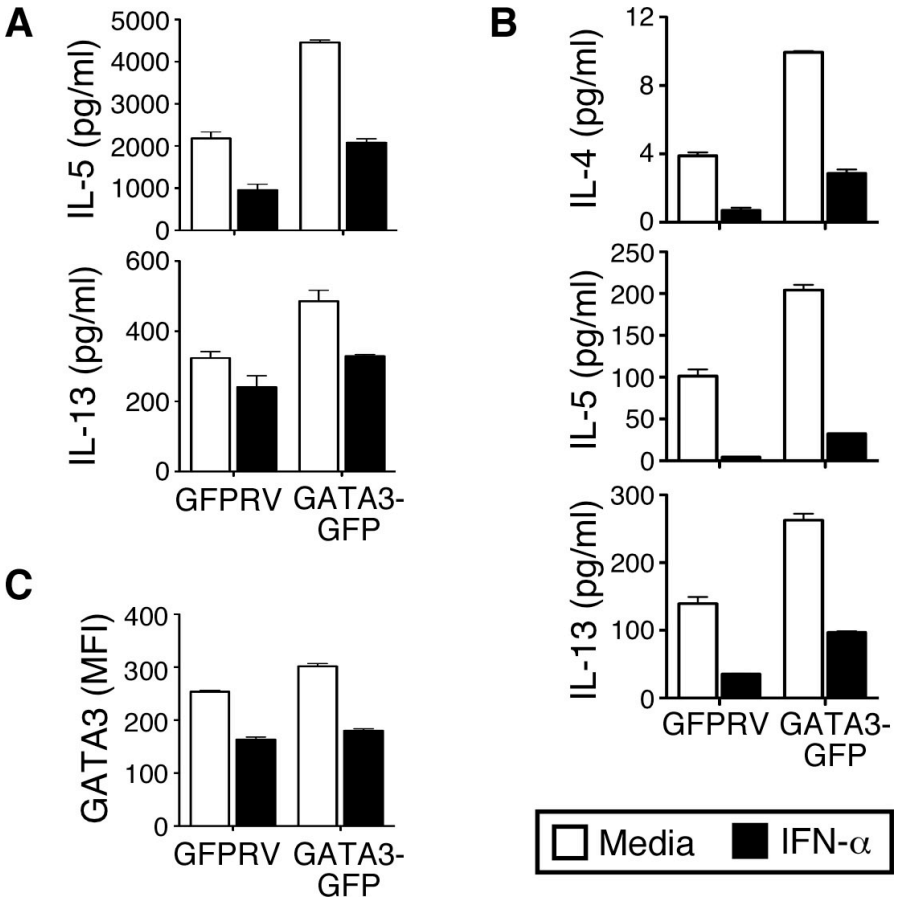

Figure 3.14. IFN-α inhibits Th2 cells that ectopically express GATA3. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 under Th2 conditions for 1 week prior to retroviral transduction with GFPRV or GATA3-GFP. GFP^+ cells were sorted on day 14 and reactivated with anti-CD3/anti-CD28 in the presence or absence with IFN-α. *A*, Cell culture supernatants were harvested on day 3 following the GFP sort and analyzed for IL-5 and IL-13 by ELISA. *B,* On day 7 following the GFP sort, cells were washed and restimulated for 48 h with anti-CD3/anti-CD28. IL-4, IL-5 and IL-13 were quantified by ELISA from the culture supernatants. *C*, Cells were harvested on day 5 following the GFP sort and GATA3 protein was measured by intracellular staining and flow cytometric detection. Data are gated on live cells and expressed as the mean fluorescence intensity of the population.

CHAPTER IV

TYPE I INTERFERON DESTABILIZES HUMAN CD4$^+$ TH2 CELLS BY SELECTIVELY INHIBITING THE GATA3 EXON 1A TRANSCRIPT

Introduction

Cellular differentiation depends on wide-ranging changes in gene expression, which includes both the expression of certain sets of genes as well as the silencing of others. These changes enable a CD4$^+$ T cell to respond in a particular manner when it receives a secondary stimulation, even though the stimulation reagent itself does not specify how the T cell should respond. For example, reactivation of Th1 and Th2 clones with anti-CD3 or ionomycin mobilizes NFAT to participate in the expression of cytokines by both subsets, namely IL-4 from Th2 cells and IFN-γ from Th1 cells (Rooney et al., 1994). However, NFAT formed a complex with AP-1 in Th1 cells but not in Th2 cells, indicating that acute transcription factor activity can be controlled by pre-programming of CD4$^+$ T cell subsets. An additional mechanism of controlling cytokine expression relies on epigenetic modifications to the chromatin itself. During differentiation, different patterns of cytokine signaling lead to regulation of the chromatin so that some genes are accessible to acutely activated transcription factors while other genes are not. This is reflected in the detection of DNase I hypersensitivity sites in the IL-4 locus in Th2 cells but not Th1, and vice versa for the IFN-γ locus (Agarwal and Rao, 1998). These mechanisms also regulate subset-specific transcription factors like GATA3 and T-bet (Wei et al., 2009). The roles of IL-4 and IFN-α/β in human Th2 cells have been discussed already in terms of functional

outcomes. This chapter will investigate in greater detail how these two cytokine signals affect GATA3 activity and the cellular machinery of epigenetics.

Epigenetics describes the multiple modifications of the chromatin that regulate its structure and function. Nucleosomes, the basic units of chromatin, consist of DNA wrapped around a protein octamer core. This protein core utilizes two copies each of four unique histone proteins: H2A, H2B, H3, and H4. When DNA is unwound from the histone proteins, it becomes sensitive to DNase treatment, a long-standing method for identifying regulatory sequences and genes capable of being transcribed. Modifications to the N-terminal tails of the histone proteins, such as methylation and acetylation, affect the way DNA wraps around the core and thus govern its accessibility. Permissive marks, such as acetylation of H4 (H4ac), trimethylation of H3 lysine 4 (H3K4me3), and acetylation of H3 lysine 9 (H3K9ac) are associated with open chromatin, the revealing of DNase hypersensitivity sites, and increased gene transcription. On the other hand, repressive marks like methylation of H3 lysine 27 (H3K27me3) or H3 lysine 9 (H3K9me2) are associated with closed chromatin and decreased transcription. Acetylation of lysines and arginines neutralizes the positive charge on these residues, freeing the negatively-charged DNA. Methylation of lysines leaves the charge intact, instead creating binding sites for chromatin-reading proteins, and is consequently associated with both open and closed chromatin states depending on the exact modification. The final state of the chromatin depends not on a single modification but on the aggregate effect of all modifications. Furthermore, daughter cells generated during mitosis inherit epigenetic patterns that stabilize the active or silent state of various genes appropriate to the cells' differentiation fate.

GATA3 function is critical for maintenance of open chromatin states at the GATA3 and Th2 cytokine loci. Part of the role that GATA3 plays in Th2 cells is to recruit proteins to modify the chromatin of target genes. One example protein, MLL, is a H3K4 methyltransferase that

maintains the accessibility of the GATA3 locus and Th2 cytokine locus in memory Th2 cells (Yamashita et al., 2006). MLL forms a complex with GATA3, c-myb, menin and increases H3K4 methylation and H3K9 acetylation of the IL-13 and GATA3 genes in human CD4+ T cells (Nakata et al., 2010; Kozuka et al., 2011). Since this activity completes the GATA3 autoregulatory loop and is crucial for stabilization of the Th2 phenotype, it is important to know how the activity of GATA3 is affected by type I interferon signals. The data presented in chapter 3 demonstrate that IFN-α/β reduces GATA3 and the Th2 cytokines to expression levels below baseline (Figure 3.2, 3.10), suggesting that IFN-α/β operates by an actively repressive mechanism rather than by simply blocking the induction normally mediated by IL-4 signaling. Therefore, it is possible that IFN-α/β signaling regulates the chromatin accessibility directly to antagonize the function of GATA3. On the other hand, IFN-α/β appears to significantly reduce GATA3 protein levels even in cells transduced with a GATA3-containing retrovirus that lacks the normal cis-regulatory DNA elements (Figure 3.14), suggesting that the protein may be targeted directly. The situation is further complicated by data indicating that the stability of the GATA3 mRNA, as well as the translation, stability, and cellular localization of the protein, are all carefully regulated (Yamashita et al., 2005; Hosokawa et al., 2006; Maneechotesuwan et al., 2007; Shinnakasu et al., 2008; Maneechotesuwan et al., 2009; Cook and Miller, 2010; Licata et al., 2010; Stellato et al., 2011). Interfering at any point in this loop would plausibly impair the rest of the cycle as well, so delineating the mechanism by which IFN-α/β interferes with GATA3 requires careful dissection of the pathway. A model of potential points of regulation is shown in Figure 4.1. The study presented in this chapter addresses these multiple points of regulation in order to uncover the mechanism(s) for the suppression. More specifically, these data indicate that normal Th2 development is accompanied by increased expression of the Th2-specific GATA3 exon 1A transcript. IFN-α/β inhibits expression of this transcript, potentially through

epigenetic silencing of an upstream cis-regulatory element in conserved non-coding sequence (CNS) I.

Results

IL-4 promotes human Th2 development during cellular activation and division

My previous results indicated that IFN-α/β suppressed Th2 cytokine by, at least in part, inhibiting the expression of GATA3. Since GATA3 expression is regulated in many different ways, there are many plausible ways that IFN-α/β could be inhibiting it. A model of these possibilities is shown in Figure 4.1 The first three steps relate to IL-4-mediated induction of GATA3: expression of the IL-4 signaling components, phosphorylation of STAT6, and induction of GATA3 by STAT6. I have attempted to examine the expression of the IL-4 receptor, but the available antibodies yielded poor detection by flow cytometry (data not shown). Acute STAT6 phosphorylation appears to be unaffected by IFN-α (Figure 3.12). I have not directly investigated DNA-binding by STAT6. However, committed Th2 cells are stabilized by GATA3 and no longer require further IL-4 signaling, yet these cells can still be inhibited by IFN-α (Figure 3.7, 3.13). Thus, it is unlikely that IFN-α operates by preventing the early steps of IL-4 signaling; IFN-α more plausibly interferes with the GATA3 auto-regulatory loop. The subsequent steps illustrated in Figure 4.1 are part of that loop: 4, GATA3 transcription; 5, transcript stability; 6, translation; 7, protein stability; 8, nuclear import; and 9, DNA-binding and gene transactivation. The data presented here examine these processes.

IL-4 signaling through GATA3 instructs Th2 differentiation (Farrar et al., 2001), and maximal expression of Th2 cytokines is achieved only when IL-4 signals are present during a primary activation (Figure 4.2). In this experiment, naïve $CD4^+$ T cells were activated for one week under neutralized, IL-4, IFN-α, or IL-4 + IFN-α conditions as described in the methods. At

day 7, cells were washed and reactivated in the presence of IL-4 for one week more. Intracellular cytokine expression was measured upon reactivation at day 14. As a control, additional cells were treated for two consecutive weeks under neutralized or IFN-α conditions (Figure 4.2, conditions 1-2), which confirmed previous results indicating that IFN-α suppresses Th2 cytokine levels below the baseline control (Figure 3.2, 3.10). As expected, cells treated for two weeks with IL-4 showed the greatest expression of both IL-4 (upper panel) and IL-13 (lower panel) (Figure 4.2, condition 4). Any condition lacking IL-4 during week 1 showed reduced cytokine expression even if treated with IL-4 during week 2 (conditions 3, 5-6). This demonstrates that maximal cytokine expression is achieved when IL-4 signals are present during the primary activation. Interestingly, cells that were treated with IFN-α prior to the IL-4 treatment showed a modest but statistically significant reduction in cytokine expression relative to cells cultured under neutralized conditions in week 1 (compare conditions 3 and 5). If this reflects the primary mechanism of suppression, it may suggest that IFN-α induces epigenetic modifications that render cells resistant to Th2 polarization. Alternatively, since the effect is modest, it could be explained by reduced signaling from IL-4. IL-4 signaling has been shown to promote IL-4Rα expression in T cells (Renz et al., 1991; Mozo et al., 1993), but this may be inhibited by IFN-α. This is unlikely to be a major mechanism for IFN-α-mediated suppression, but this experiment is not sufficient to distinguish between these two possibilities.

DNase hypersensitivity sites in the Th2 cytokine locus appear in Th2 cells but not Th1 cells, revealing the increased accessibility of the chromatin in Th2 cells (Takemoto et al., 1998; Agarwal et al., 2000; Santangelo et al., 2002). It has been suggested that the DNA synthesis (S) phase of the cell cycle may provide an opportunity for modifications to the chromatin that would alter its accessibility (Weintraub et al., 1978). Based on this hypothesis, cytokine expression in

differentiating T helper cells has been evaluated as a function of cellular division. In murine cultures, IL-2 is produced even in undivided cells, but effector cytokines like IFN-γ and IL-4 are both expressed primarily by cells that have passed through multiple rounds of proliferation (Bird et al., 1998; Ben-Sasson et al., 2001).

I have repeated this observation of cytokine production with human cells (Figure 3.4) but wished to determine whether GATA3 expression correlated directly with increased IL-4 expression at each cell division. To test this, I labeled naïve $CD4^+$ T cells with CFSE and activated them for 5 days. On day 5, cells were washed and reactivated for 6 hours with PMA + ionomycin followed by co-staining for IL-4 and GATA3 (Figure 4.3). The expression of GATA3 and IL-4 in the bulk populations followed the same patterns reported already, with lower expression values seen in the cells treated with IFN-α. As before, IL-4 production increased as a function of division. As might be expected, GATA3 also increased in dividing cells, regardless of cytokine treatment, and this trend was observed in both the restimulated cells and the resting counterparts (Figure 4.3, A and C). Interestingly, in resting cells, the induction of GATA3 by IL-4 was evident only in the most-divided population of cells, and this induction was suppressed by IFN-α (Figure 4.3, B). More surprisingly, in restimulated cells, IL-4 elevated GATA3 protein levels even in the undivided population, and this too was blocked by IFN-α. This correlates with previous studies that used cell cycle inhibitors to demonstrate that proliferation was associated with greater expression of effector cytokines but was not strictly required (Bird et al., 1998; Ben-Sasson et al., 2001).

GATA3 expression also correlates with IL-4 production, but not at the single-cell level (Figure 4.4). Since protein levels of GATA3 vary only modestly in response to varying cytokine conditions, simple detection of the protein is not sufficient to explain the larger variance in cytokine production. To determine whether Th2 commitment requires that GATA3 pass a

certain threshold level, GATA3 and IL-4 expression were compared within this same experiment. Reactivated cells from Figure 4.3 were gated on the fully divided CFSE-low populations (Figure 4.4). Interestingly, though IL-4-treated cells showed the greatest expression of GATA3 and the highest percentage of cytokine positive cells (Figure 4.4, A), the MFI of GATA3 in the IL-4-treated but cytokine-negative cells was greater than the MFI of GATA3 in the cytokine-positive cells that had been treated with IL-4 + IFN-α (Figure 4.4, B). Thus, increased GATA3 expression does not directly correlate to Th2 cytokine production. A more likely explanation is that the IL-4-treated cells, which have elevated GATA3 levels, are more likely to produce Th2 cytokines, but the cytokine expression itself is stochastic and not necessarily indicative of an individual cell's ability to make the cytokines. Thus, GATA3 and IL-4 production correlate at the population level but not at the single-cell level.

IFN-α selectively regulates the Th2-specific GATA3 exon 1A transcript

GATA3 transcription utilizes two distinct first exons (1A and 1B), each controlled by its own promoter but spliced to a common exon 2. Asnagli *et al.* showed that murine Th2 preferentially utilize the exon 1A transcript, while the exon 1B transcript is expressed only briefly (Asnagli et al., 2002). Subsequent studies demonstrated that while TCR stimulation and TCF-1 induce expression of the exon 1B transcript (Yu et al., 2009), induction of the exon 1A transcript is dependent on STAT6 (Scheinman and Avni, 2009). This two-exon system is also conserved in human cells. In fact, a DNA sequence alignment of the GATA3 locus generated by the UCSC Genome Browser shows that the original, downstream exon 1B and the more recently discovered exon 1A are both highly conserved in 17 vertebrate species (Figure 4.5). To determine whether IFN-α differentially regulates these two transcripts, I designed forward primers in either exon

1A or exon 1B that were paired with a reverse primer in exon 2. An additional primer pair spanning exons 5-6 were used to measure total GATA3 transcripts, and all three primer pairs were used to evaluate GATA3 transcripts in a single experiment (Figure 4.6). Treatment with IL-4 significantly elevated GATA3 mRNA levels, and this increase was entirely due to an increase in exon 1A. In contrast, exon 1B showed no statistically significant differences. This induction by IL-4 was completely inhibited by treatment with IFN-α. Thus, IFN-α suppresses a Th2-specific GATA3 transcript.

To investigate whether GATA3 transcript levels increased in dividing cells, naïve $CD4^+$ T cells were labeled with CFSE and activated for 5 days under different cytokine conditions as indicated (Figure 4.7). On day 5, cells were sorted based on division (panel A), and mRNA was isolated and analyzed by qPCR for GATA3 exon 1A and exon 1B transcripts (panel B). GATA3 exon 1A expression increased as a function of division, especially in response to IL-4, but this increase was markedly suppressed by IFN-α. Once again, exon 1B showed little change. Thus, GATA3 exon 1A correlated precisely with GATA3 protein levels. The exon 1A transcript is also preferentially maintained in committed Th2 cells, yet can be suppressed by reactivating these cells in the presence of IFN-α (Figure 4.8). This suggests that sustained expression of GATA3 in differentiated Th2 cells depends on maintenance of the GATA3 exon 1A transcript, and that inhibition of the transcript by IFN-α may be sufficient to reverse Th2 commitment.

The next possible point of regulation is mRNA stability (Figure 4.1, step 5). The suppression of the exon 1A transcript in IFN-α-treated cells could explained either by reduced transcription or by selective degradation of the exon 1B transcript. Studies of murine Th2 cells have shown that the two transcripts normally degrade at the same rate (Scheinman and Avni, 2009), but perhaps IFN-α could accelerate the normal turnover of the exon 1A transcript. A

number of mechanisms regulate mRNA stability, including deadenylation, endoribonucleolytic cleavage, and targeting by miRNAs, but these mechanisms act at the 3' end of the transcript (Wu and Brewer, 2012). GATA3 also appears to be regulated in this fashion, as GATA3 mRNA stability is enhanced by the activity of the protein HuR, which acts at the 3'-UTR of not only GATA3 but also IL-4, IL-13, and CRTh2 (Licata et al., 2010; Stellato et al., 2011). Since the exon 1A and exon 1B transcripts differ only at the 5' end, these mechanisms would not selectively degrade the exon 1B transcript. I have performed a single experiment looking at decay of the exon 1A and exon 1B transcripts during an 8-hour treatment with actinomycin D (data not shown). However, 8 hours proved to be too long and the cells treated with actinomycin D expressed little mRNA, so no conclusions can be made. Nevertheless, unless IFN-α initiates a novel 5'-acting mechanism, it is unlikely that the GATA3 exon 1A transcript is suppressed due to accelerated degradation.

IFN-α does not alter the stability or nuclear localization of GATA3 protein

Since GATA3 mRNA levels are regulated by IFN-α, a suppressive mechanism that worked by directly targeting the GATA3 protein would affect the mRNA mainly through loss of a feedback mechanism. This is a plausible explanation since GATA3 controls its own expression. In murine Th2 cultures, the exon 1A transcript is not highly expressed until after several days of differentiation under the control of IL-4. This expression may result from amplification of GATA3 through the autoregulatory loop. The effects of cellular division can be mimicked by treating the cells pharmacologically to increase histone acetylation and relieve the repression normally incurred by DNA methylation (Bird et al., 1998). These two modifications, when performed by the cell's own machinery, reflect the activity of transcription factors like GATA3

that makes the chromatin more accessible. Therefore, I investigated the GATA3 protein to determine whether IFN-α regulates its ability to function and promote its own expression.

The results of the retroviral transduction experiments (Figure 3.14) suggested that IFN-α may be able to GATA3 protein directly, perhaps by slowing translation (Cook and Miller, 2010) or inducing degradation of the protein (Yamashita et al., 2005) (Figure 4.1, steps 6-7). I tested protein degradation using MG132, a pharmacological inhibitor of the 26S proteasome (Figure 4.9). Naïve T cells cultured for 5 days under different cytokine conditions were treated for 2 hours with MG132 followed by flow cytometric analysis of GATA3 protein levels. Treatment with MG132 slightly increased GATA3 protein levels, but the effect was the same in both IL-4-containing cultures regardless of whether IFN-α was added to the culture, and the MG132 treatment did not restore GATA3 levels to the level of cell treated with IL-4 alone. The half-life of the GATA3 protein is approximately 1 hour (Yamashita et al., 2005), similar to GATA1 and GATA2 (Minegishi et al., 2005; Ferreira et al., 2007), so the 2-hour treatment with MG132 should have been sufficient to reveal a difference if IFN-α was affecting protein stability.

Another possible mechanism is regulation of cellular localization of GATA3 (Figure 4.1, step 8). Two reports have claimed that nuclear import of the GATA3 protein is regulated by a p38 MAPK-dependent serine phosphorylation event on an unknown amino acid of the GATA3 protein (Maneechotesuwan et al., 2007; Maneechotesuwan et al., 2009). In those reports, GATA3 could be detected in the cytoplasm of newly isolated human PBMCs, but it all localized to the nucleus within 30 minutes of stimulation with anti-CD3/anti-CD28. I examined nuclear localization of GATA3 via fluorescence microscopy (Figure 4.10). Naïve $CD4^+$ T cells were activated for 5 days under different cytokine conditions as indicated. On day 5, cells were reactivated for 2 hours with plate-bound anti-CD3/anti-CD28 in the presence or absence of IFN-α. Cells were then fixed, permeabilized, and stained for GATA3. As expected, cells cultured

with IL-4 showed the greatest detection of GATA3, while cells cultured with IFN-α showed a lower GATA3 signal than the neutralized population. GATA3 was found only in the nucleus, even in control populations that were left unstimulated on day 5 or were treated acutely with IFN-α (data not shown), which accords with other reports (Scheinman and Avni, 2009). Even Maneechotesuwan *et al.* note that cells maintained in culture for several days no longer show cytoplasmic GATA3 even when resting (Maneechotesuwan et al., 2007). Thus, while IFN-α clearly suppressed overall GATA3 protein levels, it does not appear to regulate localization of GATA3 or prevent it from entering the nucleus.

Suppression of GATA3 protein leads to reduced DNA-binding

The final potential mechanism for the suppression of GATA3 by IFN-α is regulation of the GATA3 gene itself (Figure 4.1, step 9). This could be accomplished in one of two ways. First, IFN-α might directly regulate the activity of the GATA3 protein, which feeds back on itself. Alternatively, IFN-α might affect the gene by directly altering another transcription factor or the chromatin itself. Several post-translational modifications of GATA3 have been reported, including ubiquitination (Yamashita et al., 2005), phosphorylation (Maneechotesuwan et al., 2007), and acetylation (Yamagata et al., 2000). I attempted to immunoprecipitate (IP) GATA3 and analyze it by mass spectrometry in order to determine whether IFN-α signaling induced a post-translational modification of GATA3. However, I was unable to IP sufficient quantities of the protein for mass spectrometry (data not shown) even though the antibodies used for the IP are known to bind GATA3 (see Figure 4.12). Consequently, the explanation for this poor IP may simply be the low expression of the protein. Although GATA3 can be clearly detected by

sensitive fluorescence-based assays like microscopy and flow cytometry, fluctuations in GATA3 in response to cytokines were unable to be detected by western blots (Dr. Hilario J. Ramos, unpublished observation), suggesting that biochemical analyses may not always be sensitive enough to detect protein level differences if the technical aspects of the assay do not sufficiently amplify the signal.

Despite the difficulties encountered in biochemical analyses of GATA3, it was important to know if its function was regulated by IFN-α, so I next determined whether DNA-binding by GATA3 was altered by IFN-α treatment. Although several binding sites for GATA3 have been confirmed in murine cells, few have been verified in human cells. One site confirmed in human cells lies in a conserved intronic regulatory element (CIRE) located between exon 1 and exon 2 of the IL-4 gene (Tykocinski et al., 2005), but I also wanted to know if GATA3 binding at the GATA3 locus was affected. In addition to conservation of the GATA3 exons, a number of highly conserved CNS regions are found upstream of GATA3 exon 1A (Figure 4.11). One of these, CNSI, contains many putative binding sites for GATA3 (Scheinman and Avni, 2009). Though none of them have been confirmed to be bound by GATA3, I examined the DNA sequences of other conserved regions around exon 1A and did not find any other region that shows this type of clustering. Thus, if DNA-binding by GATA3 at the GATA3 locus is important for its expression, CNSI may play a key role in the regulation of GATA3 exon 1A transcript expression. A non-canonical binding sequence for GATA3 has been identified in the proximal promoter of exon 1A (Asnagli et al., 2002). I also identified several additional sites of interest both upstream and downstream of exon 1A based on the presence of putative binding sites for the IFN-α-induced transcription factor complex ISGF3. These seven regions in the GATA3 locus (marked in Figure 4.11), along with the IL-4 CIRE, were used to interrogate DNA-binding by GATA3 using two

different assays: electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP).

EMSA was performed using nuclear extracts from naïve $CD4^+$ T cells activated for 5 days under the indicated cytokine conditions (Figure 4.12). Using a probe containing the GATA3 binding site from the IL-4 CIRE, increased DNA-binding by GATA3 was detected in cells cultured in IL-4 but suppressed in cells treated with IFN-α (panel A, conditions 2 and 4). The identity of GATA3 was confirmed by competition with an unlabeled competitor probe (condition 5), and by the super-shift seen upon addition of a GATA3-specific antibody (condition 6). A probe for EαY was used as a loading control, and the ratio of GATA3 binding to EαY is shown relative to the neutralized condition (panel B). IL-4 treatment resulted in a 3-fold increase over the baseline, and this increase was entirely ablated by IFN-α. This magnitude of increase and suppression appears to be greater than the magnitude of the changes in protein levels as detected by flow cytometry (Figure 3.10). However, it does agree visually with the differences in protein levels as detected by fluorescence microscopy. Consequently, while it is still possible that IFN-α regulates the intrinsic ability of GATA3 to bind DNA, these differences may also be accounted for simply by the differences in GATA3 protein levels.

The analysis of DNA-binding by an EMSA is limited in scope. The assay determines only whether binding to probe occurs, which may give some indication of whether a protein is *able* to bind DNA, but it cannot say whether the transcription factor actually occupies that binding site in an intact cell where binding may be affected by additional cis- and trans-regulatory elements. This question must be answered using ChIP. ChIP analysis is based on cross-linking DNA to the transcription factors bound to it and then quantifying that DNA by qPCR following immunoprecipitation of the protein. ChIP was performed using several different primer pairs: one for the IL-4 CIRE, and two for the GATA3 CNSI (primers 1 and 2 in Figure 4.11). As shown

in Figure 4.13, ChIP analysis of GATA3 showed an enrichment of the DNA from the IL-4 CIRE compared to the isotype pull-down, confirming that GATA3 binds to this site in human $CD4^+$ T cells (panel A). As expected, IL-4 treatment increased GATA3 binding relative to the neutralized condition, but this binding was inhibited by IFN-α treatment. The magnitude of increase by IL-4 and suppression by IFN-α was similar to that measured by EMSA (Figure 4.12), suggesting the changes seen by GATA3 ChIP also reflect the changes in GATA3 protein expression. Surprisingly, there were no significant changes in GATA3 binding to the putative binding sites in CNSI (Figure 4.13, B) or at the exon 1A proximal promoter (data not shown). Several other regions upstream and downstream of exon 1A are highly conserved (Figure 4.11) and may play a role in controlling GATA3 expression in Th2 cells, but other GATA3 binding sites have not been thoroughly examined. Nevertheless, the loss of DNA-binding by GATA3 at the IL-4 CIRE does reveal that IFN-α is not simply reducing the size of an excess pool of GATA3; the reduction of GATA3 protein levels by IFN-α has a functional effect on the cells. Thus, while the mechanisms for GATA3-dependent GATA3 expression in human Th2 cells remain incompletely understood, the reduction in GATA3 protein in IFN-α-treated cells may be sufficient to explain the concurrent reduction in Th2 cytokine expression.

Epigenetic regulation of GATA3

The ChIP analysis of GATA3 binding does not completely rule out the possibility that IFN-α is regulating the activity of GATA3 at the GATA3 locus. However, since I was unable to detect changes in DNA-binding by GATA3, I next considered whether IFN-α signaling was able to regulate the chromatin of the GATA3 locus in some other way. Chromatin consists of both a

protein component and a DNA component, and both can be epigenetically regulated. Regulation of the DNA occurs through methylation of CpG dinucleotides. Methylated cytosines can be deaminated to form uracil, leading to nucleotide substitutions and mutation of the DNA. Consequently, CpG DNA is largely absent from the mammalian genome, being preserved mainly in CpG islands that serve as important sites of regulation. The region between GATA3 exon 1A and 1B is a CpG island, as reported in the literature (Labastie et al., 1994) and identified by the UCSC Genome Browser. Methylation of the DNA by DNA-methyltranferases permits binding by other proteins that recruit histone methyltransferases, ultimately leading to epigenetic silencing of the chromatin. One relevant example is the recent report that the DNA methyltransferase Dnmt3a limits expression of IL-4 and IL-13 by murine Th2 cells, while deletion of Dnmt3a increases allergic inflammation in vivo (Yu et al., 2012). Therefore, these DNA methylation sites at the GATA3 locus may be significant for the control of GATA3 expression.

I investigated the role of DNA methylation in human cells using 5-azacytidine, a nucleoside analog that replaces cytosine during DNA replication but cannot be methylated (Figure 4.14). Naïve $CD4^+$ T cells were activated for 5 days in the presence or absence of 5-azacytidine added daily. At day 5, mRNA was analyzed by qPCR for GATA3 exon 1A and exon 1B transcripts. Blocking of global DNA methylation should result in greater chromatin accessibility, and transcript levels of exon 1A were correspondingly increased by 5-azacytidine regardless of which cytokines were added to the culture. In contrast, exon 1B was not sensitive to 5-azacytidine. Thus, DNA methylation plays a greater role in the accessibility of exon 1A than 1B. Nevertheless, 5-azacytidine did not restore exon 1A transcript levels in IFN-α-treated cells, indicating that IFN-α was not controlling exon 1A transcription through DNA-methylation.

I next considered whether IFN-α might regulate histone marks rather than the DNA. STAT2 plays a key role in IFN-α-induced gene expression through the formation of at least two

different complexes. Many interferon-stimulated genes (ISGs) are induced by IFN-α through the activity of a transcription factor complex ISGF3, comprised of STAT1, STAT2, and IRF-9. STAT2 heterodimers formed with STAT1 can also induce ISG expression by binding to GAS elements. In a human hepatocellular carcinoma cell line that is treated with IFN-α, STAT2 has been shown to facilitate recruitment of proteins that mediate loss of histone acetylation, increased H3K27 methylation, and reduced gene transcription (Testoni et al., 2011). Reasoning that STAT2 might perform a similar function in silencing GATA3 expression, I examined STAT2 binding at the GATA3 locus (Figure 4.15). ChIP was performed in total T cells activated for 2.5 hours in the presence or absence of IFN-α. The DNA immunoprecipitated with STAT2 was quantified by qPCR using primers specific for various points in the GATA3 locus (primers 1, 3-7 as shown in Figure 11) or at the IL-4 CIRE. These sites were chosen based on the presence of either putative GATA3 binding sites (primers 1, 5) or putative STAT2 binding sites (primers 3-4, 6-7). One additional primer pair flanking a confirmed STAT2 binding site in the ISG54 proximal promoter was used as a control. Binding of STAT2 to the ISG54 promoter showed approximately a 30-fold increase in cells treated with IFN-α compared to non-treated cells (Figure 4.15 *H*), but no binding of STAT2 was detected at the GATA3 locus or the IL-4 CIRE (Figure 4.15 *A-G*).

Finally, I investigated several specific histone modifications directly. ChIP was performed in naïve $CD4^+$ T cells activated for 5 days using antibodies against H4ac (Figure 4.16), H3K4me3 (Figure 4.17), H3K27me3 (Figure 4.18), and total H3 (Figure 4.19). H3K4me3 and H4ac are permissive marks, while H3K27me3 is a repressive mark. For example, the murine GATA3 locus shows increased H3K4me3 and decreased H3K27me3 in Th2 cells compared with other T helper subsets, while the gene encoding T-bet shows a similar pattern in Th1 cells (Wei et al., 2009). After performing ChIP with these four anti-histone antibodies, I analyzed the immunoprecipitated DNA using the same primers in the GATA3 locus and IL-4 CIRE as were

used for the STAT2 ChIP (primers 1, 3-7 as shown in Figure 4.11). The ChIP efficiency data from multiple individual human donors is expressed as a fold change relative to the IL-4-treated condition and then averaged together. The qPCR for primers #3-4 and 5-6 were performed later than the qPCR for the other three primer sets, so the total number of donors analyzed with each primer set varies, and this is noted in the figure legends for each of the histones (Figures 4.16-4.19).

Several interesting trends emerged, though unfortunately most of them are not statistically significant. IL-4 treatment enhanced H4ac at several sites regardless of whether or not IFN-α was present. H3K4me3 was enhanced at all selected sites in cells treated with IFN-α alone, which is curious since this modification is usually permissive; however, although it's not clear what this data means, it is unlikely to represent a mechanism that suppresses GATA3 expression since this methylation pattern does not occur in cells treated with both IL-4 and IFN-α. H3K27me3, a repressive mark, was slightly upregulated by IFN-α. However, since none of these trends were statistically significant, little inference can be made. Since the data shown is averaged from multiple donors, the trends may become significant if repeated with more donors. However, the ChIP results are not statistically significant within some of the individual experiments either. Furthermore, other ChIP analyses of the GATA3 locus, including data from both mice and humans, have presented representative experiments rather than data averaged from multiple experiments (Scheinman and Avni, 2009; Nakata et al., 2010; Onodera et al., 2010). This may reflect the difficulty in discerning significant trends when averaging multiple experiments, particularly when the relative differences between samples are less than 2-fold. Consequently, it is particularly interesting that ChIP analysis of total H3 showed a statistically significant effect of IFN-α (Figure 4.19). No significant changes were seen around exon 1A or the IL-4 CIRE, but IFN-α treatment increased the enrichment at CNSI regardless of whether the

cells were also treated with IL-4 (Figure 4.19 *A*). When the histone ChIP data is shown as a series of line graphs (Figure 4.20), the increased pull-down CNSI by H3 in response to IFN-α becomes even more noticeable. The data for this particular analysis is averaged from 5 separate donors, indicating a significant change in nucleosome density at this location, and may reflect the mechanism by which IFN-α inhibits GATA3 expression.

Discussion

The ability of GATA3 to promote its own expression is important for stabilization of the Th2 phenotype. The density of canonical GATA3 binding sites in CNSI suggested that this region may play an important role in the GATA3 feedback loop. Therefore, it was somewhat surprising that there were no changes in DNA-binding by GATA3 in this region. There are several possible reasons for this. One possibility is that DNA-binding by GATA3 in this region does not accurately mirror its activity. During thymocyte development, occupancy of GATA3-binding sites is quite dynamic and can change significantly between different developmental stages despite little change in GATA3 expression levels (Zhang et al., 2012). Interestingly, GATA3 also bound to sites in genes that were silenced by H3K27me3, suggesting that the presence of GATA3 does not necessarily correspond to permissive epigenetic modifications. Therefore, it's possible that the lack of change in GATA3 ChIP at CNSI reflects a more subtle regulation of activity rather than lack of binding. However, since ChIP efficiency at CNSI with an anti-GATA3 antibody was comparable to that of the isotype control, it seems likely that there is little binding by GATA3 at these sites at the time point chosen for the analysis. It may be that GATA3 activity at this region is more critical during the first few days while cells are being activated, rather than at later time points when the cells are returning to a resting state. Alternatively, feedback from GATA3 may depend on binding at other sites in the GATA3 locus but not at this particular sequence in CNSI.

Despite the fact that cytokine signals did not affect binding by GATA3 to CNSI, they did alter the nucleosome density at CNSI. H3 is sometimes assumed to remain uniformly associated with the DNA and has been used as a type of loading control for histone ChIP experiments. For example, Onodera *et al.* measured total H3 by ChIP in murine $CD4^+$ T cells and found the ChIP

efficiency to remain unchanged at multiple sites throughout the GATA3 locus as cells differentiated from naïve precursors to Th2 (Onodera et al., 2010). However, nucleosome density changes as chromatin is unwound to enable gene transcription and histone proteins are displaced by transcription factors (Segal and Widom, 2009). Furthermore, changes in nucleosome density have also 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 targeting of the histones. Thus, it is plausible that IFN-α treatment could increase nucleosome density at GATA3 CNSI. Although the histone modifications I analyzed did not show significant differences between cytokine treatments, the increased binding of DNA to H3 may reflect other modifications that have not yet been examined, such as H3K9ac or the strongly repressive mark, H3K9me2, and future experiments will investigate that possibility. Finally, it is interesting that the increased binding of GATA3 CNSI to H3 occurs only when cells are treated with IFN-α (Figure 4.19). Binding to H3 is not decreased in Th2 cells compared to the neutralized condition, suggesting that even if this particular region is important for GATA3 exon 1A transcript expression, it may not be substantially affected by IL-4 signaling. This would imply that IFN-α does not regulate Th2 cells simply by reversing IL-4-induced modifications to the GATA3 locus. Instead IFN-α may operate through distinct regions that are normally accessible even in neutralized cells.

A major limitation in the ChIP analysis of the GATA3 locus as presented here is the interrogation of a few select regions. Although the sites were carefully chosen based on potential STAT2 binding, these sites to not appear to actually bind STAT2 (Figure 4.15). Furthermore, it is impossible to say whether the activity of GATA3, STAT2, or the histone proteins at these sites adequately represents the activity across the entirety of the GATA3 locus. A number of sequences both upstream and downstream of exon 1A are highly conserved and possibly critical

both for GATA3 expression and for suppression by IFN-α. The best way to thoroughly investigate the role of IFN-α in regulating transcription factor binding throughout the locus would be to perform ChIP-seq for GATA3, STAT2, and various histone modifications. Scanning through the entire locus could determine whether the increased binding by H3 at CNSI is accompanied by increased binding elsewhere in the locus. ChIP-seq may also identify binding sites for STAT2 or GATA3 whose occupancy changes when cells are treated with IFN-α. GATA3 expression depends on the activity of several tissue-specific enhancers, including one important in T cells but located 280kb downstream of the GATA3 gene (Lakshmanan et al., 1999; Hosoya-Ohmura et al., 2011). Accordingly, IFN-α may influence GATA3 expression through regulation of a site quite distant from exon 1A itself.

My study of the GATA3 transcripts demonstrates a preferential induction of the exon 1A transcript in Th2 cells (Figures 4.6-4.8). This correlates with previous publications reporting that IL-4 signaling through STAT6 induces the exon 1A transcript in murine Th2 cells (Asnagli et al., 2002; Scheinman and Avni, 2009; Onodera et al., 2010). Interestingly, multiple reports suggest that the exon 1B transcript is still more highly expressed than the exon 1A transcript (Scheinman and Avni, 2009; Yu et al., 2009; Onodera et al., 2010). All of these studies, in addition to mine, have quantified the transcripts by PCR and show relative expression. It would be interesting to see how the absolute transcripts levels compare to each other in a northern blot or RNase protection assay. Scheinman and Avni report that the amplification of the exon 1B transcript crosses the threshold earlier in the qPCR than exon 1A, which I also have seen (Scheinman and Avni, 2009). This may reflect differences in PCR efficiency, rather than true baseline transcript levels, though the slopes of the amplification curves for the two transcripts were similar. Alternatively, it may indicate that exon 1A is responsible for only the cytokine-mediated differences. My data represents the induction of GATA3 exon 1A and 1B as a fold

change relative to a population of cells that were neutralized and activated. Comparing activated cells to non-activated cells might show a different trend, particularly for exon 1B. GATA3 expression is carefully maintained in thymocytes (Ho et al., 2009; Zhang et al., 2012), which preferentially express the exon 1B transcript (Asnagli et al., 2002). TCR signaling through TCF-1 has also been shown to increase expression of the exon 1B transcript, and this induction is independent of IL-4 and STAT6 (Yu et al., 2009). Thus, thymocytes and naïve $CD4^+$ T cells express the exon 1B transcript, while IL-4 signaling enhances GATA3 expression in differentiating Th2 cells by inducing expression of the exon 1A transcript. This model would support the GATA3 protein expression pattern (Figure 3.10 *C*). However, control of GATA3 exon 1A and exon 1B transcripts may not be completely segregated to IL-4 and the TCR. STAT6 and NFAT each bind the promoters of both exon 1A and exon 1B in mice, suggesting a bit of overlap (Scheinman and Avni, 2009). I also see that IL-4 treatment sometimes induces the exon 1B transcript slightly (Figure 4.8). Other signals, like Notch, can promote exon 1A transcript expression (Amsen et al., 2007; Fang et al., 2007), but this Notch activity works in concert with the GATA3 protein to induce the exon 1A transcript. Furthermore, I have shown that the exon 1A transcript, but not the exon 1B transcript, remains elevated in Th2 cells in the absence of further IL-4 signaling (Figure 4.8), suggesting that GATA3 feedback in human Th2 cells preferentially maintains expression of the exon 1A transcript.

The discussion of the relative importance of the GATA3 exon 1A and exon 1B transcripts has assumed that GATA3 protein is translated off of each transcript. However, one recent report claims that the exon 1A transcript is a non-coding transcript that instead plays a role in displacing epigenetic silencing complexes from the downstream exon 1b promoter in order to enhance exon 1B transcript levels (Onodera et al., 2010). Although such a system has been described for other genes (Hekimoglu and Ringrose, 2009), no evidence was given to support a

similar function of the GATA3 exon 1A transcript. This is a surprising conclusion since neither of the first exons contains protein-coding sequence. If exon 1A only regulates the epigenetics of exon 1B, then Th2 commitment should show a greater induction of exon 1B as well, particularly as exon 1A increases. This is not what I see (Figure 4.6-4.7) or what has previously been reported (Asnagli et al., 2002). It may be useful to directly test whether GATA3 can be translated from both transcripts. This could be done in murine GATA3-deficient cells using retroviral constructs to express the fully spliced exon 1A or exon 1B transcript followed by analysis of protein expression. It would also be interesting to see if the translation rates are the same, since the relative contribution of each transcript to the GATA3 protein pool may not be the same as the actual expression levels of these transcripts.

One remaining question is the relationship between Th2 commitment and cellular proliferation. One function of STAT6 in differentiating Th2 cells is the regulation of two protein family complexes at the GATA3 gene. One protein complex, the polycomb group, maintains repressive epigenetic marks like H3K27me3 on the GATA3 gene in undifferentiated cells (Onodera et al., 2010). STAT6 binds to multiple places in the GATA3 locus and mediates displacement of the polycomb proteins by the trithorax group of proteins. The activity of this complex leads to permissive histone modifications like H3K4me3 and H3K9ac that promote GATA3 transcription. After GATA3 is expressed, it interacts with trithorax proteins like menin to maintain an open chromatin state at the GATA3 locus even in the absence of further IL-4 signaling (Nakata et al., 2010; Onodera et al., 2010). IFN-α signaling through STAT2 can counter this type of regulation by recruiting EZH2, one of the polycomb proteins, leading to increased H3K27me3 and reduced transcription (Testoni et al., 2011). If IFN-α is regulating GATA3 through the activity of STAT2, a similar mechanism may be at work.

The ability of IL-4 signaling to promote permissive epigenetic modifications in Th2 cells can be simulated by treating cells with inhibitors of DNA methylation and histone deacetylation. This combination synergizes to enable IL-4 production in STAT6-deficient T cells (Bird 1998 Immunity), and treating cells with these inhibitors also enhances the increased effector cytokine production that accompanies proliferation (Bird et al., 1998). Although cell division is not a "clock" that strictly controls cytokine production (Ben-Sasson et al., 2001), it may be that S phase offers the best opportunity to modify the DNA and alter chromatin accessibility. Cells that are stimulated through the TCR and with IL-4 but treated with a cell cycle inhibitor are unable to produce IL-4 upon a recall stimulation (Richter et al., 1999). However, even if the IL-4 is washed out after 24 hours, removal of the cell cycle inhibitor for the subsequent 2 days is sufficient to permit IL-4 production in a recall response. Thus, TCR and IL-4 signals are able to program undivided cells for later action, which correlates with my data showing that GATA3 expression is increased by a secondary activation of Th2 cells even if they have not divided (Figure 4.3). Cell division provides an opportunity to enhance the epigenetic modifications initiated by IL-4, and recall stimulation provides opportunity for cells to evidence these modifications. My data suggests that IFN-α inhibits these early epigenetic modifications, leading to suppressed expression of the GATA3 exon 1A transcript and the GATA3 protein, perhaps by blocking enhancer activity at GATA3 CNSI. Whether the changes at CNSI are truly the cause or the effect of decreased GATA3 remains to be determined.

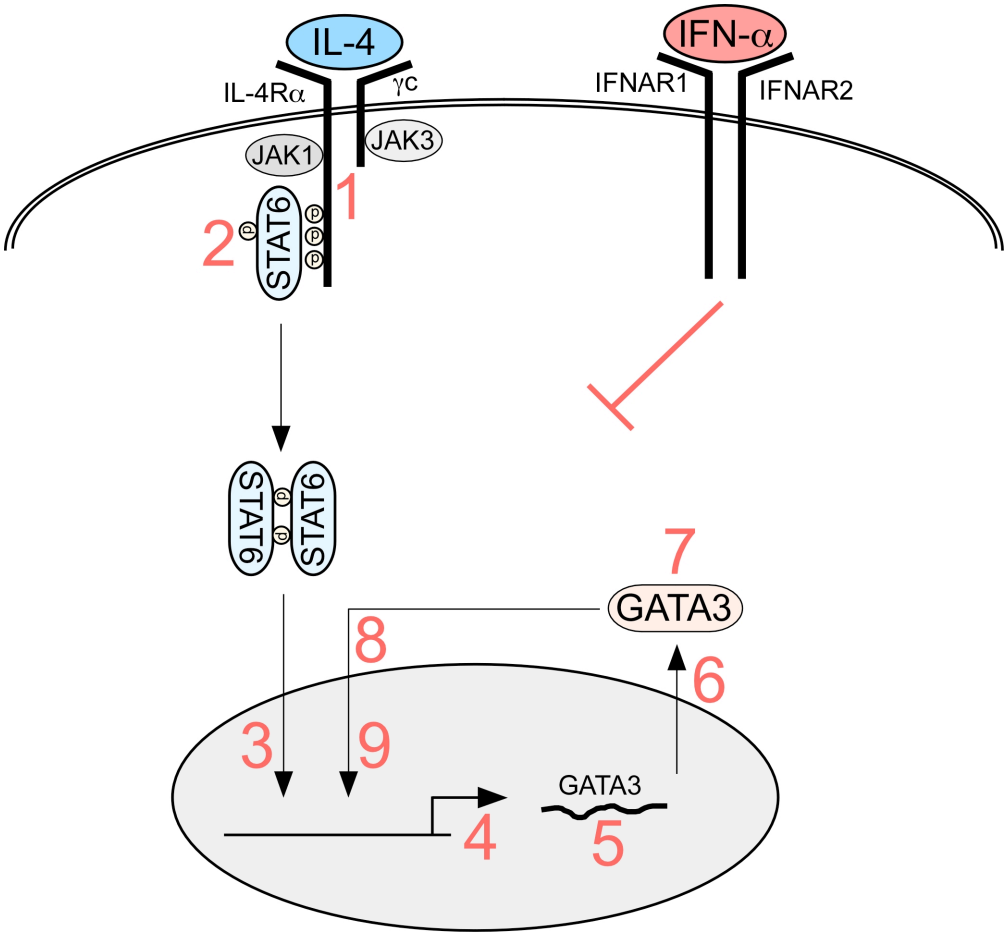

Figure 4.1. A model of possible mechanisms by which IFN-α inhibits human Th2 development. Shown here is the IL-4 signaling pathway and the GATA3 auto-regulatory loop, along with the main components of IFN-α/β signaling. The possible points of IFN-α-mediated inhibition are numbered. 1 - IL-4 receptor expression; 2 – STAT6 phosphorylation; 3 – DNA-binding by STAT6; 4 – GATA3 transcription; 5 – GATA3 transcript stability; 6 – GATA3 translation; 7 – GATA3 protein stability; 8 – nuclear import of GATA3; 9 – DNA-binding by GATA3.

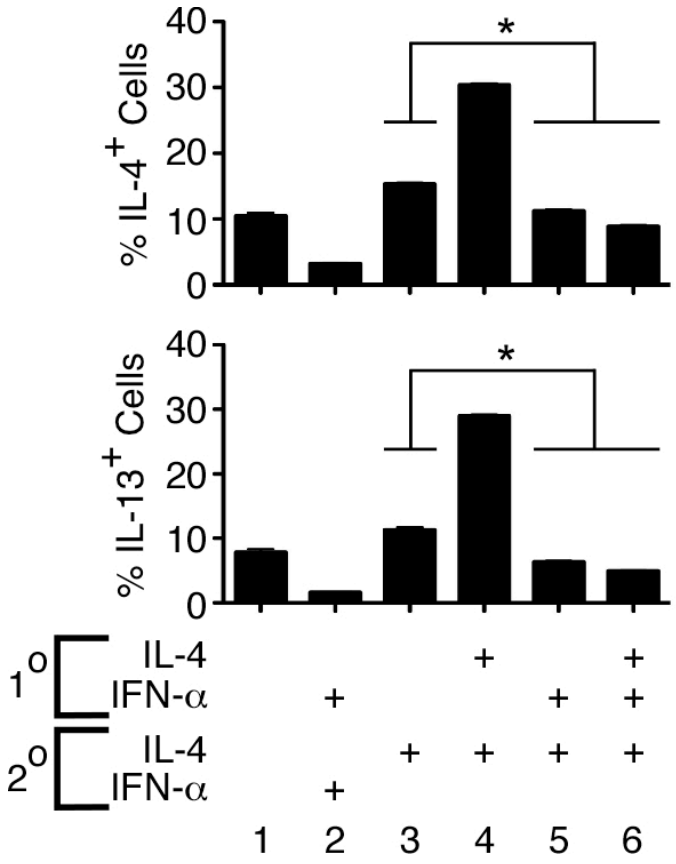

Figure 4.2. Optimal production of Th2 cytokines requires IL-4 signaling during a primary activation. Purified human $CD4^+/CD45RA^+$ T cells were activated with plate-bound anti-hCD3/anti-hCD28 for 7 days with anti-IL-4, IL-4, IFN-α, or IL-4 + IFN-α (1^o conditions). Cells were then washed and restimulated for an additional 7 days with anti-IL-4, IFN-α, or IL-4 (2^o conditions). Cells were stimulated with PMA + ionomycin for 6 h, and IL-4 and IL-13 were measured by intracellular staining. *, $p < 0.05$.

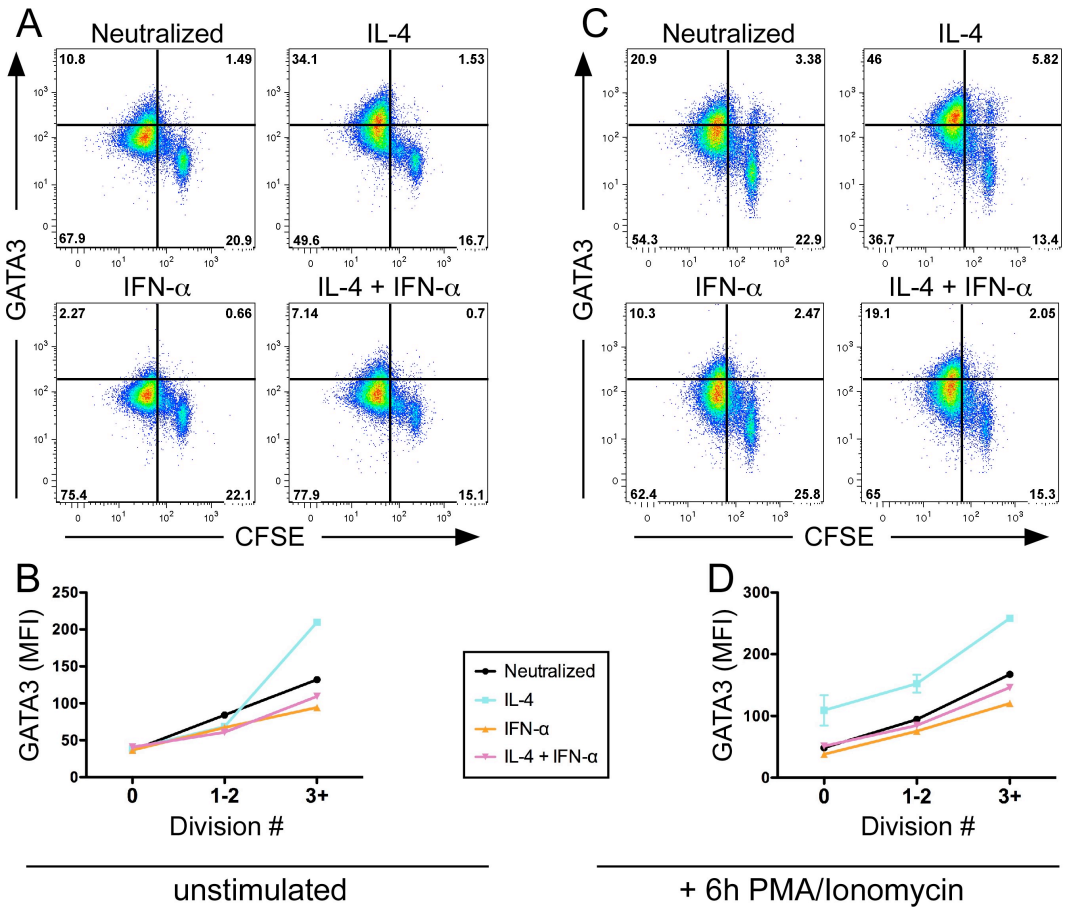

Figure 4.3. GATA3 protein expression increases as a function of cellular proliferation but is inhibited by IFN-α. Purified human $CD4^+/CD45RA^+$ cells were labeled with CFSE and activated with plate-bound anti-CD3/anti-CD28 for 5 days under the indicated cytokine conditions. Cells were left unstimulated (*A, B*) or were restimulated with PMA + ionomycin for 6 h (*C, D*), and GATA3 and IL-4 (see Figure 4.4) were measured by intracellular staining.

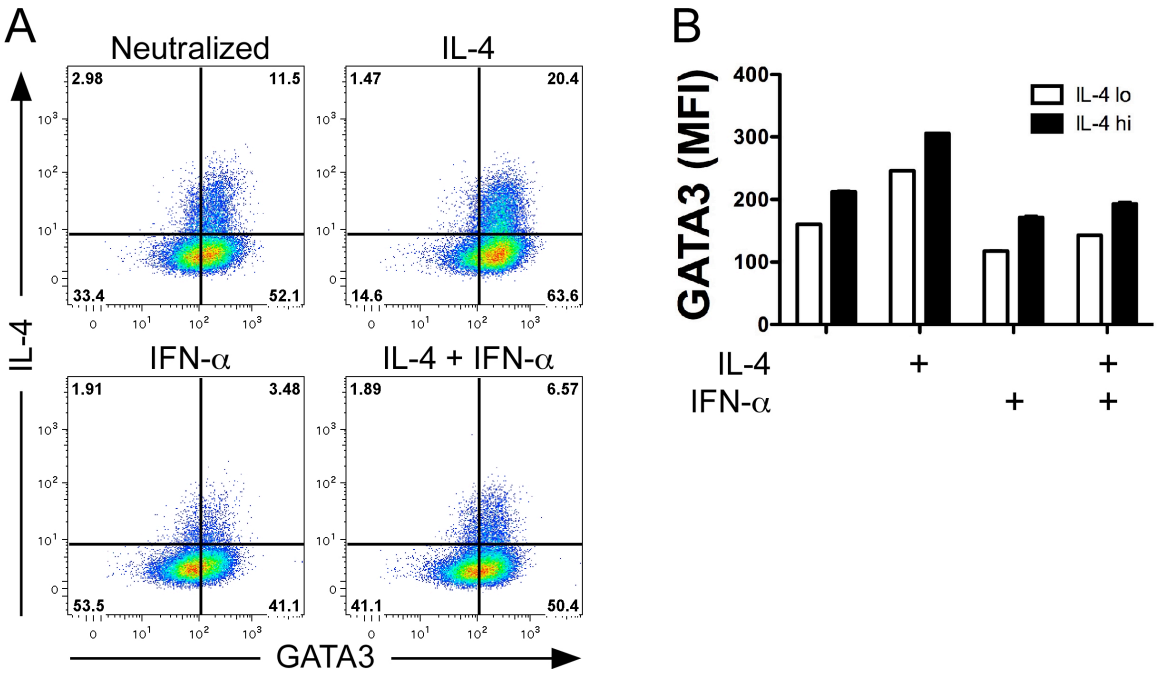

Figure 4.4. Expression of GATA3 and IL-4 correlate at the population level but not the single-cell level. *A*, Data from the reactivated cells in Figure 4.3, panel *C*, are gated on the most-divided population (CFSE low). *B*, Data from *A* are gated on IL-4-low and IL-4-high and expressed as the mean fluorescence intensity of GATA3.

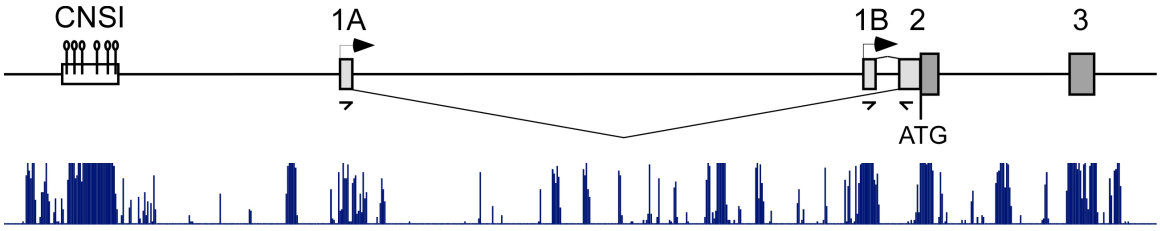

Figure 4.5. A model of the GATA3 locus. The upper panel shows coding and regulatory regions of the GATA3 locus. GATA3 transcription utilizes two distinct first exons, exon 1A and exon 1B, each controlled by its own promoter and separated by ~10kb. Each of these exons can be spliced to a common exon2, which contains the beginning of the protein coding sequence. The complete GATA3 transcript includes three more exons not shown here. Upstream of exon 1A are several large conserved non-coding sequences (CNS), including CNSI shown here. The exons and CNS regions, in addition to numerous shorter intervening sequences, are highly conserved, as shown by the visualization tool for alignment (VISTA) plot of 17 different vertebrate species (lower panel). This graph was generated by the UCSC genome browser (http://genome.ucsc.edu). GATA3 exon 1A and exon 1B utilization is analyzed by qPCR using forward primers located either in exon 1A or exon 1B and a reverse primer in exon 2.

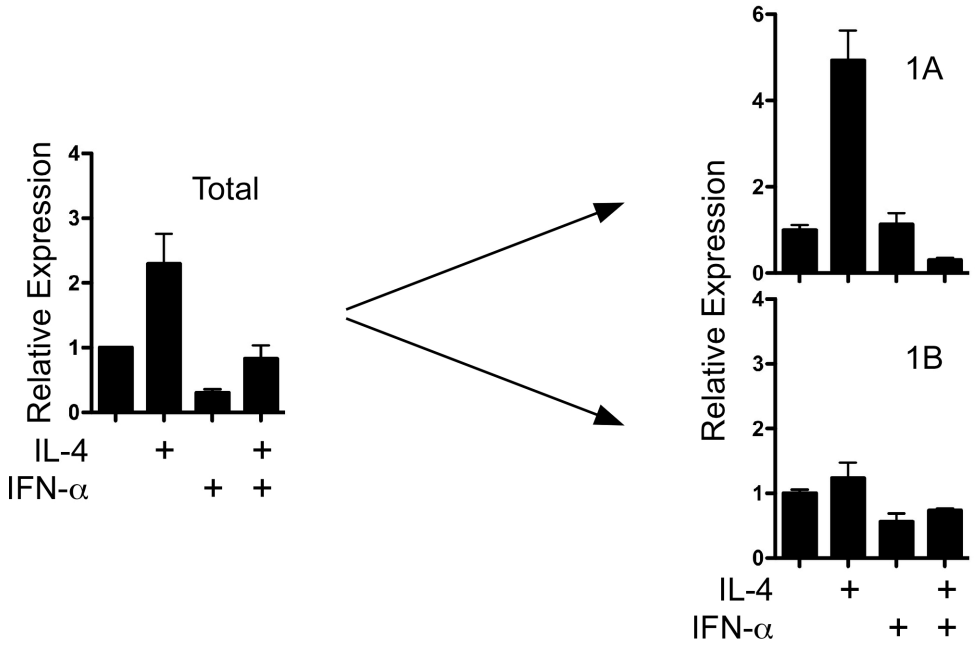

Figure 4.6. IFN-α selectively inhibits the GATA3 exon 1A transcript. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 for 72 h with IL-4, IFN-α, or IL-4 + IFN-α. mRNA was isolated from cells, and relative GATA3 mRNA was quantified by real-time PCR. Primers used to measure total GATA3 transcripts span exons 5-6, while primers used to measure exon 1A and 1B span exons 1-2 as shown in Figure 4.5. Data were normalized to PPIA expression and are shown relative to the neutralized condition for each primer set. Data for exon 1B are not statistically significant.

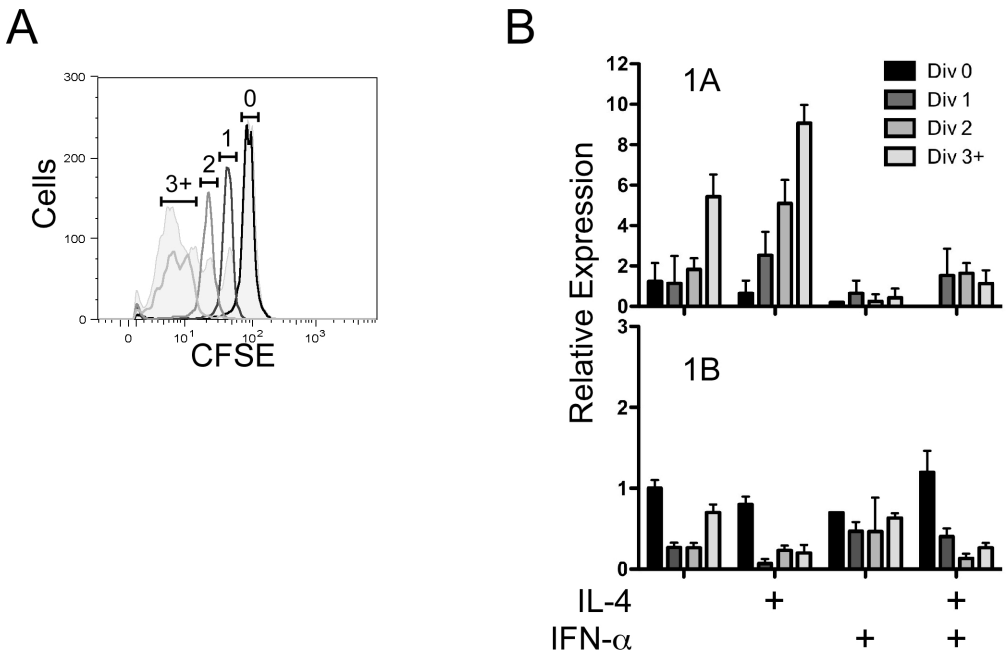

Figure 4.7. GATA3 exon 1A transcript expression increases as a function of cellular proliferation but is inhibited by IFN-α. Purified human $CD4^+/CD45RA^+$ cells were labeled with CFSE and activated with plate-bound anti-CD3/anti-CD28 for 5 days with anti-IL-4, IL-4, IFN-α, or IL-4 + IFN-α. On day five, cells were sorted based on cell divisions. *A*, the individual divisions, marked by decreasingly dark lines, are overlayed onto the light gray background representing the total pre-sort population. *B*, mRNA was isolated from cells, and relative GATA3 exon 1A or 1B mRNA was quantified by real-time PCR. Data were normalized to PPIA expression and are shown relative to the neutralized condition for each primer set.

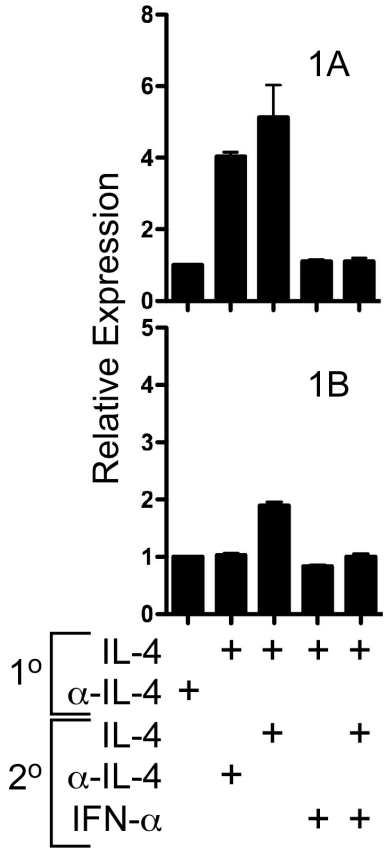

Figure 4.8. IFN-α/β suppresses GATA3 exon 1A transcript levels in committed Th2 cells. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-CD3/anti-CD28 for 7 days under neutralizing conditions or with IL-4 to promote Th2 development (1^o condition). Cells were then washed and restimulated for an additional 2 days with anti-IL-4, IL-4, IFN-α, or IL-4 + IFN-α (2^o conditions). mRNA was isolated from cells, and relative GATA3 exon 1A or 1B mRNA was quantified by real-time PCR. Data were normalized to PPIA expression and are shown relative to the neutralized condition for each primer set.

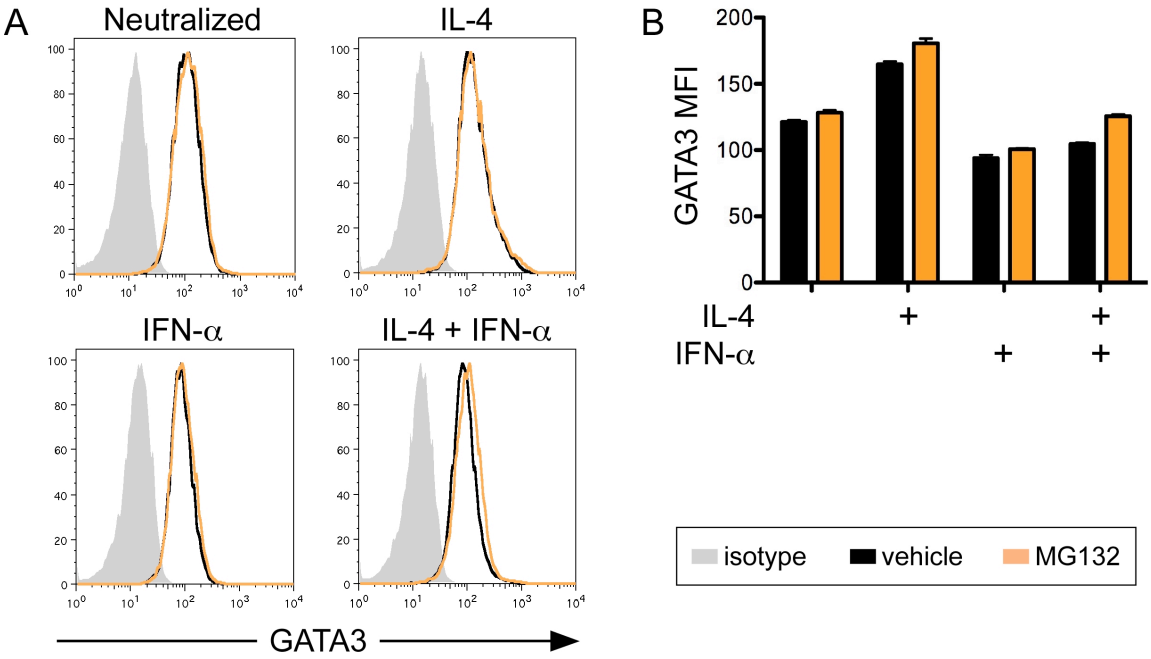

Figure 4.9. IFN-α does not affect the stability of GATA3 protein. Purified human $CD4^+/CD45RA^+$ cells were labeled with CFSE and activated with plate-bound anti-CD3/anti-CD28 for 5 days under the indicated cytokine conditions. On day 5, cells were incubated for 2 hours in the presence or absence of Mg132. GATA3 protein levels were measured by intracellular staining and expressed as histogram overlays (*A*) or mean fluorescence intensity (*B*).

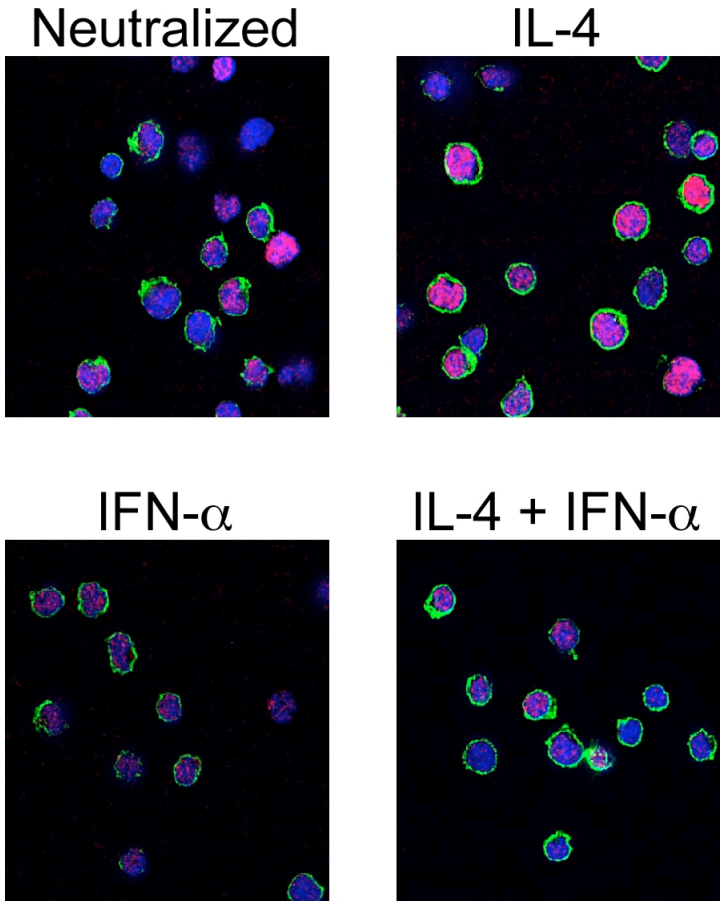

Figure 4.10. IFN-α does not inhibit nuclear localization of GATA3. Purified human $CD4^+/CD45RA^+$ cells were labeled with CFSE and activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. Cells were then stained with fluorescent antibodies for GATA3 (red) or CD4 (green) protein and were counterstained for DAPI (blue). Images were collected on a Deltavision deconvolution fluorescence microscope and normalized using ImageJ. Multiple images were collected per sample, and one representative image is shown per sample.

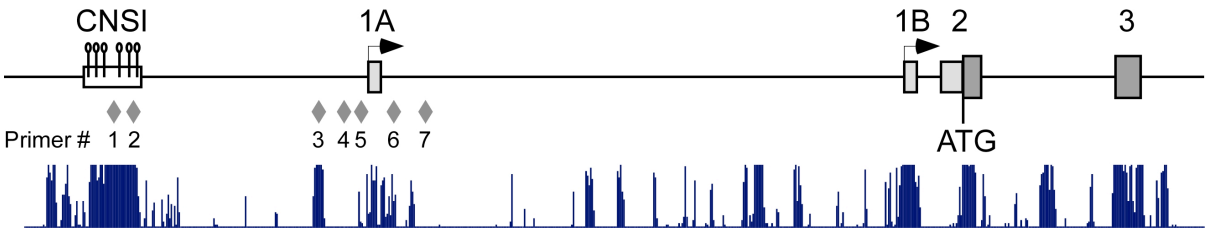

Figure 4.11. A model of the GATA3 locus showing the regions analyzed for DNA-binding by GATA3 and for histone modifications. As shown in Figure 4.5, the GATA3 locus contains many conserved coding and non-coding sequences. The highly conserved CNSI contains numerous putative GATA3 binding sites. Additional sequences around exon 1A, along with a confirmed GATA3 binding site within a conserved intronic regulatory element (CIRE) in the IL-4 gene (not shown), were used to examine GATA3 function and epigenetic modifications of the GATA3 locus. The EMSA probe corresponds to the IL-4 CIRE. Primer sets #1, #2, and #5 were used for ChIP analysis of GATA3. Primer sets #1, #3-7 were used for ChIP analysis of histones H4ac, H3K4me3, H3K27me3, and total H3.

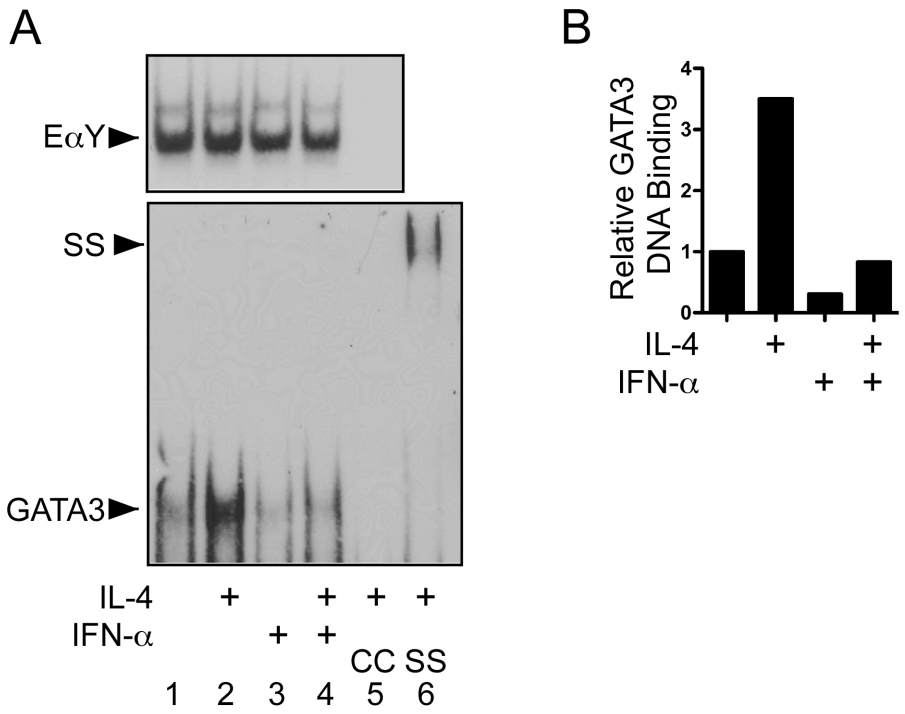

Figure 4.12. EMSA analysis shows reduced DNA-binding by GATA3 in cells treated with IFN-α. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. Nuclear lysates were prepared and tested for binding to a DNA probe corresponding to the IL-4 CIRE (*A*). The specificity of the GATA3 binding complex was confirmed using an unlabeled competitor (condition 5) and an anti-hGATA3 antibody (condition 6). *B*, The intensity of the bands shown in *A* is normalized to the background and shown relative to the neutralized condition.

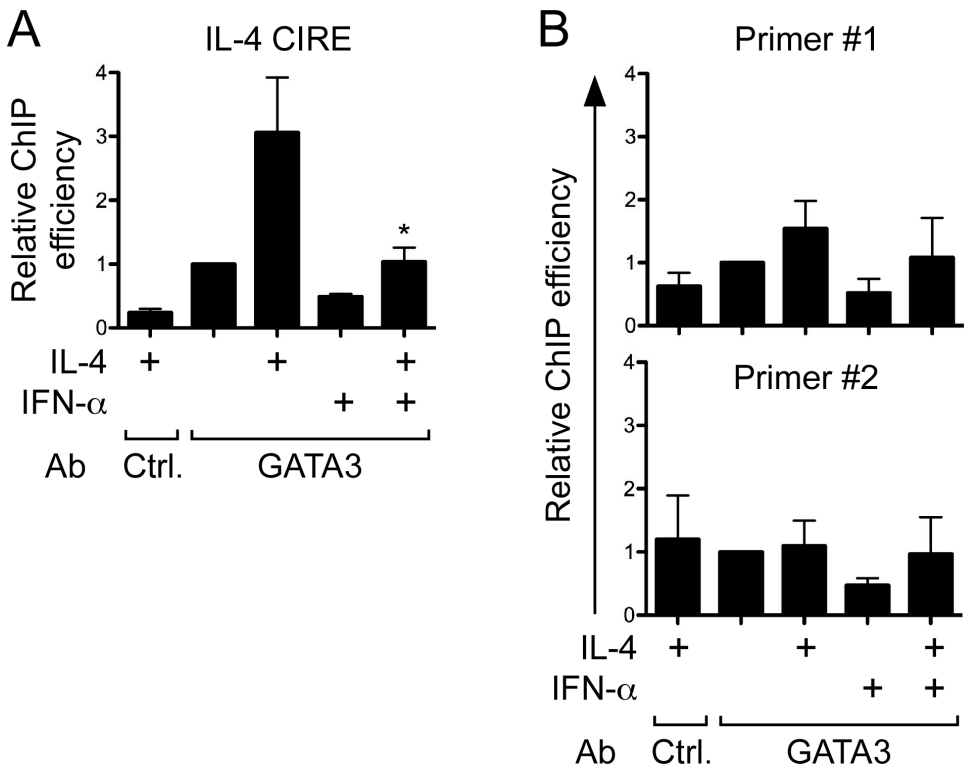

Figure 4.13. ChIP analysis shows reduced DNA-binding by GATA3 in cells treated with IFN-α. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. ChIP was performed using anti-hGATA3 or control antibodies. Immunoprecipitated DNA was quantitated by qPCR, compared to an input control, and shown as relative ChIP efficiency compared to the IL-4-treated sample. Data shown is averaged from 5 separate donors. *, $p < 0.05$ compared to the IL-4-treated sample.

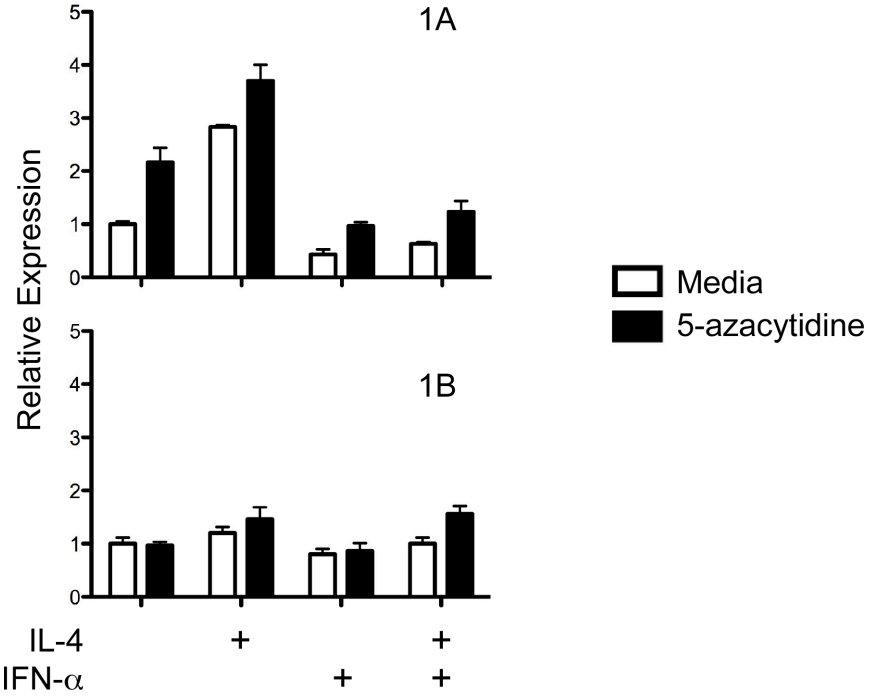

Figure 4.14. IFN-α does not regulate GATA3 by inducing DNA methylation. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. 5-azacytidine was added daily for all 5 days. On day 5, mRNA was isolated from cells, and relative GATA3 exon 1A or 1B mRNA was quantified by real-time PCR. Data were normalized to PPIA expression and are shown relative to the neutralized condition for each primer set.

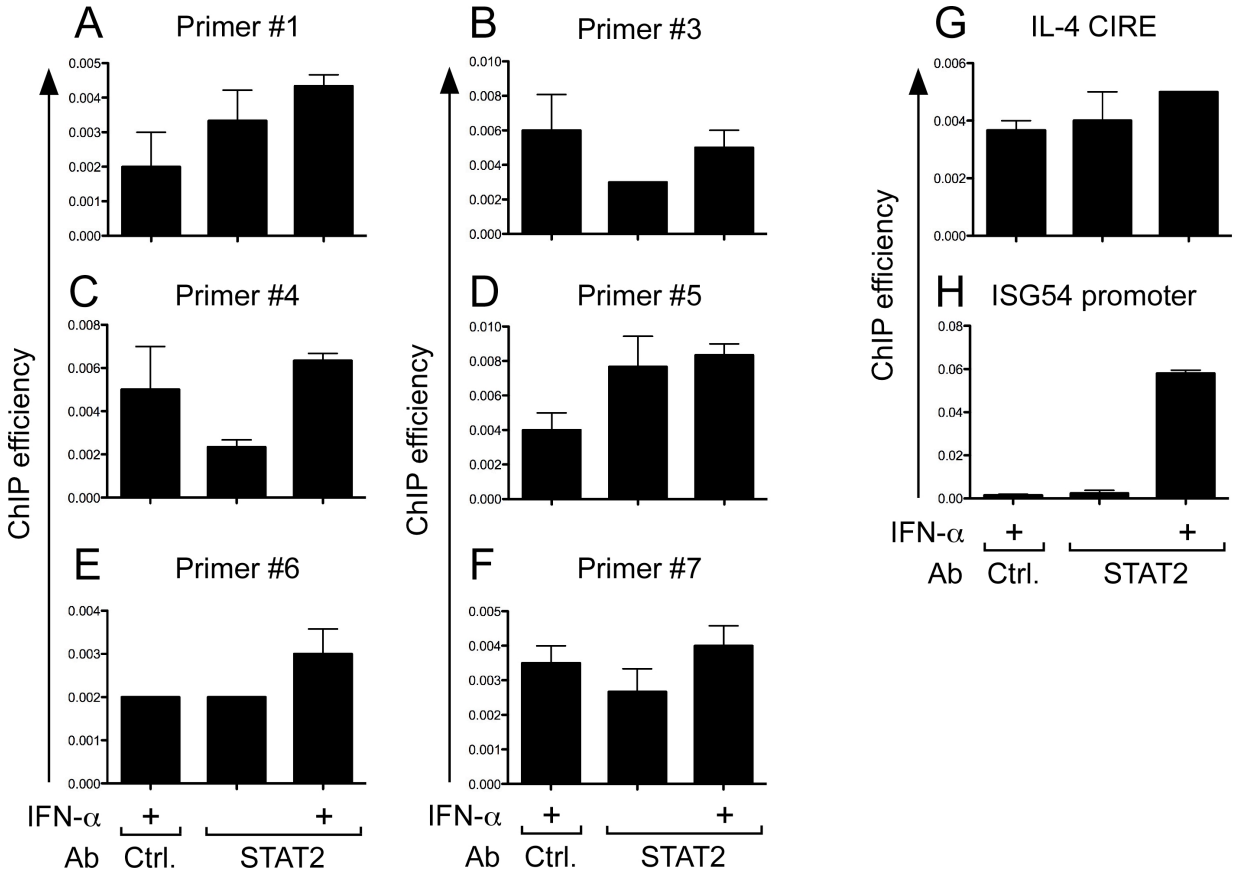

Figure 4.15. STAT2 does not bind to selected sites in the GATA3 locus. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. ChIP was performed using anti-hSTAT2 or control antibodies. Immunoprecipitated DNA was quantitated by qPCR and compared to an input control. The location of the numbered primers is shown in Figure 4.11.

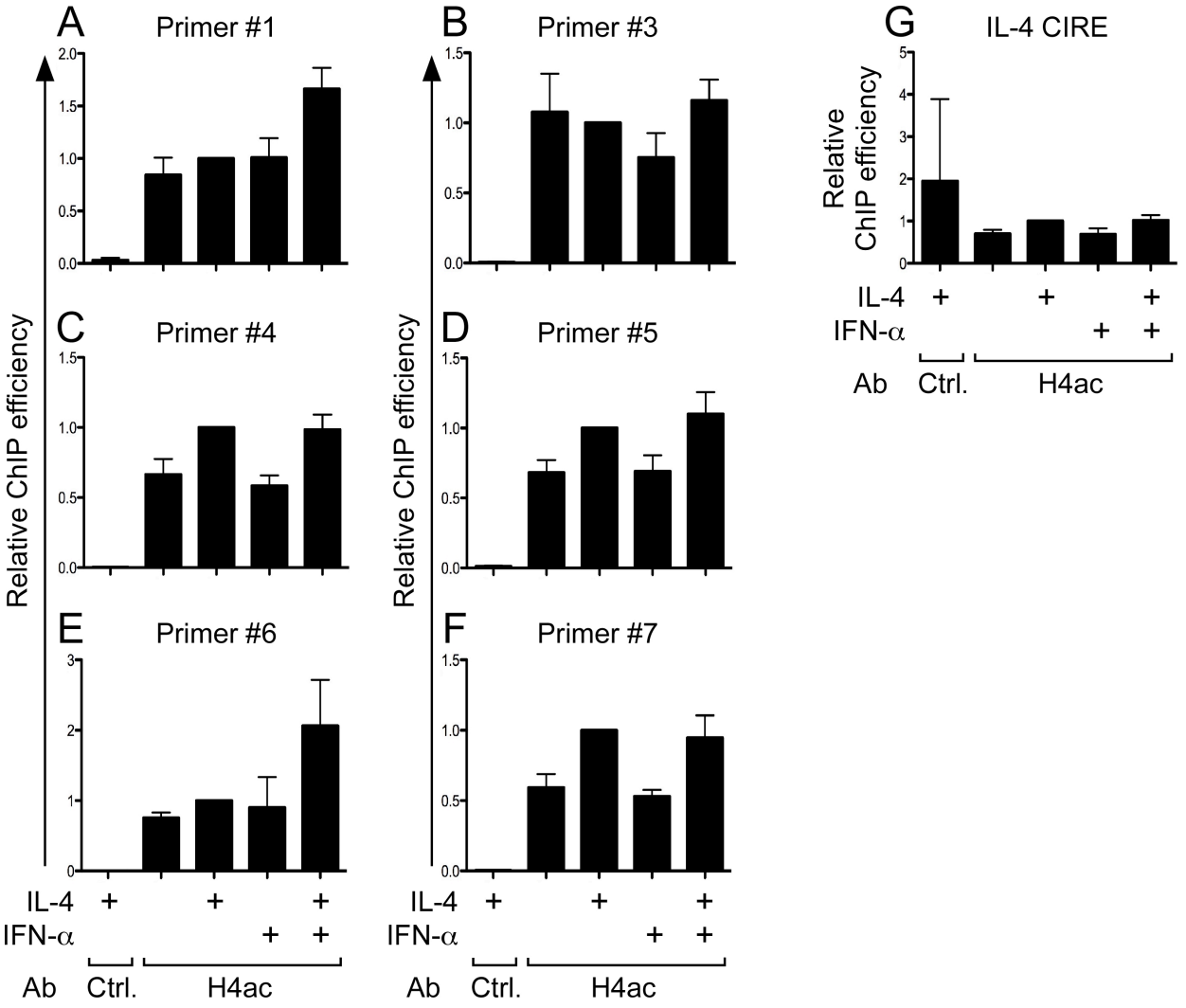

Figure 4.16. IFN-α does not affect H4 acetylation at the GATA3 locus or IL-4 CIRE. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. ChIP was performed using an anti-H4ac or control antibody. Immunoprecipitated DNA was quantitated by qPCR, compared to an input control, and shown as relative ChIP efficiency compared to the IL-4-treated sample. The location of the numbered primers is shown in Figure 4.11. Data shown is averaged from either 5 (*A, D, G*) or 3 (*B, C, E, F*) separate donors.

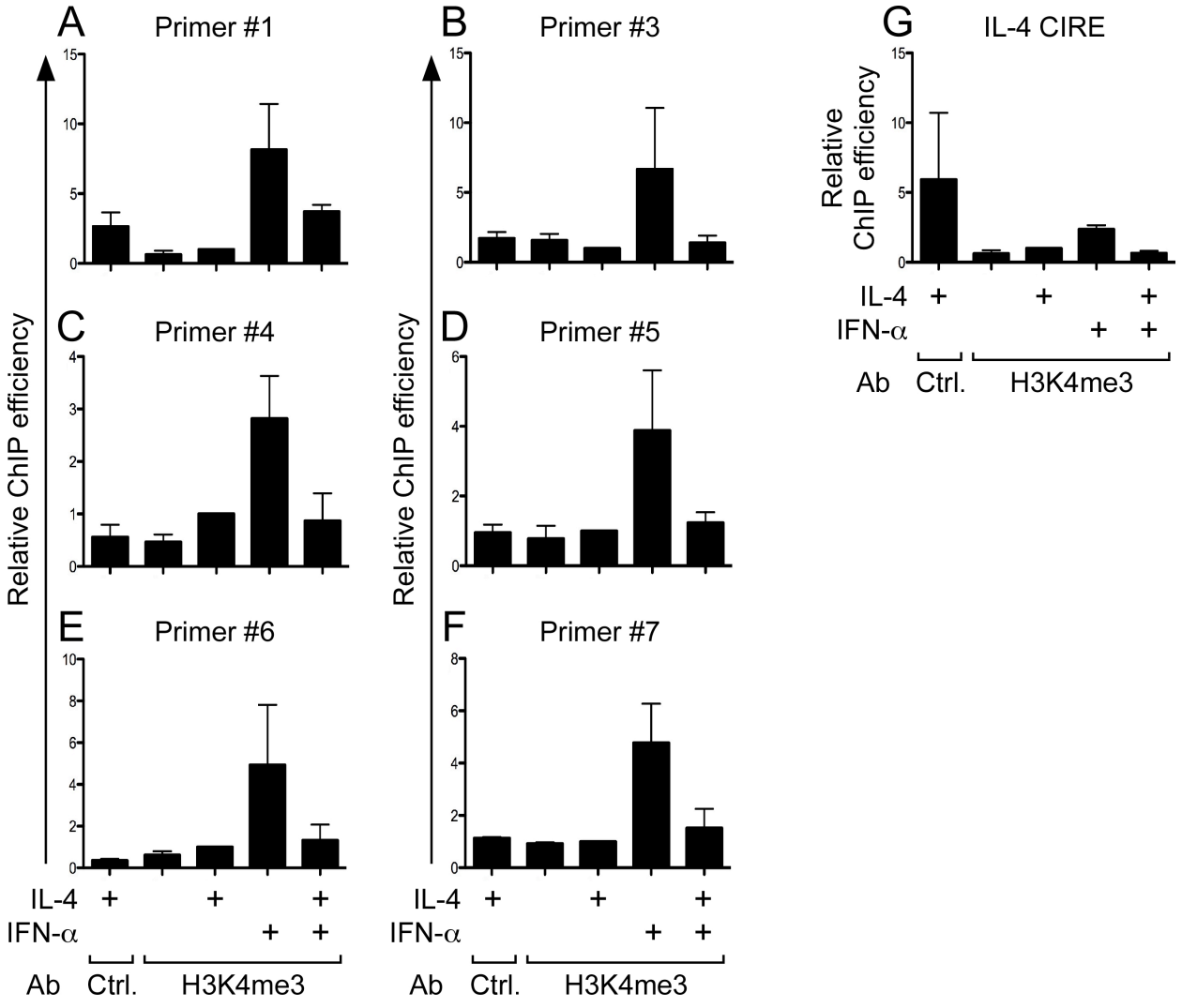

Figure 4.17. IFN-α does not affect H3K4 trimethylation at the GATA3 locus or IL-4 CIRE in IL-4-treated cells. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. ChIP was performed using an anti-H3K4me3 or control antibody. Immunoprecipitated DNA was quantitated by qPCR, compared to an input control, and shown as relative ChIP efficiency compared to the IL-4-treated sample. The location of the numbered primers is shown in Figure 4.11. Data shown is averaged from either 4 (*A, D, G*) or 2 (*B, C, E, F*) separate donors.

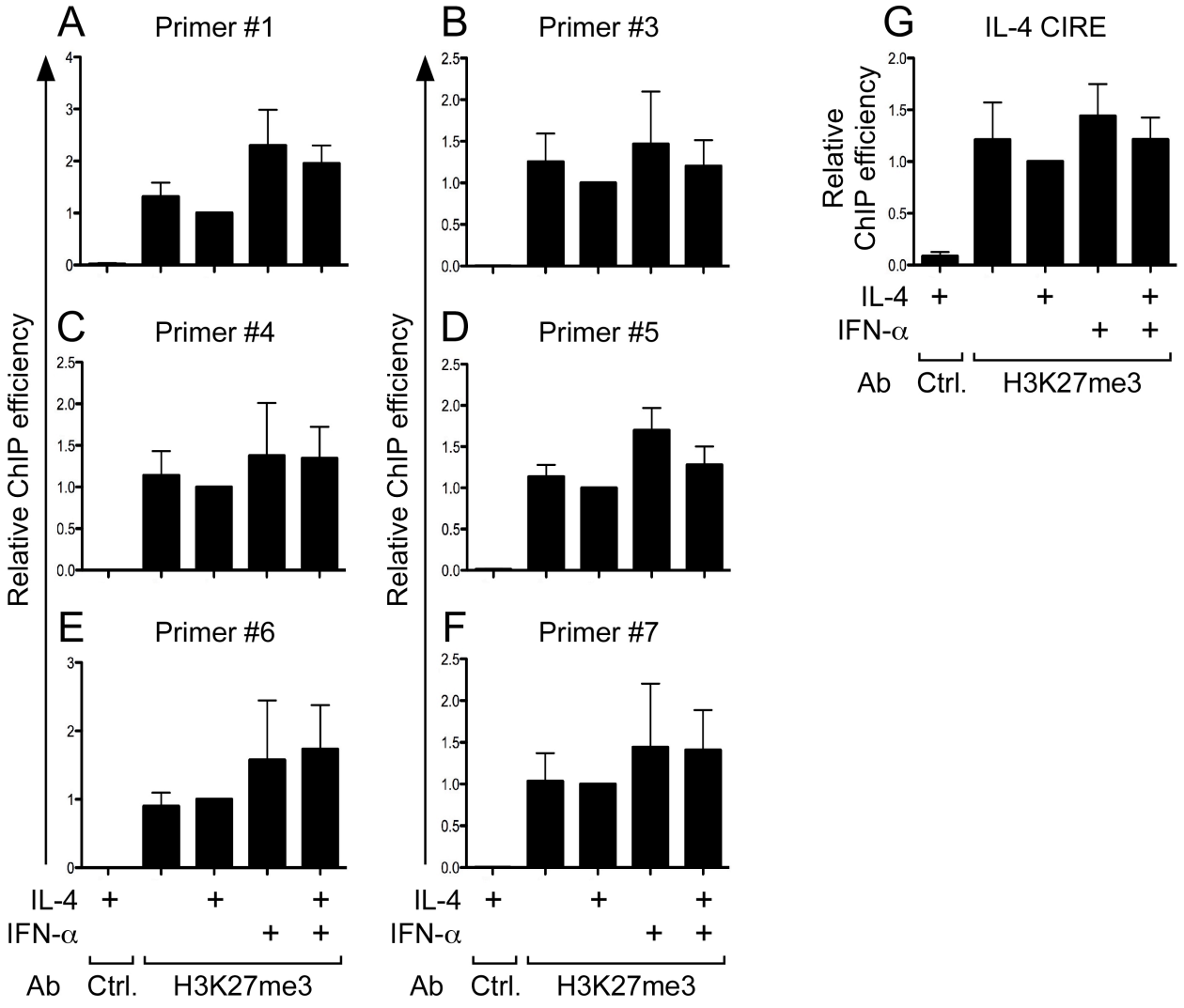

Figure 4.18. IFN-α does not affect H3K27 trimethylation at the GATA3 locus or IL-4 CIRE. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. ChIP was performed using an anti-H3K27me3 or control antibody. Immunoprecipitated DNA was quantitated by qPCR, compared to an input control, and shown as relative ChIP efficiency compared to the IL-4-treated sample. The location of the numbered primers is shown in Figure 4.11. Data shown is averaged from either 5 (*A, D, G*) or 2 (*B, C, E, F*) separate donors.

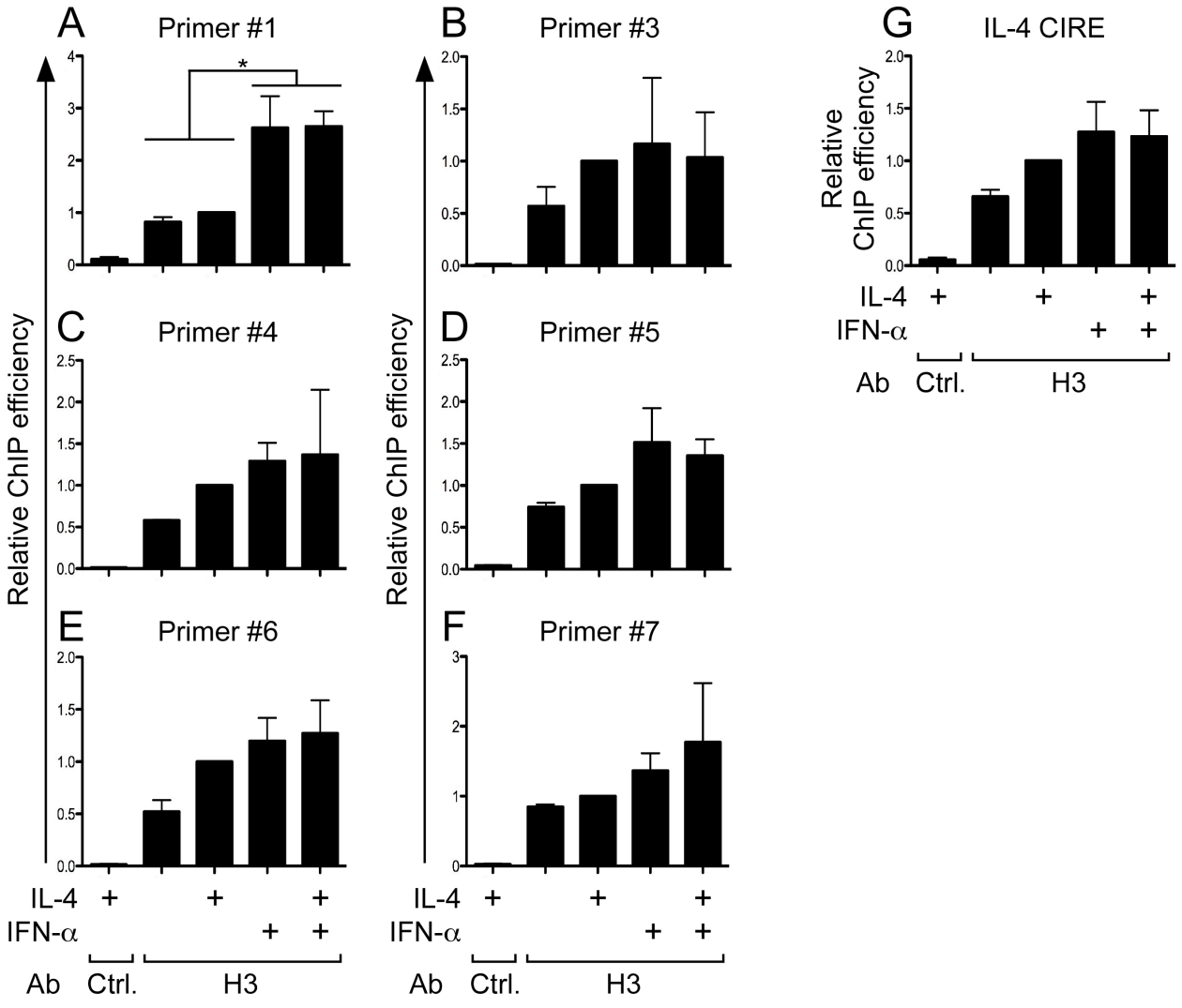

Figure 4.19. IFN-α increases the total histone H3 bound to GATA3 CNSI. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. ChIP was performed using an anti-H3 or control antibody. Immunoprecipitated DNA was quantitated by qPCR, compared to an input control, and shown as relative ChIP efficiency compared to the IL-4-treated sample. The location of the numbered primers is shown in Figure 4.11. Data shown is averaged from either 4 (*A, D, G*) or 2 (*B, C, E, F*) separate donors. *, $p < 0.05$.

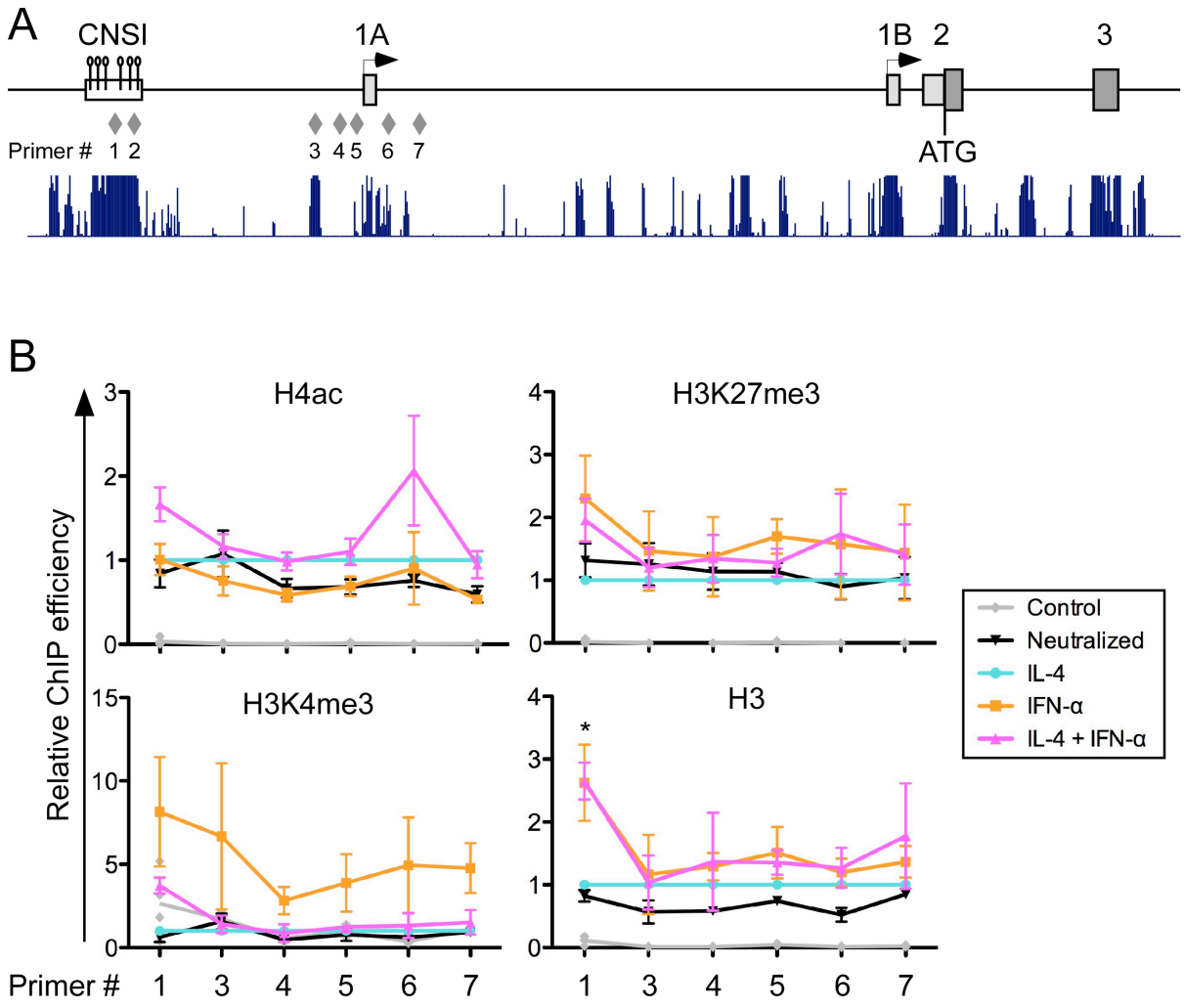

Figure 4.20. IFN-α may regulate epigenetic modifications of GATA3 CNSI. Purified human $CD4^+/CD45RA^+$ cells were activated with plate-bound anti-hCD3/anti-hCD28 for 5 days under the indicated cytokine conditions. On day 5, cells were restimulated for incubated for 2 hours with plate-bound anti-hCD3/anti-hCD28. ChIP was performed using a control antibody or antibodies against the indicated histones. Immunoprecipitated DNA was quantitated by qPCR, compared to an input control. *A*, the location of the numbered primers used for ChIP analysis. *B*, Relative ChIP efficiency is calculated compared to the IL-4-treated sample. Data shown is averaged from multiple separate donors. *, $p < 0.05$ for the IFN-α and IL-4+IFN-α samples compared to the Neutralized or IL-4 samples.

CHAPTER V

DISCUSSION

Part of this chapter is adapted from a review article published in Immunology, volume 132, pages 466-474 (Huber and Farrar, 2011).

Summary of results

My research offers insight into new roles for type I interferon both immunologically and clinically. As the first-discovered cytokine, IFN-α/β has been studied extensively for more than 50 years. The contribution of IFN-α/β to innate immunity is very well established, and more recent study has uncovered important, if somewhat controversial, roles in adaptive immunity as well. Given the number of studies already published on type I interferon, it is remarkable that new functions and potential uses for this cytokine family continue to be discovered.

The data presented here describe a novel role for type I interferon in the inhibition of human Th2 cells. Differentiation of Th2 cells from naïve precursors through the activity of IL-4 is impaired by IFN-α, as evidenced by reduced Th2 cytokine production and a loss of Th2-specific chemoattractant receptors. IFN-α/β blocks this in a dose-dependent manner by suppressing the expression of GATA3 at both the mRNA level and at the protein level. The inhibition of GATA3 destabilizes the entire Th2 phenotype, leading to a loss of Th2 cytokine production even in previously committed Th2 cells.

Skewing towards Th2 by IL-4 is most effectively accomplished when IL-4 signals are provided during a primary activation, which enables epigenetic modifications to the GATA3 and Th2 cytokine loci as cells progress through multiple rounds of cellular division. Proliferation is accompanied by a progressive increase in both GATA3 mRNA and protein expression, leading to IL-4 and IL-13 production from cells that have progressed to at the least the 3rd division. This programming occurs even in undivided cells, albeit to a lesser extent, as evidenced by enhanced GATA3 production in undivided Th2 cells upon a secondary stimulation. These IL-4- and proliferation-associated increases in GATA3 are suppressed by IFN-α.

GATA3 transcription produces two mRNAs distinguished by the utilization of distinct first exons, though these alternative exons do not contain protein-coding sequence. These two transcripts, controlled by separate promoters, allow a basal level of GATA3 in naïve CD4^{+} T cells to be supplemented during Th2 commitment by IL-4-mediated increases in the upstream, exon 1A-containing transcript. Type I interferon selectively prevents expression of this transcript. Unlike the downstream exon 1B transcript, expression of the exon 1A transcript progressively increases, matching the protein level increases and lending further support to the suggestion that the exon 1A transcript gives rise to the Th2-specific increase in GATA3 protein.

Finally, though no changes in three different histone modifications were detected around exon 1A or nearby CNSI, the loss of GATA3 protein in response to IFN-α led to a decrease in DNA-binding by GATA3, suggesting that disruption of the GATA3 autoregulatory loop may underlie the destabilization of committed Th2 cells by IFN-α. This loss of DNA-binding by GATA3 was accompanied by an increased association between the CNSI DNA and histone H3, suggesting that IFN-α may disrupt the function of an enhancer region necessary for exon 1A transcript expression and Th2 commitment.

This is the first report that type I interferon suppresses the expression of GATA3 and thereby destabilizes human Th2 cells. Other reports have considered the effect of IFN-α on baseline IL-4 expression, but not the ability of IFN-α to override IL-4-driven Th2 development. Similarly, while a growing number of reports have described the two-exon transcription system of GATA3, these studies have all focused on pathways that promote expression of the GATA3 transcripts. This is the first report that a cell-extrinsic signal can specifically inhibit Th2 cells by selectively suppressing one of these transcripts. A model of these results is shown in Figure 5.1.

A role for STAT2

At present, it is unclear what links IFN-α/β signaling to GATA3 suppression. IFN-α/β signaling activates a number of proteins, including STAT1, STAT2, STAT3, STAT4, STAT5, and even STAT6, as well as MAPK, PI3K, Akt, and NF-κB (Gupta et al., 1999; Uddin and Platanias, 2004; de Weerd and Nguyen, 2012). IFN-γ shares the ability to activate STAT1 but has no inhibitory effect on human Th2 cells. IL-12 shares the ability to activate STAT4, but has only a moderate effect on Th2 cytokine production, much less than the effect of IFN-α treatment, and IL-12 does not appear to suppress GATA3. Furthermore, IFN-α activates STAT4 much more transiently than does IL-12, making it unlikely that STAT4 mediates the suppression of Th2 cells. Activation of other STAT proteins, MAPK, PI3K, Akt, and NF-κB is common to various other signaling pathways, including the TCR and IL-4, so it seems unlikely that they could transmit a signal that is specific to IFN-α/β.

The most interesting possibility is the activation of STAT2 or a STAT2-containing complex. STAT2 activation is a unique property of type I and type III interferons. IFN-λ1, IFN-

λ2, and IFN-λ3 (also designated IL-29, IL-28A, and IL-28B respectively), collectively referred to as type III interferon, bind to a dimeric receptor comprised of the IL-28Rα and IL-10R2 chains (Kotenko et al., 2003). Though type I and type III interferons signal through distinct receptors, they share the ability to activate many of the same molecules, including STAT1 and STAT2 (Dumoutier et al., 2004). In addition, both type I and type III interferons induce formation of the ISGF3 transcription factor complex and binding of this complex to ISREs in canonical ISGs (Zhou et al., 2007). Furthermore, the inhibition of Th2 cells by IFN-α/β parallels recent work demonstrating that type III interferon (IFN-λ) can also suppress GATA3 and Th2 cytokine production (Dai et al., 2009). Interestingly, though IFN-α and IFN-λ induce a similar antiviral response, IFN-α more efficiently induces binding of ISGF3 to the ISG56 promoter as well as greater transcription of interferon response genes like ISG56 and OAS1 (Zhou et al., 2007). Unlike IFN-α, induction of these antiviral responses by IFN-λ required the additional activity of p38 and JNK, which reveals qualitative differences in the efficacy of these two signaling pathways that might explain the unique ability of type I interferon to regulate CRTh2 expression (Figure 3.6). Nevertheless, since both IFN-α/β and IFN-λ activate STAT2 and drive ISGF3 complex formation, STAT2 may play a crucial role in suppressing human Th2 development.

Although the implication of STAT2 appears to conflict with the lack of binding by STAT2 to the selected sequences of the GATA3 locus (Figure 4.15), these data do not preclude the possibility that STAT2 binds elsewhere in the GATA3 locus. An enhancer region 280kb downstream of *gata3* is critical for normal GATA3 transcription in T cells (Hosoya-Ohmura et al., 2011). The Th2 cytokine locus encompasses more than 600kb of sequence, yet these cytokines are coordinately regulated (Spilianakis and Flavell, 2004). Even more impressive is the interaction between *ifng* and *ifngr1* which, although on the same chromosome, are

separated by more than 98Mb (Deligianni and Spilianakis, 2012). Interactions between genes on separate chromosomes have also been described (Lieberman-Aiden et al., 2009; Botta et al., 2010). Thus, STAT2 could plausibly regulate GATA3 through regulation of DNA sequences quite remote from the GATA3 locus. Furthermore, STAT2, either alone or as part of the ISGF3 complex, may mediate the suppression indirectly through the upregulation of an intermediate agent. A model of potential regulation of human Th2 via STAT2 is shown in Figure 5.2

If STAT2 is responsible for mediating suppression of GATA3 by IFN-α, that could potentially explain the species-specific effect. STAT2 is highly divergent across species, and the mouse sequence harbors a unique minisatellite sequence in the C-terminus that is not found in any other species (Farrar and Murphy, 2000; Farrar et al., 2000a). In an effort to determine the significance of the species-specific difference in STAT2, a knock-in mouse was generated in which the C-terminus of murine STAT2 was replaced with the human sequence, resulting in a chimeric mouse/human STAT2 molecule (Persky et al., 2005). IFN-α/β treatment of $CD4^+$ T cells from this STAT2 knock-in mouse led to normal ISGF3 formation and ISG expression. However, the modified STAT2 molecule does not restore the ability of IFN-α to inhibit Th2 cells from this mouse (Dr. J. David Farrar, unpublished observation). Additional changes in the murine STAT2 protein may have arisen due to natural selection. STAT2 is a critical component of IFN-α/β signaling and facilitates the induction of much of the innate antiviral response. Naturally, many viruses interfere with IFN-α signaling components, particularly STAT2, which is unique to type I and type III interferon signaling pathways (Parisien et al., 2001; Jones et al., 2005). Selective pressure from viruses may have caused adaptation in murine IFN-α/β signaling that broke a connection between IFN-α and GATA3 which remained intact in humans. If this is the case, replacing other IFN-α signaling components with human counterparts may restore

suppressive capacity. On the other hand, if difference is in the GATA3 locus, then perhaps a transgene with human GATA3 locus could be placed into murine cells. Unfortunately, this may not be feasible since normal GATA3 expression requires regulatory elements quite distant from the proximal promoter (Lakshmanan et al., 1999; Hosoya-Ohmura et al., 2011). Furthermore, since the root of the species specificity is unknown, there is a significant chance that any studies in the mouse would go unrewarded.

SOCS proteins

An additional pathway to consider is the suppressor of cytokine signaling (SOCS) family of proteins (Alexander and Hilton, 2004). SOCS proteins inhibit JAK/STAT signaling, and since the expression of SOCS proteins is induced by cytokine signaling itself, these proteins create negative feedback loops that keep cytokine signaling under control. IFN-α and IFN-γ both suppress IL-4 signaling in human monocytes by upregulation of SOCS1 (Dickensheets and Donnelly, 1999; Dickensheets et al., 1999), and IFN-γ also performs a similar function in human lung epithelial cells (Heller et al., 2004). However, IL-4 itself upregulates SOCS1 in human monocytes, thus suppressing its own signaling (Dickensheets et al., 2007), and IL-4 has paradoxically been shown to suppress IFN-γ signaling in keratinocytes via induction of SOCS1 (Albanesi et al., 2007). Likewise, IFN-α-induced SOCS1 and SOCS3 suppress its own signaling (Zimmerer et al., 2007). Given this data and the fact that SOCS proteins regulate proximal IL-4 signaling, which type I interferon bypasses, it is unlikely that IFN-α/β inhibits Th2 cells through the action of SOCS proteins.

Future directions

Future investigation of the links between IFN-α and GATA3 will need to start from these two ends of the chain. I have already suggested that STAT2 may play a key role in the suppression of Th2 cells; this could be confirmed by knock-down of STAT2 via siRNA. Performing siRNA is notoriously difficult in primary human T cells, but since IFN-α inhibits GATA3 in total T cells with the same pattern as in naïve T cells (data not shown), perhaps a sufficiently large starting pool of cells could be generated. Alternatively, a dominant-negative version of STAT2 could be made through a Y690F mutation, and this mutated STAT2 could be expressed in T cells via retroviral transduction. This mutant STAT2 would compete with the endogenous STAT2 for binding to the IFNAR but the normal activation of the protein would be impaired. If STAT2 is confirmed to mediate the suppression, subsequent studies would begin with STAT2. One important caveat of this strategy is that mutating Y-690 would also block activation of STAT1, which is recruited to this site following activation of STAT2. Thus, similar studies would need to be performed with STAT1 as well in order to determine whether STAT2 was actually controlling GATA3 somehow or whether it merely served as a bridge for STAT1. If these studies confirmed a role for STAT2, subsequent work would aim to identify any places where it binds around the GATA3 locus. Rather than selecting a few specific sequences, as I have done already, DNA-binding by STAT2 should be evaluated more thoroughly by using ChIP-seq in order identify potential binding sites within the GATA3 locus. STAT2 may also play a role by inducing expression of an intermediate factor rather than binding directly at the GATA3 locus. Since STAT2 mediates induction of the many ISGs expressed following IFN-α signaling, it may be difficult to determine whether STAT2 binding to sites beyond the GATA3 locus are involved in this suppressive function, but that remains a plausible mechanism that must be considered.

A similar approach would be to use ChIP-seq to more thoroughly evaluate DNA-binding by GATA3. Although ectopic expression of GATA3 in T cells can induce expression of the endogenous GATA3, it is unclear whether this is caused by direct binding of GATA3 to the GATA3 locus. Part of the difficulty in dissecting the mechanism of suppression by IFN-α is the fact that the mechanisms governing GATA3 expression in human Th2 cells remain unclear. Thus, it would be helpful to determine where GATA3 binds at the GATA3 locus in response to IL-4 treatment and how this is affected by IFN-α. In addition, though there is some evidence that GATA3 binds at the GATA3 locus in murine T cells, it is possible that GATA3 promotes its expression by upregulating an intermediate factor. Analysis of GATA3 binding by ChIP-seq would clarify the effect of IL-4 and IFN-α on binding by GATA3 at multiple gene loci (GATA3 locus, IL-4 locus, CRTh2, others?), which could improve our understanding of GATA3 auto-regulation in Th2 cells and also shed more light on other aspects of Th2 behavior that could be altered by IFN-α.

A less-biased alternative would be to search for regions of the genome that show epigenetic changes upon IFN-α treatment. Performing ChIP-seq with antibodies for different histone modifications, such as H3K27me3 or H3K9ac, could confirm epigenetic regulation of GATA3 CNSI following IFN-α treatment and may reveal additional regions of the GATA3 locus that are epigenetically regulated. One additional option is formaldehyde-assisted isolation of regulatory elements (FAIRE), in which chromatin is cross-linked, sonicated, and isolated by phenol-chloroform extraction (Giresi et al., 2007). DNA that is cross-linked to proteins is found in the organic phase, while naked DNA is found in the aqueous phase and can then be identified via DNA arrays. DNA isolated in this manner closely corresponds with the promoters and transcription start sites of actively expressed genes. One caveat to this approach, if it mainly

identifies transcription sites, is the problem mentioned already of identifying which of the hundreds of IFN-α-induced genes might be responsible for mediating suppression.

A complementary approach would be to use regions of interest, such as GATA3 CNSI or additional regions discovered by ChIP-seq, to identify DNA-protein and DNA-DNA interactions that are selectively formed or lost when cells are treated with IFN-α. Analysis of long-range DNA interactions with these regions could be identified by chromosome conformation capture (3C) (Dekker et al., 2002; Tolhuis et al., 2002). The idea of 3C is to cross-link DNA, digest the ends and ligate them together, and then sequence through the newly ligated product for identification. 3C has been used to identify both intra- and inter-chromosomal interactions (Liu and Garrard, 2005; Lieberman-Aiden et al., 2009; Botta et al., 2010; Deligianni and Spilianakis, 2012). CCCTC-binding factor (CTCF) has been recognized as a chromosomal insulator, but more recent data reveals that it mediates these long-range DNA interactions as well (Ong and Corces, 2009; Phillips and Corces, 2009; Botta et al., 2010). CTCF has been shown to bind to multiple sites of immunological interest, including MHC I and II genes, the IG κ locus, and the Th2 cytokine locus (Liu and Garrard, 2005; Ribeiro de Almeida et al., 2009; Ottaviani et al., 2012). CTCF is required for optimal Th2 cytokine expression (Ribeiro de Almeida et al., 2009), which fits with reports of long-range chromosomal interactions at the Th2 cytokine locus (Spilianakis and Flavell, 2004). Consequently, it would also be useful to determine whether CTCF binding is altered by IFN-α.

Finally, new DNA-protein interactions could be identified using a DNA affinity precipitation assay, a clever technique that relies on biotinylated DNA to capture proteins from cellular lysates and then isolate them by a pull-down with streptavidin-coated beads (Yu et al., 2007; Pham et al., 2012). Bound proteins could then be separated by gel electrophoresis and

identified by mass spectrometry. Proteins differentially pulled down in the presence or absence of IFN-α could then be used as the basis of further study.

Balancing T helper cell responses: revisited

The resolution of an infection is highly dependent on the ability of the immune system to appropriately instruct $CD4^+$ T cell differentiation, and innate cytokines ensure proper differentiation by both instructing differentiation to one fate and inhibiting the development of other subsets. IL-12, IFN-γ, and IL-4, cytokines strongly connected with Th1 and Th2 development, have been shown to cross-regulate each other. However, the balance of this cross-regulatory activity seems to favor Th2 cells, as IL-4-induced GATA3 overrides IL-12 signaling, redirects Th1 cells to Th2, and stabilizes the Th2 phenotype (Ouyang et al., 1998; Usui et al., 2003; Kaminuma et al., 2004; Zhu et al., 2006). Consequently, additional counter-regulation of Th2 cells may come from another source in order to even the balance, particularly since $CD4^+$ T cells can produce their own IL-4. My data indicates that IFN-α/β may fill that role by suppressing Th2 responses. IFN-α is produced during viral and some bacterial infections (Katze et al., 2002; Monroe et al., 2010; Parker and Prince, 2011), which are cleared through the activity of $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells. IFN-α is not sufficient in vitro to drive development of either Th1 cells or cytotoxic $CD8^+$ T cells (Ramos et al., 2007; Davis et al., 2008; Ramos et al., 2009), but it may support development of these subsets indirectly by preventing the alternative options. $CD8^+$ Tc2 cells that produce IL-4 have been described (Seder et al., 1992a; Kelso and Groves, 1997), but IFN-α has not yet been studied in Tc2 cultures.

Nevertheless, IFN-α may provide counter-regulatory signals needed to control proper differentiation during a Th1-type immune response.

If Th2 cells lose GATA3 and Th2 cytokine expression when treated with IFN-α, what do they become? IFN-α does not directly drive Th1 development (Ramos et al., 2007; Davis et al., 2008). It suppresses Th17 development (Harrington et al., 2005; Moschen et al., 2008). I have not examined FoxP3, but IFN-α did not increase IL-10 secretion (data not shown). Antiviral responses are controlled by Th1 and $CD8^+$ cytotoxic T cells, so there is no apparent need for IFN-α to induce a new T helper phenotype. However, IFN-α/β may play a broader role in $CD4^+$ T cell functions by regulating the development and stability of long-lived memory cells. Although IFN-α/β may promote cell cycle arrest and, in some cases, apoptosis in certain cell types (Krishnaswamy et al., 1996; Gujer et al., 2011), $CD4^+$ T cells respond quite differently depending upon their activation status. Marrack *et al.* demonstrated that IFN-α/β protected cells from undergoing acute activation-induced cell death (Marrack et al., 1999). Though not directly driving proliferation, IFN-α/β seemed to block apoptosis following antigen stimulation *in vitro*, which may be related to the development of long-lived central memory cells. As central memory cells were first described as having decreased effector capabilities, they display enhanced recall proliferation coincident with elevated secretion of IL-2 (Sallusto et al., 1999). Recently, Davis *et al.* demonstrated a direct role for IFN-α/β in promoting the development of human central memory-like $CD4^+$ T cells and preserving elevated IL-2 expression preferentially within these cells versus their effector cell counterparts (Davis et al., 2008). Yet, these paradigms of effector and memory T helper populations have been developed largely in the context of Th1 development, and it seems unlikely that a Th1-associated cytokine like IFN-α would contribute to Th2 memory development. Therefore, I conclude that the effect of IFN-α on human Th2 cells

is just one part of a network of cytokine signaling that is carefully balanced to ensure appropriate T helper differentiation. In this role, IFN-α promotes memory characteristics in Th1 cells, and also provides important counter-regulatory signals that suppress GATA3 in order to allow other cytokines to drive Th1 effector cell differentiation.

While IFN-α suppresses key components of Th2 cells, it does not prevent all functions of IL-4 signaling. For example, by activating IRS1/2, triggering PI3K and Akt signaling, IL-4 regulates cell growth, proliferation, and survival, and these pathways may be required even in non-Th2 cells. I have also shown that T-bet expression is suppressed in cells treated with both IL-4 and IFN-α (Figure 3.11). T-bet expression cannot be sustained by IFN-α signaling (Ramos et al., 2007), so it is not surprising that T-bet is increased at d3 but back down to baseline levels at d7. What is a bit surprising is that IL-4 overrides even the transient induction of T-bet. GATA3 regulates the expression of IL-12Rβ2 (Ouyang et al., 1998), STAT4 (Usui et al., 2003), and T-bet (Chen et al., 2011), suggesting that the reduction of GATA3 in IFN-α-treated cells would hinder their ability to prevent T-bet expression. Thus, IL-4 may regulate T-bet function independently of GATA3. IL-4 can inhibit T-bet transcription in a STAT6-dependent manner (Mullen et al., 2001), though whether that also requires GATA3 is unknown. Since IFN-α inhibits IL-4 signaling downstream of STAT6, it's possible that activated STAT6 is sufficient to limit T-bet expression even while IFN-α inhibits expression of GATA3.

One last point should be made about T cell plasticity. Instruction and counter-regulation play important roles in guiding appropriate T cell function during an immune response. Plasticity describes the loss of these functions *after* the cell has differentiated. T helper subsets were once thought to be terminally differentiated, but that no longer appears to be entirely true (Zhou et al., 2009). Naïve $CD4^+$ T cells show both H3K4me3 and H3K27me3 histone

modifications, one permissive and one suppressive, at subset-specific genes like *tbx21* and *gata3* (Wei et al., 2009). Upon differentiation to Th1, cells lose the H3K27me3 markings and increase the H3K4me3 marking at *tbx21*. Similar patterns were found with *gata3* and Th2 cells. Cells left in the naïve state or differentiated to other fates, while retaining or increasing the H3K27me3 marking, also retain some of the H3K4me3 marking. This suggests that these genes are not entirely converted to a permanently silenced state. Accordingly, Th17 cells are quite unstable (Lee et al., 2009), and Th1 cells can be converted to Th2 (Panzer et al., 2012). More recently, Hegazy *et al.* showed that murine Th2 cells can be directed to begin producing IFN-γ when treated with a combination of with IL-12 + IFN-γ + IFN-α (Hegazy et al., 2010). Although this combination did not restore IFN-γ production in my experiments with human Th2 cultures, my data does concur with the ability of cytokines to at least counter-regulate Th2 functions in differentiated cells. The plasticity of cells raises a very important question for immunological memory. If T cell responses are carefully matched to a pathogen via antigen specificity, what is the value of undermining that specificity by converting the behavior of the cell? This question is highlighted further by reports that $CD8^+$ T cells can exchange TCRs from their surface and thus acquire new antigen specificity for a short time until the TCR is turned over (Chaudhri et al., 2009). These conversions may grant the immune system greater flexibility when responding to a challenge, but they also reshape our understanding of immunological memory and the complexity in T cell development and function. How is functional specificity retained in memory populations? Can virus-specific Th1 cells convert to an army of activated Th2 cells in the lung of an infected patient with allergic asthma? Could allergen-specific Th2 cells lose functional ability following an IFN-α-inducing infection? Are these concerns only a problem in vitro, and phenotypes are regulated by spatial and temporal dynamics in vivo? Hopefully future studies will answer these questions.

Tissue-specific regulation of GATA3: revisited

Since GATA3 expression is critical in other tissues besides Th2 cells, it is important to consider how suppression of GATA3 by IFN-α might effect its function in those tissues. Asnagli *et al.* has shown that, at least in mice, GATA3 expression in the thymus is derived from the exon 1B transcript rather than the 1A transcript (Asnagli et al., 2002). If this selectivity is preserved in humans as well, it would suggest that IFN-α would not affect GATA3 expression in thymocytes since IFN-α selectively inhibits exon 1A expression. It would be detrimental to an antiviral immune response if thymocyte development was inhibited by circulating type I interferons. However, by selectively inhibiting the exon 1A but not 1B transcript, IFN-α is able to protect Th1 development by suppressing mature Th2 cells without adversely affecting the basal levels of GATA3 necessary for the generation of new T cells.

The reverse situation is found in cells of the nervous system, which express GATA3 exon 1A (Asnagli et al., 2002). GATA3 promotes expression of dopamine β-hydroxylase, an enzyme that converts dopamine to norepinephrine (Hong et al., 2008). Consequently, GATA3 deficiency is embryonically lethal due to loss of norepinephrine production and a consequent failure in the development of the sympathetic nervous system (Lim et al., 2000). GATA3 expression is also required for survival of sympathetic neurons in adult mice (Tsarovina et al., 2010). This may hold immunological significance, as it has become increasingly clear that the immune system and nervous system communicate with each other. Norepinephrine has been shown to suppress the secretion of inflammatory cytokines and chemokines, as well as the expression of MHC II and NOS2, by microglial cells (Feinstein et al., 2002; Mori et al., 2002; Heneka et al., 2010). Furthermore, lymph nodes are innervated with sympathetic neurons (Giron et al., 1980; Felten et al., 1984), and norepinephrine signaling through adrenergic receptors in adaptive immune

cells is increasingly recognized as immunomodulatory (Kohm and Sanders, 2001). The β2-adrenergic receptor is selectively expressed Th1 but not Th2 cells, and treatment of Th1 and Th2 clones with the β2-adrenergic receptor agonist terbutaline suppressed IFN-γ production but had no effect on IL-4 (Sanders et al., 1997). These data suggest that IFN-α signaling in sympathetic neurons could enhance Th1 function by regulating GATA3-controlled norepinephrine production. IFN-α is reported to both increase and decrease norepinephrine production from different cells of the nervous system (Schaefer et al., 2003). However, bovine adrenal chromaffin cells, a model of sympathetic neurons, make less norepinephrine when treated with IFN-α (Tachikawa et al., 1997). In addition to the natural context of viral infections, there are also clinical contexts such as the use of IFN-β to treat the neurodegenerative disease multiple sclerosis. In such situations, IFN-α/β may plausibly suppress the expression of GATA3 in the brain with significant effects on the immune system.

Antiviral immunity and atopic diseases: reciprocal antagonism

The role of Th2 cells in allergic diseases has been discussed already, but there is increasing evidence that Th17 cells also contribute to a variety of inflammatory processes involved in autoimmunity and allergic diseases (Ciprandi et al., 2009). IFN-α/β has been demonstrated to negatively regulate Th17 development in mice (Harrington et al., 2005), and the suppression of Th17 development by IFN-α/β has recently been extended to human Th17 cells (Moschen et al., 2008; Ramgolam et al., 2009). Given the use of IFN-β clinically for the treatment of multiple sclerosis, a disease associated with increased inflammation and IL-17 levels in the CNS (Bennett and Stuve, 2009), the ability of IFN-α/β to limit Th17 cells may

explain the effectiveness of this treatment (Chen et al., 2009). Furthermore, the ability of IFN-α/β to inhibit Th2 and Th17 cells suggests that it may play a key role in controlling allergic responses.

The importance of IFN-α/β-mediated suppression of allergic T cell subsets is underscored by studies demonstrating that pDCs from asthma patients secrete less IFN-α/β than healthy donor pDCs in response to viral infections and TLR ligands (Bufe et al., 2002; Gehlhar et al., 2006; Tversky et al., 2008). Likewise, Gill *et al.* compared the induction of IFN-α by influenza virus in pDCs isolated from asthma patients or healthy subjects and found that flu infection promoted significantly less IFN-α secretion by pDCs from asthma patients (Gill et al., 2010). Thus, considering my observations that IFN-α blocks Th2 development and stability, I propose that the defect in IFN-α production in asthmatic pDCs may skew T cell priming toward Th2 development. It has been suggested that the reduction in IFN-α/β secretion during upper respiratory viral infections may lead to exacerbated lung pathology in asthmatics due to the inability of innate secretion of IFN-α/β to control viral replication in the lungs (Gehlhar et al., 2006). While this is possible, asthma exacerbation by viruses may also be attributed to the lack of counter-regulation normally provided by IFN-α/β. Given that respiratory viral infections, such as RSV, have been linked to the induction of asthma, it is possible that the inflammation accompanying these infections supports priming of bystander allergen-specific Th2 cells. Further, as asthmatics encounter recurrent infections, the lack of IFN-α secretion may allow additional Th2 priming.

While pDCs are a significant source of IFN-α/β secretion during viral infections, these cells also express relatively elevated levels of the high affinity IgE receptor (FcεRI). Although it is not clear what specific role pDCs may play in allergen-induced asthma via IgE-mediated

activation, Lui and colleagues recently demonstrated a reciprocal regulation of TLR9 and FcεRI upon receptor-ligand engagement (Schroeder et al., 2005). Here, IgE cross-linking significantly reduced TLR9 expression, resulting in decreased IFN-α production in response to CpG DNA. These results are intriguing because they suggest that sensitization with allergens may block IFN-α secretion during viral infections. Moreover, Gill *et al.* demonstrated that IgE, but not IgG, cross-linking significantly reduced IFN-α secretion from pDCs in response to both influenza A and B infection (Gill et al., 2010). Collectively, these results demonstrate that pDCs from asthma patients secrete significantly less IFN-α, and IgE cross-linking blocks IFN-α secretion even in healthy control pDCs in response to flu, suggesting both an intrinsic and extrinsic mechanism for IFN-α suppression. Thus, IFN-α/β seems to be a key focal point of reciprocal antagonism by antiviral and allergic responses. IFN-α/β also promotes IL-21 secretion (Strengell et al., 2004), which is reported to negatively regulate both IgE production and allergic rhinitis (Ozaki et al., 2002; Shang et al., 2006; Hiromura et al., 2007). These findings are supported by early studies demonstrating that IFN-α/β can suppress IgE class switching during B cell priming (Pene et al., 1988a; Finkelman et al., 1991). In summary, IFN-α/β may prove to be a potent cross-regulatory signal to block Th2/Th17 development as well as IgE production, thus underscoring its potential therapeutic use in atopic diseases. The role of IFN-α/β in modulating $CD4^+$ Th responses is summarized in Figure 5.3.

Clinical applications

One of the most significant aspects of my work is the fact that it has all been done with primary human T cells. Though murine models provide important in vivo systems, research

findings in mice often do not translate into humans due to biological differences between the two species. This is illustrated by the data presented here showing that IFN-α/β inhibits Th2 cells from humans but not mice. Recent studies by Upham and colleagues confirm the ability of type I interferons to suppress human Th2 cells (Pritchard et al., 2012a; Pritchard et al., 2012b). Importantly, though cells from asthmatics produce less IFN-α/β in response to a respiratory viruses (Wark et al., 2005), IFN-β is able to induce ISG expression and inhibit rhinovirus replication in PBMCs from asthmatics (Cakebread et al., 2011), indicating that the immune cells remain sensitive to IFN-α/β signaling despite the defect in IFN-α/β production. While these in vitro experiments certainly do not guarantee that IFN-α would inhibit human Th2 responses in vivo, they nevertheless raise the possibility that IFN-α/β could be useful as a novel therapy for allergic diseases.

Many current therapies for allergic diseases treat the symptoms of allergic inflammation rather than addressing the underlying allergen sensitivities. Nonspecific therapies include antihistamines, as well as inhaled β2-adrenergic receptor agonists and glucocorticoids. Antihistamines block receptor binding of histamine, which is only one of the many secreted inflammatory mediators secreted by mast cells and eosinophils. β2-adrenergic receptor agonists, commonly used in inhalers as well as more concentrated nebulizer formulas, relax the smooth muscle that wraps around the airways and constricts them during allergen exposure. Glucocorticoids function as general immunosuppressants, which reduce the allergic inflammation but also leave patients with increase vulnerability to infections. Furthermore, these therapies require indefinite treatment, cause adverse effects, and are ineffective for some patients.

More specific treatments include a variety of antibody-based therapies targeting IgE (omalizumab) (Busse et al., 2001), IL-4Rα (AMG 317, pitrakinra) (Wenzel et al., 2007), IL-5 (mepolizumab, rezlizumab) (Kips et al., 2003; Flood-Page et al., 2007; Corren, 2011, 2012), and IL-13 (IMA638, tralokinumab) (Bree et al., 2007; Walsh, 2010; Hodsman et al., 2012). While a few of these therapies are somewhat helpful for the treatment of asthma, many relieve only certain symptoms since they again fail to address the underlying allergen sensitivity. A more promising therapy is allergen immunotherapy, which induces tolerance to specific allergens through a series of controlled cutaneous or sublingual exposures. Unfortunately, though this therapy can be highly effective in some situations, particularly cases of allergic rhinitis, its efficacy in cases of allergic asthma is still uncertain (Incorvaia et al., 2010). Furthermore, it is available for a limited number of allergens and may be less feasible for atopic individuals sensitive to multiple allergens.

Consequently, there is potentially room for an additional therapy that could suppress the activity of the underlying allergen specific Th2 cells and B cells regardless of antigen-specificity and without global immunosuppression. IFN-α may prove to do exactly that. My research demonstrates the effectiveness of IFN-α in suppressing Th2 cells, both during development and after commitment to the Th2 lineage. Furthermore, IFN-α inhibits Th2 cells without a concomitant induction of inflammatory cytokines like IFN-γ. Whether IFN-α-treated Th2 cells remain suppressed after IFN-α is removed is not yet known. Nor is it not known if IFN-α must be administered while Th2 cells are being activated or, conversely, whether IFN-α would be effective if given in the absence of active allergic inflammation. These two aspects would be particularly relevant to practical clinical application and need to be addressed in future experiments. The persistence of the inhibition by IFN-α could be tested utilizing the redirection

experimental design shown in Figure 3.7, followed by a third week of culture under Th2 conditions. The requirement of cellular activation during IFN-α treatment could be tested by setting up the redirection experiment using two parallel sets of cells, one that is activated during IFN-α treatment and one set that remains unactivated during the treatment. One such preliminary experiment suggests that IFN-α inhibits Th2 cells even when given in the absence of TCR stimulation, but this needs to be repeated (data not shown). An additional consideration is that IFN-α treatment comes with a number of side effects (Corssmit et al., 2000), which could negate the benefit of IFN-α for patients with only mild allergies. However, for patients with severe allergies, especially in cases where patients have become resistant to other therapies, IFN-α may be a useful alternative. Delivering IFN-α via an inhaler may minimize the systemic effects, especially since IFN-α suppresses Th2 cells even when provided at doses lower than standard research concentrations (Figure 3.5). Furthermore, if the mechanism of suppression by IFN-α could be determined, it may be possible to develop a drug that mimics its suppressive activity but without the side effects. Nevertheless, for patients with asthma exacerbation caused by respiratory viral infections, IFN-α therapy might help not only the allergic inflammation but also compensate for the deficient IFN-α production in these patients. As type I interferon is already approved for clinical treatment of HCV infections, multiple sclerosis, and several cancers, its safety and side effects have already been determined. The only remaining question is the efficacy of IFN-α in vivo for treatment of allergic diseases.

An assortment of clinical data suggests that IFN-α would be effective in vivo. No clinical trial has yet been done, though we are interested in starting one. Nevertheless, several case studies have shown IFN-α to be effective at reducing the symptoms of several Th2-mediated diseases. Hypereosinophilia has been successfully treated with IFN-α (Zielinski and Lawrence,

1990; Butterfield and Gleich, 1994). IFN-α has also been used in several case studies of patients with allergic asthma, including patients resistant to corticosteroids, and found to be effective (Gratzl et al., 2000; Simon et al., 2003; Kroegel et al., 2006; Kroegel et al., 2009). IFN-α has also been used successfully for treatment of eosinophilia and asthma associated with Churg-Strauss syndrome (Tatsis et al., 1998; Metzler et al., 2010). Finally, patients treated with IFN-α for chronic HCV infections have reported improvement in their eosinophilia and asthma (Kanazawa et al., 2003; Yamamoto et al., 2005). Thus, IFN-α may prove to be useful as a novel therapy for allergic diseases.

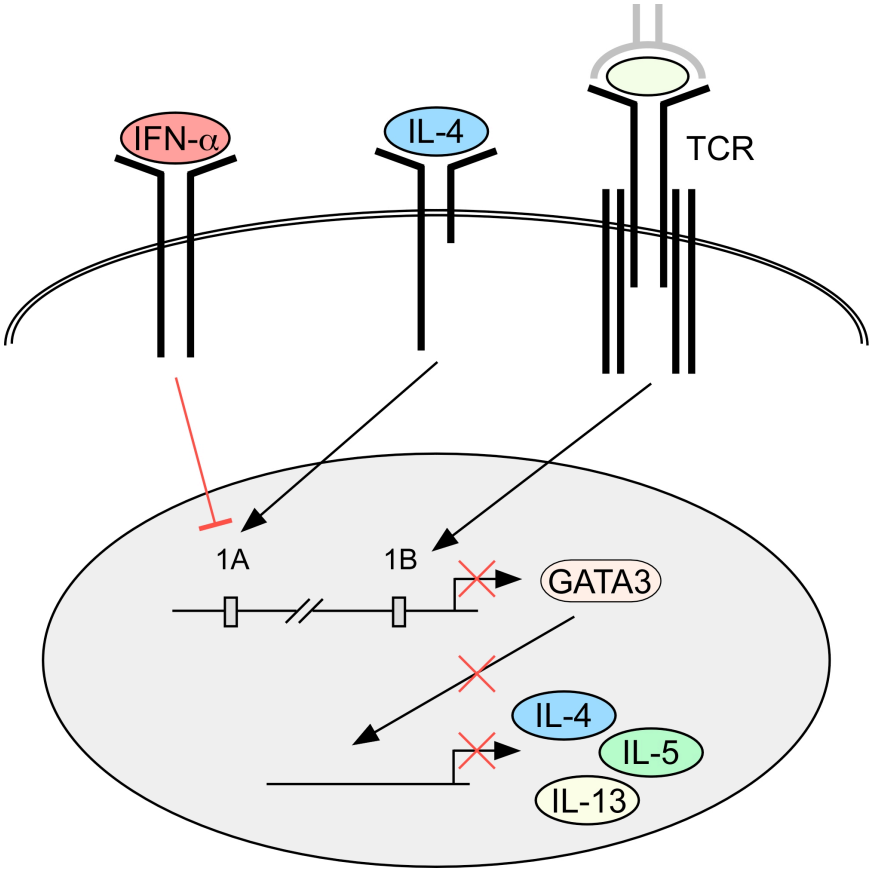

Figure 5.1. IFN-α regulates human CD4⁺ T helper 2 by suppressing expression of the Th2-specific GATA3 exon 1A transcript. GATA3 mRNA expression comprises two transcripts differing in the first exon. While TCR signaling induces expression of the exon 1B transcript, IL-4 signaling during Th2 development leads to increased expression of the exon 1A transcript. IFN-α suppresses this transcript in developing and committed Th2 cells, leading to decreased GATA3 protein levels. Consequently, GATA3 binding at the Th2 cytokine locus is reduced and expression of the Th2 cytokines is impaired.

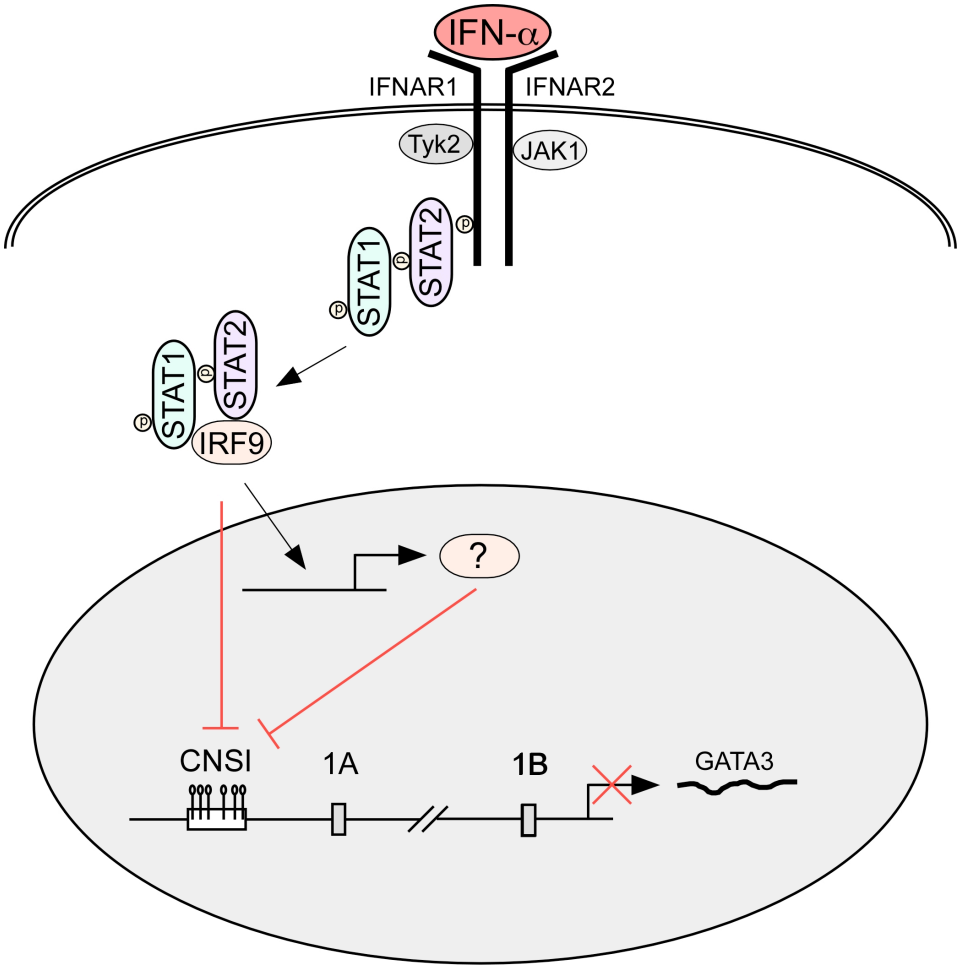

Figure 5.2. IFN-α may regulate GATA3 expression via STAT2-mediated epigenetic silencing of CNSI. Optimal GATA3 expression requires the function of cell-specific enhancers. Conserved non-coding sequence I (CNSI) upstream of GATA3 exon 1A shows increased association with histone H3 in response to treatment with IFN-α. I propose that IFN-α signaling through STAT2, induces epigenetic silencing of this region, which subsequently leads to reduced GATA3 exon 1A transcription and impaired GATA3 function in human Th2 cells.

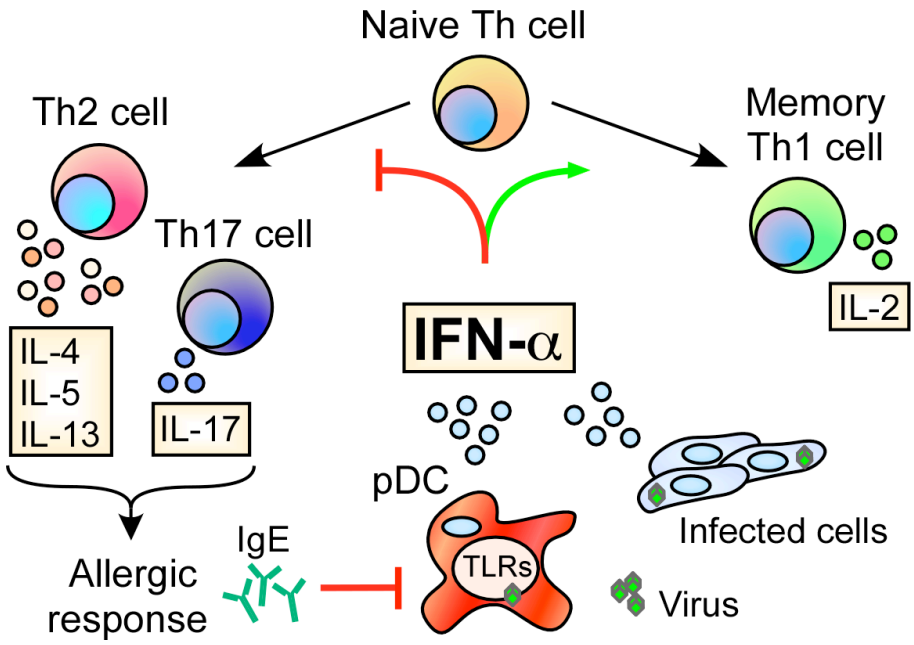

Figure 5.3. Type I interferon regulates CD4⁺ T helper cell development. IFN-α/β contributes to various functions of Th1 cells, particularly the secretion of IL-2 by memory cells. Conversely, IFN-α/β restricts the development of alternative populations such as Th2 and Th17. (This figure was previously published in Immunology, volume 132, pages 466-474 (Huber and Farrar, 2011). Copyright 2011 Blackwell Publishing Ltd.)

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