INSTABILITY OF AN EPIGENETIC MARK: T-BET AND STAT4 INFLUENCE THE SYMMETRY AND PLASTICITY OF DNA METHYLATION AT THE *IFNG* PROMOTER IN EFFECTOR AND MEMORY TH2 LYMPHOCYTES

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To Laura and my parents

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3me-H3K4 – Histone 3 trimethylated on lysine 4

Ac-H3(K9) – Histone 3 acetylated on lysine 9

AKT/PKB – Protein kinase B

APC – Antigen presenting cell

APL – Altered peptide ligand

Bcl – B cell lymphoma

BCR – B cell receptor

cAMP – Cyclic AMP

CFDA-SE – 5 (and 6)-carboxyfluorescein diacetate-succinimidyl ester

ChIP – Chromatin immunoprecipitation

CRE – Cyclic AMP response element

CREB1 – cAMP response element binding protein 1

CTL – Cytotoxic T lymphocyte

CTLA-4 – Cytotoxic T lymphocyte antigen 4

db-cAMP – Dibutyrl-cAMP

DNMT –DNA methyltransferase

FOXO1 – Forkhead box –containing O family 1

GEF –Guanine exchange factor

GFP – Green fluorescent protein

ICOS – Inducible T cell costimulator

Ifng –Interferon gamma gene

IFN-γ – Interferon gamma protein

IKK – IκB kinase

IL-12Rβ – IL-12 receptor beta subunit

IL-4 – Interleukin 4

IRES –Internal ribosomal entry site

ITAM – Immunoreceptor tyrosine-based activation motif

IκB – Inhibitor of NF-κB

JAK – Janus kinase

KO – Knockout

LCR – Locus control region

MAPK – Mitogen activated protein kinase

me – methyl

MHC – Major histocompatibility complex

NFAT – Nuclear factor of activated T cells

NF-κB – Nuclear factor kappa light polypeptide gene enhancer in B cells

NKT cells – Natural killer T cells

PAMP – Pathogen associated molecular pattern

PI3K – Phosphatidylinositide 3-kinase

PKA – Protein kinase A

PKC – protein kinase C

PLC – phospholipase C

pMHC – Peptide containing MHC complex

RAG – Recombination activating gene

SCID – Severe combined immune deficiency

SEM – Standard error of the mean

SH2 – Src homology domain 2

STAT – Signal transducer and activator of transcription

T-bet – T box expressed in T cells

TCR –T cell receptor

T_{FH} – T follicular helper

TGF-β – Transforming growth factor beta

Th – T helper

Treg – Regulatory T cells

I. INTRODUCTION

The mammalian immune system has two main components: innate and adaptive. The innate immune system is composed of cells such as neutrophils, dendritic cells, natural killer (NK) cells, and macrophages. These cells respond to broad categories of molecules such as double-stranded RNA and lipopolysaccharides via pathogen-associated molecular pattern (PAMP) receptors such as the Toll-like receptors (Medzhitov et al., 1997; Rock et al., 1998, 1998), or the absence of MHC (major histocompatibility complex) class I on host cells (Santoli et al., 1978). The adaptive component of the immune system allows for pathogen-specific responses based on the recognition of a specific molecule (Diener and Paetkau, 1972). Some infectious challenges can be resolved by the innate system before the antigen level reaches a threshold that leads to the activation of the adaptive immune system.

When the adaptive immune system is activated signals from the innate immune system are integrated by the cells of the adaptive immune system and are used to inform the development of an immune response tailored to the specific threat. The adaptive immune system aids in pathogen clearance directly by mechanisms such as antibody production by B cells (Bjorneboe et al., 1947; Fagraeus, 1947; Harris et al., 1945) and the recognition and destruction of virus-infected cells by cytolytic ($CD8^+$) T cells (Berke and Amos, 1973; Lindahl and Wilson, 1977; Lohmann-Matthes and Fischer, 1973). Indirectly, cytokines produced by cells of the adaptive immune system serve to direct the responses of cells both in the adaptive and innate compartments of the immune system (Abbas et al., 1996; Dennert, 1974; Snapper and Paul, 1987).

Cells of the adaptive immune system detect their cognate antigen via an antigen receptor complex. These receptors include one or more constant subunits along with a heterodimer of variable chains, consisting of a heavy chain and light chain in the B cell

receptor (BCR) (Nemazee, 2000; Schatz et al., 1992). In T cells the most often occurring subunits are the alpha and beta chains, although there is a proportionally smaller population defined by having a T cell receptor (TCR) composed of a gamma chain and a delta chain (Bluestone and Matis, 1989; Chien et al., 1987; Davis and Bjorkman, 1988). These receptor components are encoded by genes consisting of multiple gene segments that must be recombined to form a functioning receptor subunit (Alt et al., 1984, 1986; Schatz et al., 1992). This rearrangement is primarily carried out by the enzymes RAG1 and RAG2 along with multiple ligases and DNA repair enzymes (Schatz et al., 1989). Humans and mice deficient in enzymes required for receptor rearrangement cannot form B or T cells and are severely immune-compromised (Schuler et al., 1986), demonstrating the centrality of the antigen receptor to the adaptive immune response. The joining of gene fragments to make a functional protein is random but always includes one V (variable) segment, one D (diversity) segment, and one J (joining) subunit (Alt et al., 1986; Honjo et al., 1981; Schatz et al., 1992). The junctions between the segments are composed of a variable number of nucleotides from each segment (Pollok et al., 1984; Schatz et al., 1992). This variability increases the number of unique receptors that can be generated, but also creates the potential to create non-functioning genes by creating shifts in the reading frame (Pollok et al., 1984; Schatz et al., 1992). The combinations of the V, D, and J transcriptional cassettes, along with their quasi-random joining allows for the generation of an almost infinite number of unique antigen receptors. This means that a given pathogen, introducing multiple antigens, will only activate a fraction of the antigen receptors in the host repertoire. This diversity of receptor specificity gives rise to the adaptive immune system's ability to treat one antigen differently from another, and thus be adaptive.

T cell ontogeny: Thymic development

T cells are a leukocyte population that matures from hematopoietic stem cells in the thymus. Unlike the B cell receptor, the T cell receptor can only recognize its cognate antigen in the context of an MHC. Due to the random generation of TCRα and TCRβ chains, it is likely that many generated TCRs will not be able to interact with or 'recognize' host MHC molecules (Alt et al., 1986; Pollok et al., 1984). This random generation also entails that many TCRs generated will recognize epitopes belonging to the host in the context of MHCs (Alt et al., 1986; Kappler et al., 1987; Klein et al., 2009). The development of T cells in the thymus, termed thymic education, serves to minimize the impact of non-reactive and auto-reactive clones.

Immature cells begin as $CD4^-$, $CD8^-$ thymocytes lacking a T cell receptor (Ikuta et al., 1992; Shortman and Wu, 1996). During the course of development these double-negative thymocytes rearrange the TCRβ gene to form a functioning beta chain of the T cell receptor. A functioning TCRβ molecule will form a complex with CD3 molecules, the invariant TCRζ chain, and an invariant pre-TCRα chain. Signaling through this pre-T cell receptor leads to clonal expansion, expression of the co-receptors CD4 and CD8, and initiation of TCRα rearrangement (Starr et al., 2003). Double-positive ($CD4^+$,$CD8^+$) thymocyte survival is dependent first on assembling a functional TCRα chain that will form an MHC-restricted dimer with the expressed TCRβ chain. The TCRα rearrangement is started with the J fragments at the 5' end of the locus and proceeds to the 3' end. This allows multiple recombinations of the TCRα gene to occur, with the rearrangements only ceasing after the successful generation of an MHC-recognizing TCR or upon cell death. The proliferation after pre-TCR signaling along with the multiple attempts at rearranging the TCRα gene allowes each cell to maximize the possibility of a successful TCRβ rearrangement leading to the generation of at least one functional TCR (Petrie et al., 1993).

The generation of a functioning TCR is required for T cell survival, but it is not sufficient. Further developmental checkpoints must be passed before thymic education is complete. Signaling from a functional TCRα/β dimer-containing receptor interacting with an MHC-peptide (pMHC) complex protects against apoptosis in a process termed positive selection (Borgulya et al., 1992; Jameson et al., 1995; Starr et al., 2003). Cells failing to achieve positive selection eventually undergo death by neglect. Cells that have undergone positive selection repress the expression of RAG1/2 (Brändle et al., 1992), and restrict themselves to either CD4 or CD8 expression, depending on which class of MHC the TCR recognized (Hogquist et al., 1993; Jameson et al., 1995; MacDonald et al., 1988; Starr et al., 2003). This cessation of recombination means that the vast majority of T cells generated will express only one TCR, and thus have a single specificity. The single antigen-specificity of each TCR has important ramifications on the development of prophylactic immune responses that will be discussed later in this work. While the ability to recognize an MHC is required for survival and further development, more screening must be carried out in the thymus.

The random generation of TCRs means that some TCRs will react strongly to self-antigens in the context of MHCs. In order to prevent auto-immunity, T cells are also screened for reactivity for self-antigens. Cells with TCRs that bind with high affinity to self-pMHC complexes are either deleted from the repertoire in a process termed negative selection (Guidos et al., 1990; Kappler et al., 1987; Sha et al., 1988) or are induced into becoming natural T regulatory cells (Kawahata et al., 2002; Liston et al., 2008). The deletion of auto-reactive clones is accomplished by an active process involving the induction of apoptotic pathways (Guidos et al., 1990; Schönrich et al., 1993; Sha et al., 1988). Both positive and negative selection require an interaction between the antigen receptor and self-antigens in the context of MHC. As this became evident, it was hypothesized that the difference between TCR signaling in positive selection and

negative selection was due to the affinity of a TCR for a pMHC expressed in the thymus (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). Alternatively, it was hypothesized that peptides displayed within the thymus were different than those encountered elsewhere (Moran and Hogquist, 2012), although further research found that this was not likely (Hogquist et al., 1997; Lo et al., 2009; Marrack et al., 1993; Moran and Hogquist, 2012). One powerful tool used to test the affinity hypothesis was the use of mice expressing transgenic TCRs.

Thymic development: Insights from transgenic TCR models

The number of different TCRs being generated and tested in the thymus raises significant problems in investigating how a TCR's interaction with self-antigen-MHC complexes regulates its survival and development. To circumvent this, successfully recombined Tcra and Tcrb genes were inserted into a host genome. This allows for the majority of T cells to express the same TCR. These transgenic TCR models have been used to investigate the role of TCR signaling in T cell development (von Boehmer, 1990; Jameson et al., 1995; Starr et al., 2003). Transgenic systems have been used to investigate the effects of antigen concentration and affinity on thymic development. Commonly, the transgenic TCR's antigen and MHC specificity is known, allowing for the stimulation of the receptor by its cognate antigen. The use of peptide-specific TCR transgenic mice has been a powerful tool in understanding TCR signaling in positive and negative selection.

Studies using an MHC class I-restricted transgenic TCR provided key initial insights into role of concentration in thymic selection. The addition of a low concentration of cognate antigen to thymocytes from a TCR transgenic mouse was found to induce positive selection, whereas a higher concentration induced negative selection

(Ashton-Rickardt et al., 1994; Sebzda et al., 1994). Furthermore, changing residues of the antigen peptide that contact the TCR was found to alter the response of thymocytes (Evavold et al., 1993; Nicholson et al., 1995). Interestingly, it was also found that some agonist could promote positive selection across a range of concentrations, but were unable to trigger negative selection (Kraj et al., 2001; Sebzda et al., 1996). Taken together, these results suggested that affinity of a ligand for a TCR, as well as the concentration, was important for determining the response of thymocytes to antigen.

The known antigen specificity of transgenic TCRs allowed for investigation into the effects of a TCR's affinity for its cognate pMHC specifically via the use of altered peptide ligands (APL). The alteration of one or more residues of a TCR's cognate peptide to increase or decrease its binding affinity for the TCR has been shown to produce profound effects on the development of transgenic TCR-expressing T cells (Hsu et al., 1995; Sebzda et al., 1994). The effect of these altered peptide ligands depends on several variables such as the location of expression within the thymus, the concentration of ligand used (Alam et al., 1996; Sebzda et al., 1994), and which population of thymic cells presents the antigen (Aschenbrenner et al., 2007), but a few key principles can be simply articulated. Introduction of a high-affinity peptide into the thymic environment leads to an increase in negative selection of TCR transgenic cells in many cases, but exceptions have been reported (Ashton-Rickardt et al., 1994; Kraj et al., 2001; Moran and Hogquist, 2012; Spain et al., 1994). Conversely, low affinity 'antagonist' peptides has been demonstrated to promote the positive selection of thymocytes (Alam et al., 1996; Hogquist et al., 1994, 1997; Hu et al., 1997). The interplay between antigen density and antigen affinity also has significant impact, as a low affinity antigen may be positively selecting at high concentrations, but not at lower concentrations (Liu et al., 1998a).

Further work showed that the time a thymocyte spent in contact with a thymic antigen presenting cell (APC) had a strong predictive value as to whether it would

undergo positive or negative selection (Williams et al., 1999). The duration of APC-T cell interaction also was found to be a determining factor in whether a presented antigen induced activation or tolerance (Katzman et al., 2010) Combined with the observations about the effects of ligand concentration and affinity, this suggest a model in which the strength of and number of pMHC-TCR interactions determines binding time, and therefore cell survival and function upon antigen recognition in the periphery. . Interestingly, recent work has demonstrated that the signaling strength of TCRs after exposure to self-pHMC during thymic education correlates with its strength of signaling upon exposure to its cognate exogenous antigen (Mandl et al., 2013). This suggests that positive selection can also serve to promote the survival of cells which will respond most vigorously to pathogens (Mandl et al., 2013).

To summarize: A TCR bound with high affinity to a pMHC signals through pathways that are not engaged by the lower affinity interactions leading to positive selection (Morris and Allen, 2012). T cell survival is dictated by the ability of a TCR to react to MHC/self-peptide complexes at a much lower signaling intensity than is seen in T cells responding to their cognate exogenous antigen (Ashton-Rickardt et al., 1994; Starr et al., 2003). To pass through thymic education with the potential to become an effector T cell, a TCR's affinity for self-pMHC must fall within certain tolerances.

Thymic development: MHC specificity and coreceptor selection

As previously discussed, a key requirement of thymic education is the recognition of self-peptides in the context of an MHC molecule. There are two predominant types of MHC, MHC class I and MHC class II (Benacerraf, 1988; Braciale et al., 1987). Each MHC type samples peptides derived from different cellular processes although there are mechanisms that allow for cross-presentation (Joffre et al., 2012; Rock et al., 2010).

MHC class I molecules are expressed on most cell types and primarily present peptides derived from proteins created by the MHC class I-bearing cell (Apcher et al., 2012; Procko and Gaudet, 2009; Vigneron and Van den Eynde, 2012). Production of proteins due to viral infection or mutation will create non-self-antigens detectable by T cells. MHC class II molecules are displayed on antigen presenting cells of the immune system such as macrophages, dendritic cells, and B cells. These molecules typically display peptides derived from proteins taken up from the local environment, such as those found on a phagoctyosed bacterium (Goldszmid and Sher, 2010; Ramachandra et al., 2009). Further studies would determine that a TCR's specificity goes beyond the class of MHC it recognizes.

The use of a transgenic TCR with a known MHC requirement allowed for the investigation of the role of MHC expression in T cell development(von Boehmer, 1990). One key experiment involved placing thymocytes expressing a TCR reactive to antigen in the context of one MHC II allele into cultures of cell expressing other MHC class II alleles. This experiment demonstrated that recognition of the host MHC by a TCR was required for positive selection and development past the $CD4^+$, $CD8^+$ double-positive stage (Berg et al., 1990). Furthermore, the use of an MHC class I restricted transgenic TCR in CD8-deficient animals was found to lead to deficiencies in T cell development (von Boehmer, 1990; Jameson et al., 1995; Killeen et al., 1992; Schönrich et al., 1993).

MHC molecules are recognized by the coreceptors CD4 and CD8. As discussed later, these surface molecules play an important role in the initiation of TCR signaling. The expression of CD4 or CD8 is on a T cell's surface is determined by the class of MHC recognized by its TCR. Double positive thyomcytes that recognize peptide in the context of MHC class I develop into $CD8^+$ single positive T cells. These cells, also known as CTLs (cytotoxic T lymphocytes), primarily serve to induce cell death in cells displaying non-self antigen on their surface MHC class I (Hogquist et al., 1993). By

surveying peptides produced within a given cell CTLs provide protection against viruses (Long and Jacobson, 1989; Morrison et al., 1986; Zinkernagel and Doherty, 1979) and also form an important part of the immune system's response to cancer (Garcia-Lora et al., 2003; Seliger et al., 2006).

Thymocytes recognizing peptide in the context of MHC class II molecules develop into $CD4^+$ single positive cells (Klein et al., 2009; Kraj et al., 2001; MacDonald et al., 1988; Starr et al., 2003). $CD4^+$ T cells (T helper cells) are integral to the ability of the adaptive immune system to deploy pathogen-appropriate responses. T helper cells serve to process signals present at the time of their activation and respond by initiating one of several programs of cytokine production that serves to drive the immune system's response to a given pathogen type (Abbas et al., 1996). At the completion of thymic education, a T cell is an MHC class-restricted naïve cell capable of responding to its cognate antigen.

NKT cells: Lipid reactive T cells

While most T cells react only with peptide antigens presented by MHC molecules, an important exception does exist. A small subset of thymus-derived lymphocytes recognize lipid antigens in the context of the CD1d molecule in mice, or any of the CD1 family in humans (Brossay et al., 1998; Kawano et al., 1998; Tsuji, 2006). These cells are termed natural killer T cells (NKT cells) due to the presence of NK cell surface markers alongside a TCR in many subsets. The largest NKT cell subset is characterized by a semi-invariant αβ TCR and is termed iNKT (invariant NKT) cells. Other populations of NKT cells have diverse αβ or γδ TCRs, but all are CD1-restricted (Godfrey et al., 2004; Van Kaer, 2007; Van Kaer and Joyce, 2005). As with αβ T cells, NKT cells undergo positive and negative selection (D'Cruz et al., 2010; Hu et al., 2011).

NKT cells can be activated by bacterial-derived glycolipids and are important early responders to many pathogens such as *Mycobacterium tuberculosis*, various species of *Leishmania*, and other pathogenic microorganisms (Brigl et al., 2003; Cohen et al., 2009; Ishikawa et al., 2000; Moody et al., 2004; Wiethe et al., 2008). Activation of NKT cells via exogenous lipids such as α-GalCer can modulate immune responses to tumors (Kawano et al., 1997, 1998; Motohashi et al., 2011; Vivier et al., 2012) or serve to enhance vaccines (Cerundolo et al., 2009; Joyce et al., 2011; Padte et al., 2011). While NKT cell cells are a relatively small population of lymphocytes (Van Kaer, 2007), they occupy an important niche between innate and adaptive immunity and can provide an early release of cytokines that contributes to shaping a developing immune response (Ishikawa et al., 2000; Stanley et al., 2008).

T helper subsets: General

In order to respond to different types of pathogens, $CD4^+$ T cells are able to differentiate into several different effector types after being activated through their TCR. Each effector type (also called lineage or subset) produces a set of hallmark cytokines, while inhibiting the transcription of genes associated with other effector sets (Grogan et al., 2001). Each subset also expresses different patterns of chemokine receptors (Breitfeld et al., 2000; Campbell et al., 2003; Kim, 2005; Schaerli et al., 2000), meaning that different subsets will migrate to a site in response to different stimuli. The effector programming that a T helper cell adopts is, in large part, determined by the local cytokine milieu at the time of activation. Cytokine signaling drives the expression of lineage-specific transcription factors sometimes referred to as master regulators (Kanhere et al., 2012; Nakayama and Yamashita, 2008; Vahedi et al., 2013). The combined signals from the TCR and cytokine receptors, along with the activity of the master regulators, drive the lineage-specific cytokine expression which characterizes the T helper subsets (Corn et al.,

2005; Kaplan et al., 1996a; Nakayama and Yamashita, 2010; Rooney et al., 1994; Zhang and Boothby, 2006; Zhu and Paul, 2010).

The two best studied effector programs are the T helper 1 (Th1) and T helper 2 (Th2) responses (Mosmann et al., 1986) (Fig. 1.1). Th1 cells express the master regulator T-bet and produce IFN-γ. IFN-γ is a type II interferon which activates macrophages to promote the clearance of intracellular pathogens such as *Listeria monocytogenes* (Hsieh et al., 1993) and *Mycobacterium tuberculosis* (Barnes et al., 1993; Cooper et al., 1993; Orme et al., 1993). IFN-γ also triggers B cells to class switch to the IgG2a isotype. The constant regions of IgG2a antibodies interact with receptors on phagocytes leading to increased uptake of antigens bound to the variable region of the antibody (Bolland, 2005; Cohen-Solal et al., 2004; Kimberly et al., 1989; Nimmerjahn et al., 2005). This antibody-mediated phagocytosis mechanism is termed opsonization (Mudd et al., 1929; Noguchi, 1907), and further enhances the effectiveness of phagocytes in a Th1 response (Cohen et al., 2011; Oishi et al., 2013; Schlageter and Kozel, 1990). By inducing the production of IgG2a and activating macrophages, the IFN-γ produced by Th1 effectors guides the immune response of cells from both the innate and adaptive arms of the immune to clear pathogens.

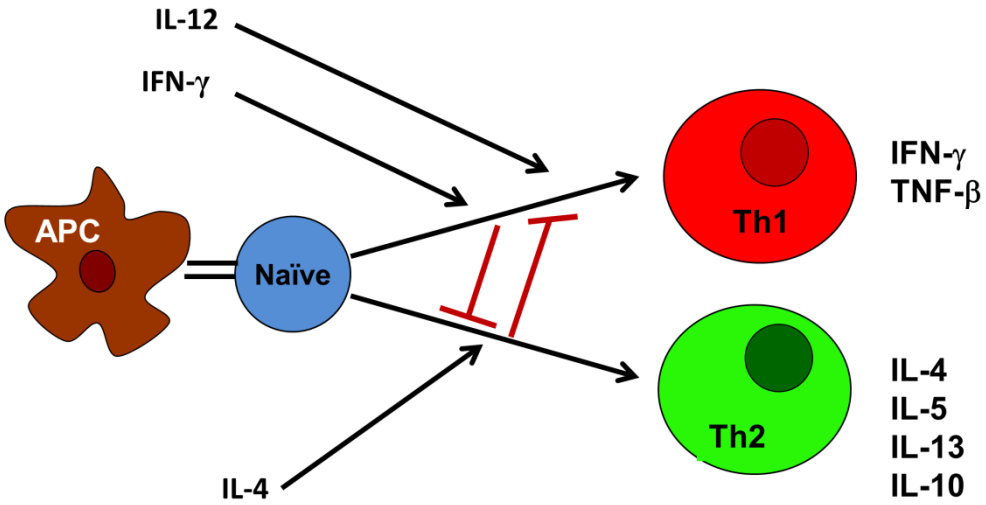


Figure 1.1. Th1 and Th2 development following antigen stimulation. After activation via the T cell receptor, cytokines in the local environment steer commitment to one of several mutually exclusive effector lineages. Shown above are two best studied effector programs: Th1 and Th2.

The importance of the Th1 response and IFN-γ in human health can be seen in patients lacking a functional IFN-γ receptor. These patients are highly susceptible to mycobacterial infections and other intracellular bacteria (Arend et al., 2001; Dorman et al., 2004; Jouanguy et al., 1996; Pierre-Audigier et al., 1997; Roesler et al., 1999; Vinh et al., 2009). Th1 effectors have also been found to play a major role in the pathology of autoimmune diseases such as type I diabetes (Haskins and Cooke, 2011; Katz et al., 1995; Öling et al., 2012; Trembleau et al., 1995). The Th1 subset is therefore a very important but potentially very destructive lineage.

The Th2 effector program (Fig. 1.1) is characterized by expression of the master regulator GATA3 (Pai et al., 2004; Ranganath et al., 1998; Zhang et al., 1997a; Zheng and Flavell, 1997) and production of IL-3, IL-4, and IL-13 (Abbas et al., 1996; Heinzel, 1989). Th2 effectors direct immune responses against helminthic parasites (Finkelman et al., 2004; Panzer et al., 2012; Svetic et al., 1993). The humoral aspect of a Th2 response is characterized by the class switching of B cells to IgG1 and IgE (Liu et al., 2003; Matsumoto et al., 2013; Snapper and Paul, 1987), while the cell mediated effects stem from the recruitment of eosinophils and mast cells to the site of inflammation (Hagan et al., 1985; Hepworth et al., 2012; Masure et al., 2013; Shintoku et al., 2013). IL-4 produced by Th2s also provides anti-apoptotic and proliferative signals to B cells (Howard and Paul, 1983; Liao et al., 2011a; Swain and Dutton, 1985), and many other cell types (Crosby and Waters, 2010; Hallett et al., 2012). The IgE produced under Th2 conditions binds and cross-links Fc receptors on the surface of mast cells, which triggers release of histamines and cytokines (Ishizaka et al., 1972; König and Ishizaka, 1974; König et al., 1974). Protection from helminthes has been important throughout evolutionary history of mammals (Ilic et al., 2012; Maizels et al., 2009; Pillai and Bix, 2011; Pulendran and Artis, 2012), but in the developed world, the Th2 response is most

commonly seen in atopic diseases such as allergic asthma (Holgate, 2012; Romagnani, 1994; Wambre et al., 2012).

Beyond Th1 and Th2 effector programs, several other $CD4^+$ T cell subsets have been identified. As with Th1 and Th2 lineages, each of the other T helper types regulates distinct processes in the immune system. The Th17 subset produces IL-17, which serves to recruit neutrophils to clear extracellular bacterial and fungal infections (Drewniak et al., 2013; Hernández-Santos et al., 2012; Higgins et al., 2006; Puel et al., 2011). Development from a naïve $CD4^+$ T cell to a Th17 effector is mediated by TGF-β and IL-6 signaling and the master regulator RORγt (Harrington et al., 2006; Ivanov et al., 2006; Weaver et al., 2006). Th17 polarization is opposed by signaling from IL-4, IL-12, IL-2, and IFN-γ (Zhu and Paul, 2010). The Th17 effector program also has an etiologic role in the autoimmune diseases multiple sclerosis (Maddur et al., 2012; Saresella et al., 2013; Wang et al., 2013), psoriasis (Cauli and Mathieu, 2012; Chiu et al., 2012), and rheumatoid arthritis (Komatsu and Takayanagi, 2012; Maddur et al., 2012; Miossec and Kolls, 2012). Th17 cells demonstrate some flexibility in cytokine expression, as fully differentiated cells can be made to produce IFN-γ under physiological conditions while maintaining production of IL-17 (Basu et al., 2013; Lexberg et al., 2010; Mukasa et al., 2010).

The T follicular helper subset (T_{FH}) secretes IL-21(Schaerli et al., 2000) and specializes in the formation and maintenance of germinal centers (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000). Development along the T_{FH} program in humans requires IL-12 signaling (Schmitt et al., 2009), the expression of Bcl6 and the repression of Blimp-1 (Johnston et al., 2009; Yu et al., 2009). In mice, Bcl6 expression, along with IL-21, IL-6, and STAT3 signaling drive T_{FH} development (Nurieva et al., 2008). In both humans and mice the inducible costimulator ICOS is required for effective T_{FH} polarization (Bauquet et al., 2008; Breitfeld et al., 2000). T_{FH} effectors can express the

master regulators and cytokines associated with one of the other effector subsets: i.e. in a Th1 dominated response, T_{FH} cells can express T-bet and produce *Ifng* (Crotty, 2011; Johnston et al., 2009), whereas in a Th2 response they would express GATA3 and produce Th2 cytokines (King and Mohrs, 2009; Reinhardt et al., 2009; Yusuf et al., 2010). While T_{FH} cells play an important role in generating effective humoral responses against antigens, they are also implicated in autoimmune disorders, especially in those characterized by autoreactive antibodies such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (King et al., 2008; Vinuesa et al., 2005).

Functionally distinct from the T helper lineages are the $CD4^+$ regulatory T cells (Tregs). Tregs are divided into two categories based on how the regulatory function was acquired. Tregs which develop from the recognition of self-antigens in the thymus are termed thymic Tregs (tTreg) or natural Tregs (nTregs) (Aschenbrenner et al., 2007; Kawahata et al., 2002; Olivares-Villagómez et al., 1998). T cells acquiring regulatory function after exiting the thymus are termed induced Tregs (iTregs) (Apostolou et al., 2002; Josefowicz et al., 2012). Regulatory T cells have the ability to suppress the effector responses of other T cells, both by the secretion of IL-10 and TGF-β, and by contact dependent inhibitory mechanisms (Kumar et al., 1997). The transcription factor Foxp3 is the master regulator of the Treg program (Fontenot et al., 2003), and in cases where humans or mice lack functional Foxp3, widespread autoimmunity develops (Bennett et al., 2001; Josefowicz et al., 2012; Wildin et al., 2001). The ability to generate and maintain a functional population of regulatory T cells has repeatedly been shown to be vital for the prevention of autoimmunity.

T cell activation: TCR signaling

$CD4^+$ T cells exit the thymus capable of adopting any one of several effector or regulatory functions. In this naïve state, the cells do not produce cytokines or provide help to other cells of the immune system. For a helper T cell to transition from naïve to an effector, it must experience its cognate antigen in the context of an antigen presenting cell's expressed MHC class II. TCR signaling regulates proliferation, survival, and the effector function of a T cell (Lanzavecchia et al., 1999). To carry this out, the receptor complex is able to invoke multiple signaling pathways and exert control over a host of genes.

The α and β chains of the T cell receptor lack signaling components, and instead rely on other components of the receptor complex for signal transduction. The signaling components of the TCR are the invariant CD3 proteins and the TCRζ chain (sometimes called CD3ζ). The CD3 complex is composed of multiple subunits: CD3δ, CD3ε, and CD3γ. These three subunits form into two heterodimers, and, together with a TCRζ homodimer form the signaling component of the TCR (Guy and Vignali, 2009; Shores and Love, 1997; Zehn et al., 2012). Each invariant chain of the TCR contains one or more ITAM (immunoreceptor tyrosine-based activation motif) domains. The ITAMs become phosphorylated during the course of TCR stimulation, and this phosphorylation allows for the recruitment and activation of multiple downstream effector proteins and adaptors during TCR stimulation (Guy and Vignali, 2009; Humphrey et al., 2005; Salmond et al., 2009; Au-Yeung et al., 2009; Zhang et al., 1998b).

After the αβ dimer binds to its cognate antigen in the context of MHC, either the CD4 or the CD8 coreceptor binds to the MHC molecule, drawing its cytoplasmic domain into closer proximity to ITAMS of the CD3 and TCRζ complex (Leitenberg et al., 1998; Veillette et al., 1988, 1989). Associated with the cytoplasmic tail of the coreceptor (CD4

or CD8) is the Src family kinase Lck. Together with the Src family kinase Fyn, Lck then phosphorylates the ITAMs of the CD3 and TCRζ dimers (Guy and Vignali, 2009; Palacios and Weiss, 2004). The phosphorylation of TCRζ allows for the recruitment and phosphorylation of the Syk-family tyrosine kinase ZAP-70 (Salmond et al., 2009; Au-Yeung et al., 2009). Phosphorylated ZAP-70 interacts with phospholipase Cγ1 (PLCγ1), which is activated by the Tec kinase Itk (Berg et al., 2005; Liu et al., 1998b; Park et al., 1991; Reynolds et al., 2002; Shan and Wange, 1999; Smith-Garvin et al., 2009). PLCγ1 cleaves the membrane phospholipid $PI(4,5)P_2$ to produce inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) (Choi et al., 2007; Patterson et al., 2005). Each of these molecules activates a separate set of signaling pathways.

IP_3 binds to receptors on the endoplasmic reticulum which triggers a release of calcium into the cytoplasm (Berridge, 2009; Berridge and Patel, 1968; Prentki et al., 1985; Streb et al., 1983, 1984; Williamson et al., 1985). This Ca^{++} ion flux leads to the activation of the phosphatase calcineurin, which dephosphorylates members of the NFAT (nuclear factor of activated T cells) family of transcription factors (Loh et al., 1996; Luo et al., 1996; Park et al., 1995). Once dephosphorylated, these transcription factors migrate to the nucleus and transactivate multiple target genes (Hogan et al., 2003; Kiani et al., 2001; Macian, 2005; Rooney et al., 1994). NFAT signaling is required for the function of several T helper subsets (Agarwal et al., 2000; Kiani et al., 2001; Porter and Clipstone, 2002; Rooney et al., 1994), and is required for the proliferation of activated effector $CD4^+$ T cells and the production of IL-2 (Jain et al., 1993; McCaffrey et al., 1993; Novak et al., 1990; Shaw et al., 1988).

The DAG produced by PLCγ1 binds to and activates a member of the protein kinase C (PKC) family of kinases, PKCθ (Altman and Villalba, 2002, 2003; Baier et al., 1993). The activation of PKCθ leads to the activation of members of the NF-κB family of transcription factors (Altman and Villalba, 2002; Coudronniere et al., 2000). The

NF-κB family of transcription factors is involved in the regulation of many genes in lymphocytes and can be activated by multiple receptors including the TCR (Baldwin, 2012; Karin and Ben-Neriah, 2000; Oh and Ghosh, 2013; Ruan and Chen, 2012; Vallabhapurapu and Karin, 2009; Visekruna et al., 2012). These transcription factors are characterized by an N-terminal Rel homology domain (RHD) required for dimerization and site-specific DNA binding, and each dimer has either a transactivating or transrepressing domain at the C- terminus (Hoffmann et al., 2006; Vallabhapurapu and Karin, 2009). The NF-κB family members NF-κB1 (p50/p105) and NF-κB2 (p52/p100) are found in an inactive precursor state (p105 and p100) and are processed by the proteasome into their active forms (p50 and p52) (Blank et al., 1991; Fan and Maniatis, 1991; Mercurio et al., 1992; Neri et al., 1991; Sen and Baltimore, 1986). A second set of NF-κB transcription factors is composed of RelA (p65) (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1991), RelB (Ryseck et al., 1992), and c-Rel (Brownell et al., 1987; Kieran et al., 1990; Sica et al., 1992). NF-κB transcription factors function as either homodimers or heterodimers (Oeckinghaus and Ghosh, 2009; Rattner et al., 1991; Urban et al., 1991). These proteins are kept inactive via interactions with inhibitory factors termed IκBs (inhibitors of NF-κB), which prevent nuclear translocation of dimerized NF-κBs (Baeuerle and Baltimore, 1988; Davis et al., 1991; Ganchi et al., 1992; Inoue et al., 1992; Vallabhapurapu and Karin, 2009). During activation, IKK (IκB kinase) complexes phosphorlate IκBs, leading to the release and nuclear localization of NF-κB dimers (Rothwarf et al., 1998; Zandi et al., 1997, 1998).

In the case of signaling from the TCR, PKCθ is able to phosphorylate the CARD (caspase recruitment domain) domain protein Carma1 (Blonska and Lin, 2009, 2011; Lin and Wang, 2004; Matsumoto et al., 2005; Sommer et al., 2005; Wang et al., 2004). The phosphorylation of Carma1 allows for its binding to Bcl10 and Malt1 at the plasma membrane (Cheng et al., 2011; Hara et al., 2004; Shinohara et al., 2007; Wang et al.,

2004). This protein complex, sometimes called the CBM complex, is essential for the ubiquitination of IKKγ (also known as NEMO) (Li et al., 2001; Poyet et al., 2000; Yamaoka et al., 1998). The ubiquitination of NEMO and PKCθ-mediated phosphorylation activates the IKK complex (Blonska and Lin, 2011; Lee et al., 1998; Ling et al., 1998). Activated IKK complexes phosphorylate IκB proteins, leading to their disassociation from NF-κB dimers, allowing the NF-κB dimers to translocate to the nucleus and regulate the transcription of multiple genes (Ganchi et al., 1992; Zandi et al., 1998).

The TCR signals through multiple pathways, each of which controls multiple genes. Signaling through the TCR provides signals that are necessary for T cell survival, proliferation, and differentiation. Stimulation of the TCR alone will not, however allow for a functional immune response. Another set of signaling pathways must be activated to achieve T cell activation.

T cell activation: Costimulators and co-repressors

Early studies in TCR signaling found that stimulation of the TCR was insufficient to induce proliferation (Green et al., 1994; Jenkins and Schwartz, 1987; Quill and Schwartz, 1987; Smith et al., 1997). It was found that when T cells were stimulated solely via the TCR, clonal anergy or cell death would occur (Bartik et al., 1994; Boise et al., 1995; Koulova et al., 1991; Linsley et al., 1990; Watts, 2010). This led to the hypothesis that a second signal was needed to successfully activate lymphocytes (Bretscher and Cohn, 1970; Jenkins and Schwartz, 1987; Lafferty and Woolnough, 1977; Quill and Schwartz, 1987). The requirement for a second signal provides some safeguard against inappropriate immune responses such as autoimmunity as well as

modulating and enhancing signaling through the TCR (Boise et al., 1995; Chambers, 2001; Lafferty and Gill, 1993; Lenschow et al., 1996).

The second, or costimulatory, signal is typically generated by membrane bound receptors on the T cell interacting with ligands on antigen presenting cells. The membrane-bound costimulator CD28 is the main source of this second signal during T cell activation (Boise et al., 1995; June et al., 1987; Lesslauer et al., 1986). CD28 is a homo-dimeric Ig-superfamily member protein that binds to B7 molecules expressed on antigen presenting cells (Koulova et al., 1991; Linsley et al., 1990, 1991). Upon the interaction of CD28 with B7, the YMNM motif of the intracellular domain of CD28 is tyrosine phosphorylated via Lck and Fyn (Bjørgo and Taskén, 2010; Lenschow et al., 1996; Raab et al., 1995; Rudd et al., 2009; Salmond et al., 2009). PI3K and Grb2 then bind to CD28 via SH3 domain interactions (Okkenhaug and Rottapel, 1998).

PI3K produces $PIP_{3,}$ which binds to PDK1 via the pleckstrin homology (PH) domain (Bayascas, 2011). PDK1 phosphorylates Akt on T308, resulting in partial activation (Fayard et al., 2011; Parry et al., 1997; Rudd et al., 2009). Signaling from CD28 via Akt leads to mammalian target of rapamycin (mTOR)-aided translation (Wu et al., 2005), activation of cyclin-dependent kinases (Appleman et al., 2000; Boonen et al., 1999; Rowell et al., 2005), and the inhibition of FAS-induced apoptotic signaling (Jones et al., 2002).

The activation induced binding of Grb2 to CD28 facilitates the recruitment of guanine exchange factors (GEFs) such as Vav1 and SOS (Son of Sevenless) (Buday et al., 1994; Gogishvili et al., 2008; Schneider and Rudd, 2008; Schneider et al., 1995) to CD28. These GEFs activate small GTPases such as Rac1 and $p21^{ras}$. These GTPases activate the JNK and MAPK kinase cascades, leading to the activation of AP-1

transcription factors and the transcription of genes such as IL-2 (Izquierdo et al., 1993; Schneider and Rudd, 2008; Schneider et al., 1995; Su et al., 1994).

The overall effect of CD28 signaling is to promote survival and proliferation during T cell activation (Boise et al., 1995; Linsley et al., 1991; Noel et al., 1996a; Rudd et al., 2009). This is accomplished via up-regulating the expression of Bcl-$_{XL}$ (Boise et al., 1995; Wu et al., 2005), increasing the production of IL-2 (Chen et al., 1998; Jenkins et al., 1991; June et al., 1987; Linsley et al., 1991), promoting the activity of cyclin-dependent kinases (Appleman et al., 2000; Boonen et al., 1999; Rowell et al., 2005), and the alteration of cell energetics to accommodate increased energy requirements of activation (Frauwirth et al., 2002; Jacobs et al., 2008; Marko et al., 2010). The signaling pathways engaged by CD28 synergize with those initiated by the TCR to enable a functional T cell response by promoting clonal expansion and preventing apoptosis.

A second important costimulatory molecule in the B7 receptor family is inducible co-stimulatory molecule (ICOS) (Hutloff et al., 1999; Rudd and Schneider, 2003; Yoshinaga et al., 1999). As its name suggests, ICOS is only faintly expressed in naïve T cells, but its expression is induced during TCR/CD28-mediate activation (McAdam et al., 2000; Simpson et al., 2010). ICOS is activated by ligation with B7RP-1, which is expressed on antigen presenting cells (Yoshinaga et al., 1999). Stimulation via ICOS promotes survival and proliferations, but does not induce IL-2 (Hutloff et al., 1999). ICOS signaling can promote development along several different T lineages, and is especially important in the development of T_{FH} cells, with ICOS deficiency resulting in impaired germinal center formation and an absence of T_{FH} memory cells (Crotty, 2011; Simpson et al., 2010). Signaling through ICOS provides pro-survival and proliferative signals through pathways downstream of AKT (Rudd and Schneider, 2003; Simpson et al., 2010) and also modulates the effector responses of multiple T lineages by increasing cytokine secretion or providing tolerogenic signals (Bauquet et al., 2008; Gao et al.,

2012; Simpson et al., 2010). By of regulating and increasing T helper responses, promoting cell survival, and promoting the T_{FH} lineage, ICOS serves as a key co-stimulatory molecule in $CD4^+$ T cell activation and development.

In the same family as ICOS and CD28 but possessing significantly different functionality is cytotoxic T lymphocyte antigen 4 (CTLA-4). Like ICOS, CTLA-4 is upregulated after T cell activation (Alegre et al., 1996; Freeman et al., 1992; Sansom, 2000). However, CTLA-4 signaling is antagonistic towards signals from TCR/CD3 and CD28 (Noel et al., 1996b; Walunas et al., 1994, 1996). While the exact mechanisms of signal-dampening by CTLA-4 are unclear, and several mechanisms have been proposed (Bour-Jordan et al., 2011; Noel et al., 1996b; Rudd and Schneider, 2003; Rudd et al., 2009). There is evidence to support competition for PI3K, dephosphorylation of CD3 and CD28 complexes via PP2a, and disruption of cytoskeletal architecture at the immune synapse as likely mechanisms of CTLA-4 function (Bour-Jordan et al., 2011; Greenwald et al., 2002; Rudd and Schneider, 2003; Rudd et al., 2009; Scalapino and Daikh, 2008).

While the mechanisms underpinning CTLA-4's function remain unclear, the importance of its function has been demonstrated in a knockout mouse model. Mice deficient in CTLA-4 suffer from severe lymphoproliferative disorders as well as autoimmunity and fall fatally ill at a young age (Krummel and Allison, 1995; Tivol et al., 1995; Waterhouse et al., 1995). CTLA-4 plays a crucial role in tolerance and immune regulation by raising the signaling threshold of the TCR complex and CD28. Without this dampening effect, lower levels of TCR/CD28 stimulation would be sufficient to drive strong immune responses.

Differentiation of naïve $CD4^+$ cells into effector subsets: Cytokine signaling

While the T cell receptor complex and costimulators are required for activation, a substantial portion of the signaling input that controls differentiation and survival after stimulation comes from cytokines. Cytokines bind to membrane-bound receptors on their target cell. The cytokine receptor is then able to initiate signaling through multiple pathways, and can influence proliferation (Liao et al., 2011a; Yoo et al., 2002), survival, and acquisition of effector function (Bradley et al., 1995; Hsieh et al., 1993; Liao et al., 2011b; Manetti et al., 1993). Cytokine receptors are dimeric membrane spanning proteins that are expressed in many immune and non-immune cell types.

One pathway common among many cytokine receptors is the JAK-STAT pathway (Shuai and Liu, 2003). The cytoplasmic tails of cytokine receptors are associated with tyrosine kinases called Janus kinases (JAKs) (Murray, 2007; Watling et al., 1993; Wilks et al., 1991). Upon receptor-ligand interaction, JAKs phosphorylate tyrosines on the cytoplasmic domains of the receptor subunits(Beadling et al., 1994; Silva et al., 1994; Silvennoinen et al., 1993). These phosphotyrosines allow STAT molecules to bind to the receptor via SH2 domains and undergo tyrosine phosphorylation (Jacobson et al., 1995; Shuai et al., 1993; Silva et al., 1994). This phosphorylation allows for STAT molecules to form homo- and hetero-dimers, translocate to the nucleus, bind specific DNA elements, and facilitate transcription of target genes (Jacobson et al., 1995; Li et al., 1996; Murray, 2007; Shuai et al., 1994). While the activation of STAT proteins is an important function, JAKs are also involved in other signal transduction pathways such as the PI3K/AKT pathway (Sharfe et al., 1995). The importance of JAK3 in particular can be seen in humans or mice lacking a functional gene produce. The absence of functional JAK3 results in severe combined immune deficiency (SCID) due to defective signal transduction from IL-2 and IL-7 (Macchi et al., 1995; Russell et al., 1995).

One set of genes upregulated by STATs are the master regulator transcription factors (Afkarian et al., 2002; Kurata et al., 1999; Onodera et al., 2010). The early activation of STAT proteins has been shown to be an important first step in the initiation of effector programming. The combined actions of the master regulator transcription factors and signaling through the TCR and cytokine receptors enhances the transcription of genes associated with one subset and represses genes from other effector programs.

Cytokine signaling: Th1 development

Development into a Th1 effector (Fig. 1.2B) is dependent upon signaling from IL-12 via its receptor (Carl et al., 1993; Jacobson et al., 1995; Manetti et al., 1993; Presky et al., 1996). Interestingly, expression of the IL-12rβ subunit is induced by signaling from CD28, IFN-γ and IL-2 during T cell activation, and can be blocked by IL-4 signaling (Afkarian et al., 2002; Elloso and Scott, 2001; Rogge et al., 1997; Szabo et al., 1995, 1997; Wu et al., 1997) The IL-12 receptor is capable of signaling both in a STAT-dependent and STAT-independent manner (Lund et al., 2004) (Fig. 1.3). Upon IL-12 binding, JAK2 and TYK2 associated with IL-12 receptor phosphorylates tyrosines on the cytoplasmic tail of the IL-12rβ chain, allowing for the recruitment and activation of STAT4 (Bacon et al., 1995a, 1995b; Jacobson et al., 1995). Phosphorylated STAT4 dimerizes, translocates to the nucleus, and drives the transcription of *Ifng* (Morinobu et al., 2002; Nishikomori et al., 2002; Zhang and Boothby, 2006). STAT4 also enhances and maintains the expression of T-bet (White et al., 2001). While many effects of IL-12 signaling are mediated by STAT4, there are other pathways invoked by the IL-12 receptor (Fig. 1.3).

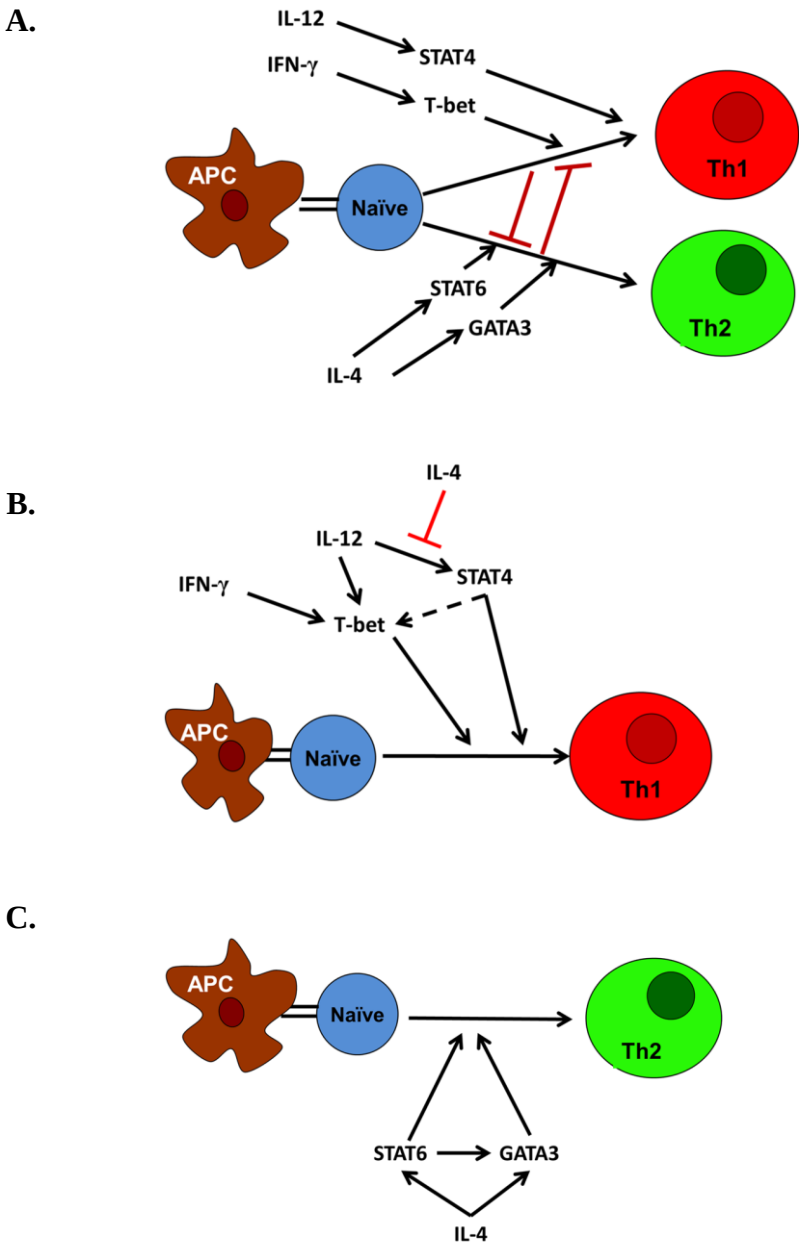


Figure 1.2 Master regulators and STATs drive T helper polarization. Shown are the STATs and master regulators downstream of polarizing cytokine for Th1 and Th2 development (A). The interplay of different signaling pathways is shown for Th1(B) and Th2 (C) development.

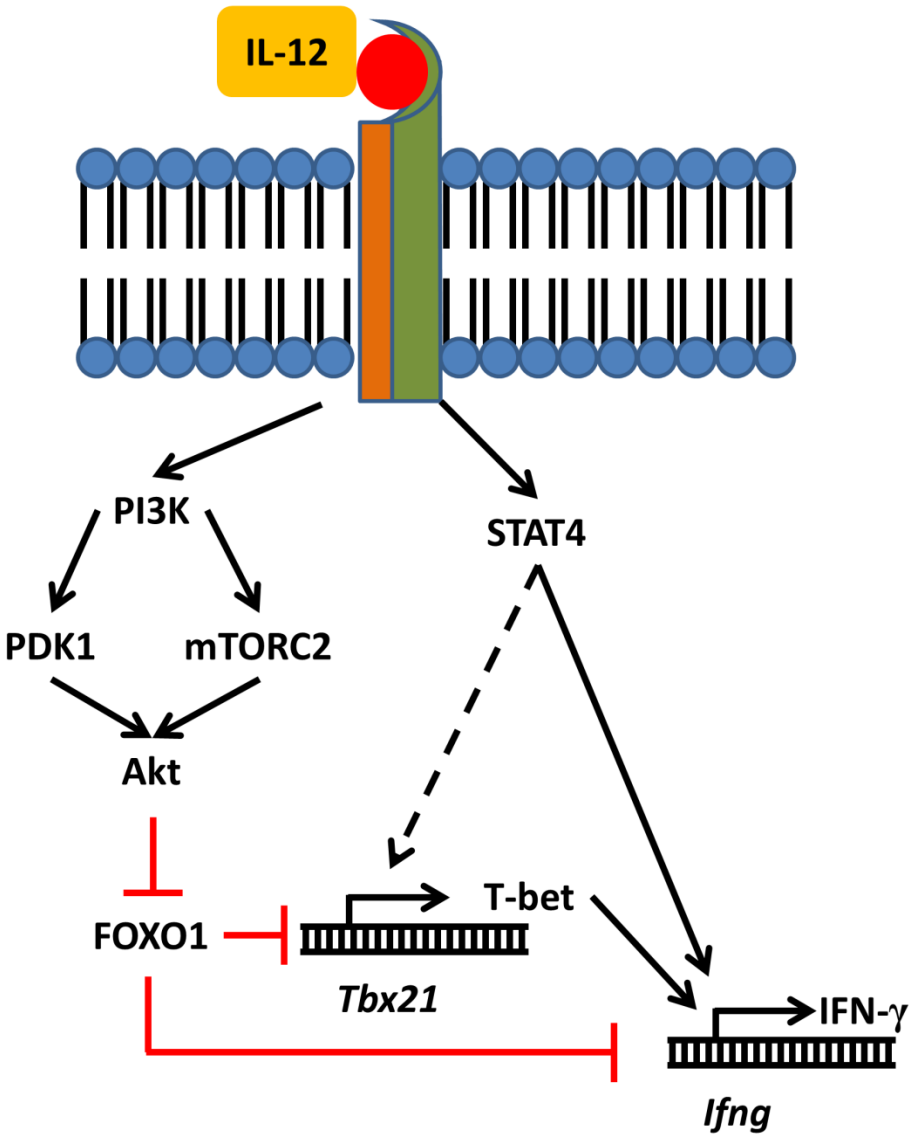


Figure 1.3 IL-12 signaling by STAT4-dependent and independent mechanisms. IL-12 binding to its receptor induces *Ifng* expression via STAT4-dependent and STAT4-independent mechanisms. Gene names are in italics.

The IL-12 receptor also signals through p38 MAPK (Lu et al., 1999; Rincón et al., 1998) and the PI3K/Akt pathway (Rao et al., 2010; Yoo et al., 2002). Akt phosphorylates the forkhead transcription factor FOXO1. This phosphorylation inactivates FOXO1 and leads to its translocation to the cytoplasm (Brunet et al., 1999; Guo et al., 1999; Rena et al., 1999). The inactivation of FOXO1 leads to an increased expression of T-bet (Rao et al., 2012) as well as possibly enhancing the transcription of *Ifng* (Ouyang et al., 2012). T-bet, as well as being required for *Ifng* transcription (Szabo et al., 2000, 2002) also inhibits the transcription of the Th2 master regulator GATA3 (Usui et al., 2006), preserving Th1 lineage commitment and function (Ferber et al., 1999). These STAT4-independent signals also account for proliferative effects of IL-12 (Morinobu et al., 2002)

Other cytokine important for developing Th1 cells include IL-2 and IFN-γ. IFN-γ signaling via its membrane-bound receptor activates STAT1. Active STAT1 drives the expression of T-bet, which is essential for IFN-γ production and IL-12rβ expression (Afkarian et al., 2002; Szabo et al., 2000). IL-2, aside from its proliferative and anti-apoptotic effects (Bödeker et al., 1980; Ma et al., 2006), has also been shown to be essential for efficient transcription of *Ifng* via activation of STAT5 (Shi et al., 2008). Development along the Th1 lineage is opposed by Th2 conditions, and exposure to IL-4 can prevent the transduction of Th1 polarizing signals via repression of *Il12rβ* transcription (Afkarian et al., 2002; Ferber et al., 1999; Szabo et al., 1995).

Cytokine signaling: Th2 development

Th2 polarization (Fig. 1.2C) is driven by IL-4 signaling through the IL-4 receptor (IL-4R). The IL-4 receptor signals through multiple pathways to regulate gene expression in $CD4^+$ T cells. Activation of STAT6 by IL-4 receptor-associated JAKs is one of the primary pathways by which IL-4 can exert transcriptional control (Hou et al.,

1994; Kaplan et al., 1996a; Kuperman et al., 1998). STAT6 up-regulates the expression of the Th2 master regulator GATA3 (Kaplan et al., 1996a; Kurata et al., 1999; Onodera et al., 2010; Ranganath et al., 1998; Zheng and Flavell, 1997). GATA3 and STAT6 then enhance the transcription of Th2 cytokines (Kaplan et al., 1996a; Ranganath et al., 1998; Zheng and Flavell, 1997). GATA3 also represses the expression of T-bet, the IL-12 receptor, and interferon gamma (Ferber et al., 1999; Nakayama and Yamashita, 2008; Ouyang et al., 1998; Usui et al., 2003).

Unlike the IL-12 receptor, the IL-4 receptor is expressed before TCR stimulation (Kupper et al., 1987; Lowenthal et al., 1988), allowing IL-4 signaling to preempt other effector programs. Foundational work found that a mouse strain-specific susceptibility to *Leishmania* infection was due to a surge in IL-4 production in the first 16 hours post infection (Launois et al., 1995). This initial IL-4 inhibited the signaling of IL-12 and the development of a Th1 response (Aseffa et al., 2002; Carl et al., 1993; Himmelrich et al., 2000). In human health, it has been hypothesized that Treg and Th2 responses to helminthic pathogens could inhibit a prophylactic immune response against *Mycobacterium tuberculosis* as these pathogens have a relatively high rate of co-infection but require different opposed immune responses for clearance (Elias et al., 2006; Ezenwa et al., 2010; Rafi et al., 2012; Resende Co et al., 2007). The dominance of the Th2 response over the more essential Th1 response may sometimes pose a significant challenge in the clearance of certain pathogens in the context of a helminth infection.

T cell activation and differentiation

While cytokine signaling plays a central role in directing T helper fate decisions, variables in TCR stimulation and costimulation can significantly impact the adoption of an effector program. While the TCR and costimulatory molecules signal mainly through

subset-independent factors, subtle differences in the relative strength of these signals can skew a $CD4^+$ T cell towards development into one subset or another. One demonstration of this phenomenon is found in studies using altered peptide ligands. Depending on the exact peptide, altered peptide ligands can induce anergy or lead to different effector programs being adopted under the same stimulation conditions (Constant and Bottomly, 1997; Nicholson et al., 1995; Windhagen et al., 1995). Specifically, altered ligands that demonstrated increased affinity for the TCR generated a much higher proportion of IFN-γ producing cells than lower affinity ligands (Kumar et al., 1995; Murray et al., 1989; Windhagen et al., 1995) The use of lower affinity altered ligands has been shown to lead to lower levels of tyrosine phosphorylation on TCRζ and ZAP-70, as well as a different pattern of activation-induced calcium flux (Boutin et al., 1997). The sustained increase of cytosolic calcium induced by the agonist peptide allowed for development along the Th1 lineage, while the brief calcium concentration increase brought on by the low-affinity altered peptide ligand was sufficient for Th2, but not Th1, development (Boutin et al., 1997). This demonstrates that the affinity of an antigen for the TCR can alter the pathways by which the TCR complex signals, and thus, can skew cells towards one effector program over another.

The binding of any ligand to a receptor is controlled not only by the affinity of the receptor, but also the concentration of the ligand relative to the receptor. Just as altering the affinity peptide-TCR binding can modulate the strength of TCR signaling, so too can altering the concentration of antigen (Constant and Bottomly, 1997; Parish and Liew, 1972). The effects of antigen concentration on T helper cell differentiation are in part dictated by the type of antigen used (Constant and Bottomly, 1997). Soluble protein antigens have been shown to exhibit different properties than attenuated or killed parasites such as *Trichuris muris* (Bancroft et al., 1994) or *Leishmania major* (Bretscher et al., 1992). Parasites generally evoke a Th1 response when administered in a low dose

and a Th2 response when given in a high dose. Conversely, a low dose of protein antigen will typically cause a Th2 response, whereas a higher dose will create a Th1 response (Constant and Bottomly, 1997). A very high dose of protein antigen will however generate a Th2 response (Hosken et al., 1995). This discrepancy may be due to the fact that post-translational modifications of peptides derived from bacteria or other pathogens can greatly increase APC uptake and antigen display (Sallusto et al., 1995). Therefore, higher doses of a whole pathogen may lead to overstimulation and death of Th1, but not Th2 effectors (Ramsdell et al., 1994; Zhang et al., 1997b). The fact that higher concentrations of soluble protein antigens evoke a Th2 response may also be due in part to the greater resistance of Th2 cells to activation-induced cell death (Constant and Bottomly, 1997; Zhang et al., 1997b).

Epigenetics and transcription in cytokine genes

Every nucleated somatic cell contains its entire genome. For cells that have begun to differentiate, this entails that large portions of the genome contain genes no longer relevant to the cells function or development. Conversely, genes that were unnecessary in progenitor stages may become necessary as the cell progressively makes fate decisions. Much as a bookstore will have the books most in demand at the front of the store and less popular items pushed to the back, so too are genes necessary for a cells function kept accessible to transcriptional machinery while genes associated with other cell fates are rendered inaccessible.

DNA in the eukaryotic nucleus is in the form of chromatin (Levene, 1903). At its most basic, chromatin is a double-stranded DNA molecule wound around histone octomers. Between 145 and 147 nucleotides are wrapped around each histone in a super-helical turn followed by up to eighty base pairs of DNA until the next histone

octomer (Crick, 1976; Fuller, 1971; Luger and Richmond, 1998; Luger et al., 2012). Chromatin is classified in two broad categories: euchromatin and heterochromatin (Jost et al., 2012; Mazzio and Soliman, 2012). While the terms "heterochromatin" and "euchromatin" originates in early histological studies (Heitz, 1928; Jost et al., 2012; Levene, 1903), later studies have allowed for a definition based on molecular structure. Electron microscopy revealed that euchromatin is a linear array of nucleosomes without significant secondary structure or compaction of the linker DNA, and this state is often compared to "beads on string" (Jost et al., 2012; Mazzio and Soliman, 2012; Olins and Olins, 1974; Oudet et al., 1975; Woodcock and Dimitrov, 2001). Euchromatic DNA can be readily accessed by transcription factors and ribosomes. The heterochromatin state consist of nucleosomes compacted together in higher order structures reducing the amount of space taken up by the bound DNA (Bednar et al., 1998; Jost et al., 2012; Woodcock and Dimitrov, 2001). Generation of heterochromatin often involves the use of linker histones, which bridge between nucleosomes, to form and maintain a compacted structure (Bednar et al., 1998; Hamiche et al., 1996; Martins et al., 2012; Routh et al., 2008; Whitlock and Simpson, 1976). Aside from saving space, this compaction also serves as a layer of transcriptional repression (Lelli et al., 2012; Martins et al., 2012; Mazzio and Soliman, 2012). This level of transcriptional regulation, mediated by the organization of DNA within the nucleus, is part of the field of epigenetics.

Epigenetics refers to heritable modifications in the nature or structure of chromatin at a given locus (Mazzio and Soliman, 2012). This does not involve changing the sequence of DNA, but rather changing the degree by which it can be accessed by transcriptional machinery. Epigenetic changes can include chromatin remodeling, the changes in the conformation of the gene, and changes in the position of a gene within the nucleus. The regulation of these conditions is an important determinant of the transcriptional activity of a given gene.

The process of altering or removing nucleosomes to alter transcriptional accessibility is known as chromatin remodeling (Fry and Peterson, 2001; Mazzio and Soliman, 2012; Peterson, 2002). Chromatin remodeling is carried out primarily by two classes of enzymes. The first group modifies histone tails by the covalent attachment of methyl, phosphoryl, or acetyl groups (Allfrey et al., 1964; Cao et al., 2002; Langan, 1969; Nislow et al., 1997; Nohara et al., 1968; Roguev et al., 2001). The effects of histone modifications on the transcriptional activity of a locus depend on which group or groups have been added and which residue of the histone is subject to modification (Mazzio and Soliman, 2012). The acetylation of histones on lysine residues is a transcriptionally permissive modification (Fields et al., 2002; Hassan et al., 2002), and the effects of histone tail methylation vary depending on the residue and the number of methyl groups added. Tri-methylation of histone 3 at lysine 4 (3me-H3K4) is found at histones of active promoters, and this modification is able to associate with TFIID and thus enhance transcription (Lauberth et al., 2013; Varier et al., 2010; Vermeulen et al., 2007). Conversely, tri-methylation of histone 3 at lysine 27 (3me-H3K27) is a strongly repressive mark able to recruit repressive transcription factors (Cao et al., 2002; Cavalli and Paro, 1998; Sewalt et al., 2002; Su et al., 2003). The acquisition of histone modifications on nucleosomes associated with cytokine genes is a lineage specific event that occurs during T cell differentiation (Ansel et al., 2003; Fields et al., 2002).

A second class of chromatin remodeling enzymes consists of ATPases that execute the modification of DNA-histone interactions. This remodeling process can involve loosening the wrapping of DNA around a histone core, repositioning of a nucleosome by sliding it to an adjacent section of DNA, or eviction of a nucleosome from the DNA strand entirely (Gutiérrez et al., 2007; Hargreaves and Crabtree, 2011; Peterson and Workman, 2000; Steger and Workman, 1996; Yodh, 2013). Nucleosome repositioning preceding transcription can be demonstrated by the appearance of DNAse

hypersensitivity sites (HSS) and new micrococcal nuclease digestion product in cytokine genes being switched on during polarization (Takemoto et al., 2000; Zhang and Boothby, 2006). The appearance of these sites was shown to be due to the repositioning of nucleosomes at the inducible HSSs to allow for greater access by transcription factors and also to the DNAse enzyme (Almer et al., 1986; Narlikar et al., 2002; Peterson, 2002).

Nucleosome repositioning is accomplished by multi-protein complexes typically containing a central ATPase, as well as multiple accessory proteins (Hargreaves and Crabtree, 2011). Chromatin remodeling complexes can exert both activating and repressing influences on transcription via compacting or opening up chromatin. Several families of chromatin remodeling complexes have been reported (Fazzio and Rando, 2012; Hota et al., 2013; Peterson and Workman, 2000; Yodh, 2013). One family important in the regulation of *Ifng* transcription in T cells is the Swi/Snf family of chromatin remodeling complexes (Zhang and Boothby, 2006). This chromatin remodeling complex can bind directly to DNA (Wang et al., 1998), or to acetylated histone tails (Chandrasekaran and Thompson, 2007; Horn and Peterson, 2001; Singh et al., 2007). The Swi/Snf complex is required to transcribe some genes, but also is crucial in the silencing of other genes such as CD4 (Chi et al., 2002). In terms of cytokine gene regulation, Swi/Snf is typically associated with creating chromatin conditions favorable to transcription.

One of the most studied repressive epigenetic modifications in the regulation of cytokine genes is the methylation of cytosine DNA bases in CpG dinucleotides. CpG methylation can serve to recruit methyl-CpG-binding domain proteins (MBD proteins) (Defossez and Stancheva, 2011; Dhasarathy and Wade, 2008; Hung and Shen, 2003). MBD family members can then bind the NuRD complex to enact further repressive modifications such as increased nucleosome density and deacetylation of histone tails (Allen et al., 2013; Li et al., 2010; Saito and Ishikawa, 2002; Zhang et al., 1999). CpG

methylation within the recognition sites for certain transcription factors can also inhibit transcription factor binding and transactivation (Jones and Chen, 2006; Sunahori et al., 2009). CpG methylation of regulatory regions of DNA is able to silence transcription by both direct and indirect mechanisms.

Methylation of CpG dinucleotides begins with the recruitment and activity of a *de novo* methyltransferase. Two enzymes fill this role in mammalian cells, DNMT3a and DNMT3b (Deaton and Bird, 2011; Robertson et al., 1999). In T cells, *de novo* methylation is carried out mainly by DNMT3a (Gamper et al., 2009). Activation via the TCR greatly increases both the activity and absolute concentration of DNMT3a in the responding cell (Gamper et al., 2009). The *de novo* methylation is carried out on one strand of the target sequence, but this allows the activity of the symmetry enforcing DNMT, DNMT1 (Bacolla et al., 1999; Bird and Wolffe, 1999; Pradhan et al., 1999). DNMT1 establishes symmetrical methylation during the initial methylation and is required to maintain the heritability of CpG methylation in actively cycling cells.

The differentiation-dependent CPG methylation of a cytokine gene was first reported in the proximal *Ifng* promoter in Th2, but not Th1, cells (Fitzpatrick et al., 1998, 1999; Melvin et al., 1995; Winders et al., 2004; Young et al., 1994). The importance of DNA methylation in the silencing of cytokines from other effector lineages has been illustrated by transgenic mouse models defective in DNMT3a or DNMT1. $CD4^+$ T cells deficient in DNMT3a are unable to silence cytokine genes from other lineages, resulting in Th1 cells producing IL-4 or Th2 cells producing IFN-γ (Gamper et al., 2009; Thomas et al., 2012). Methylation patterns in Th2 cells deficient for DNMT3a are very similar to the hypomethylated state found in naïve cells (Thomas et al., 2012). Similarly, naïve cells deficient in DNMT1 produce exponentially more *Ifng* transcript than DNMT1-sufficient cells upon *ex vivo* stimulation (Lee et al., 2001). These experiments

demonstrate that the ability to repress cytokines from opposed lineages requires the ability to sustainably methylate cytokine genes.

Regulation of the transcription of *Ifng* and Th2 cytokine genes in primary effectors: The *Ifng* gene

For interferon gamma to be produced, STAT4 and T-bet signaling are insufficient. Signaling pathways originating from the T cell receptor also are required for cytokine production. The NFAT family of calcium-flux activated transcription factors has been shown to be essential for Th1 development and the production of IFN-γ, and the binding of NFAT1 isoforms to the *Ifng* promoter has been demonstrated via ChIP (Chromatin immunoprecipitation) (Avni et al., 2002; Luo et al., 1996; Rooney et al., 1994). NF-κB signaling from the TCR is also required for Th1 differentiation and function. Use of a dominant negative IκB protein (IκBαΔN) showed T cell-intrinsic defects in IFN-γ production, as well as decreases in T-bet expression and STAT4 activation (Corn et al., 2003). Likewise, cells from mice deficient in the NF-κB subunit RelB had marked decreases in the ability to become functioning Th1 effectors, with defects in T-bet (Corn et al., 2005).

A common element in many promoters, including the *Ifng* promoter, is the cAMP response element (CRE) which serves as a binding site for CREB/ATF transcription factors (Montminy et al., 1986). CREB/ATF factors bind DNA as either homodimers or heterodimers, and can serve to activate or repress transcription of target genes (Cha-Molstad et al., 2004; Wen et al., 2010). Among the CREB/ATF factors which regulate *Ifng* transcription is CREB1. Canonically, CREB1 is activated by PKA (protein kinase A), which has been shown to have a repressive effect on IFN-γ production (Shin et al., 1998), but PKA-independent mechanisms of CREB phosphorylation have been

described, and two downstream effectors of the IL-12 receptor, p38 MAPK and Akt, have been shown to activate CREB (Johannessen et al., 2004; Pugazhenthi et al., 1999, 2000; Shankar et al., 2010). Studies in mice or Jurkat cells produced conflicting results on the role of CREB1 in *Ifng* transcription (Cippitelli et al., 1995; Penix et al., 1996; Zhang et al., 1998a). Work carried out in human $CD4^+$ T cells responding to mycobacterial antigens showed CREB1 phosphorylation in cells actively producing IFN-γ (Liu et al., 2010; Pasquinelli et al., 2009; Samten et al., 2005), and phospo-CREB1 has been shown to bind the *Ifng* promoter of human Th1cells in response to *Mycobacterium tuberculosis* (Samten et al., 2008). This evidence supports a model in which CREB1 phosphorylation is carried out in a PKA-independent manner in Th1 effectors and can serve to transactivate the *Ifng* promoter in humans.

Signaling from the TCR and CD28 also allows for the expression of the beta chain of the IL-12 receptor (IL-12rβ) (Wu et al., 1997). The expression of a functional IL-12 receptor in turn allows for the activation of STAT4 (Jacobson et al., 1995; Manetti et al., 1993; Morinobu et al., 2002; Nishikomori et al., 2002) as well as the other effects of IL-12 receptor signaling discussed earlier.

A naïve CD4+ T cell stimulated by its TCR under Th1 conditions does not immediately begin to produce IFN-γ. A period of polarization is required to become a functioning effector cell (Lanzavecchia et al., 1999). During this time multiple signaling networks and their downstream effectors act upon the *Ifng* promoter to prepare for transcription. Signals from the TCR and co-receptors provide the initial burst of T-bet expression, as well as a transient peak of *Ifng* transcription (Ariga et al., 2007). Early in the process of differentiation, histone acetylation and other transcriptionally-permissive histone modifications occur at the *Ifng* promoter (Avni et al., 2002; Fields et al., 2002). These permissive Th1-specific histone modifications require the actions of T-bet and STAT4 for initiation and maintenance (Fields et al., 2002).

One effect of signaling through the aforementioned transcription factors is the chromatin remodeling of the *Ifng* promoter. During Th1 polarization, the Swi/Snf central ATPase Brg1 is recruited to the *Ifng* promoter (Zhang and Boothby, 2006), and it is known that both NFAT and STAT4 are essential for this remodeling to occur at the *Ifng* promoter in $CD4^+$ T cells (Zhang and Boothby, 2006). It may be that this remodeling is required for T-bet binding to the *Ifng* promoter. Regulation of the activity of the *Ifng* promoter is accomplished by the synthesis of signals from the TCR and the cytokine receptors through the actions of multiple downstream pathways. There is however another layer of control for the transcription of *Ifng*.

The interferon gamma gene is not controlled solely by the promoter. Numerous elements flanking the coding regions also exert control over the transcription of *Ifng*. These conserved non-coding sequences (CNSs) serve to regulate the expression of *Ifng*. During Th1 polarization epigenetic changes can be observed at many of these elements, and several CNS regions have been found to bind to both subset-dependent and subset-independent transcription factors (Hatton et al., 2006; Schoenborn et al., 2007). Several of these elements also regulate *Ifng* transcription in a cell type-specific manner (Collins et al., 2012).

Activation and repression of the *Ifng* gene both result from multiple signaling pathways initiated by the TCR, membrane-bound costimulatory receptors, and cytokine receptors. These pathways trigger epigenetic changes at the promoter and multiple distal regulatory sites in order to promote gene expression or repression.

During the course of Th2 polarization, the *Ifng* gene is silenced by a variety of direct and indirect mechanisms. Indirect silencing mechanisms include the repression of the IL-12 receptor and T-bet mediated by STAT6 and GATA3 (Ferber et al., 1999; Hosoya et al., 2010; Ouyang et al., 1998). One of the best studied direct mechanisms for

silencing *Ifng* transcription is CpG methylation of the proximal *Ifng* promoter (Fitzpatrick et al., 1998, 1999; Melvin et al., 1995; Winders et al., 2004; Young et al., 1994). As discussed previously, methylation of the *Ifng* promoter allows for repression by multiple mechanisms.

Importantly, CpG methylation is able to directly inhibit the activity of the *Ifng* promoter, likely by inhibiting the binding of transcription factors required to transactivate the *Ifng* gene (cite). Most notably, methylation of a single CpG dinucleotide located 53 base pairs upstream from the transcription start site (C-53) was able to completely silence transcription from an *Ifng*-promoter construct (Jones and Chen, 2006). The -53 CpG dinucleotide sits in the middle of a CREB/ATF binding site, and transcription factors unable to bind the methylated promoter were identified as CREB/ATF family members (Jones and Chen, 2006). This demonstrates that promoter methylation is capable of regulating transcription in the absence bound inhibitory transcription factors.

Regulation of the transcription of *Ifng* and Th2 cytokine genes in primary effectors: The Th2 cytokine locus

The regulation of the Th2 cytokine locus differs from what is seen at the *Ifng* gene in many key details. The mechanisms controlling the production of IL-3, IL-4, IL-5, and IL-13 are a fertile area of research which will only be touched upon briefly here. As opposed to Th1 development, Th2 polarization involves the activation of several cytokine genes within the Th2 cyokine locus (Ansel et al., 2006; Fields et al., 2004). IL-3, IL-4, IL-5, and IL-13 each have their own regulatory regions including promoters, but also are under the control of a locus control region (LCR) located at the 3' end of the RAD50 gene (Lee and Rao, 2004; Lee et al., 2003). This provides a central control for execution of the Th2 effector program.

A second difference between *Ifng* and the Th2 cytokine locus is that the *Il4* promoter, the region of the LCR, and regions between the *Il4* and *Il13* genes are methylated in naïve T helper cells (Ansel et al., 2006; Lee et al., 2002). During Th2 polarization, this methylation is lost, possibly as a consequence of cell division without enforcement of symmetry (Ansel et al., 2006; Kim et al., 2007; Lee et al., 2002). The regulation of Th2 cytokine genes therefore has differences far beyond the requirements for lineage-specific transcription factors.

T cell memory

One key feature of the adaptive immune system is the ability to 'remember' antigens by allowing a small number of responding B and T cells to survive past the point of pathogen clearance and antigen withdraw. In the post-effector state, activated T cell clone populations contract, and surviving cells reach a semi-quiescent state. The onset of the memory phenotype appears to occur soon after antigen withdraw, although the exact time required for an active cell to become a true memory cell remains unknown (Dooms and Abbas, 2006; Hu et al., 2001; Swain, 1994)

Memory T cells quickly reacquire an active phenotype upon re-exposure to antigen, and begin expanding and producing cytokines days before antigen responsive naïve cells (Croft et al., 1994). These memory cells allow the immune system to respond rapidly to subsequent exposures to a given antigen and provide prophylaxis against repeat infections with the same pathogen. The initial response taken by $CD4^+$ memory cells upon restimulation is to produce the cytokines associated with the effector subset from which it is derived, demonstrating that some commitment to a given cell fate is maintained in memory cells (Cerottini and MacDonald, 1989; London et al., 1999, 2000).

The formation and behavior of memory population is central to the development of vaccines and to the general function of the adaptive immune system (Kaech et al., 2002).

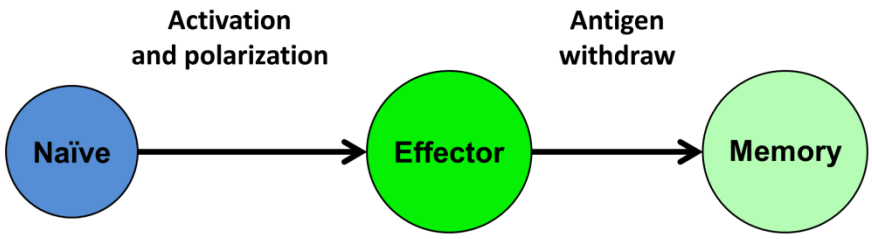
Two models are used to explain the development of memory cells from an antigen stimulated population of T cells (Fig. 1.4). The first model, called the linear differentiation model (Fig. 1.4A) has a memory population arising from fully differentiated effector cells. The partial differentiation model (Fig. 1.4B) has two populations developing during the course of polarization (Wu et al., 2002). The first is an effector population which dies off during clonal contraction following antigen clearance. The second population does not have as robust effector function, but maintains the epigenetic programing of the effector population. Cells of this partially-differentiated population survive to form a memory population.

For CTL memory cells, the evidence supports a model of development from partially-differentiated precursor cells. Division of $CD8^+$ T cells upon interaction with an APC expressing cognate antigen is reported to occur asymmetrically, with the proximal daughter cell expressing higher levels of CD8 and effector cytokines (Chang et al., 2007). The distal daughter cells were smaller and exhibited expression of surface markers associated with $CD8^+$ memory cells (Chang et al., 2007). This asymmetric division of activated $CD8^+$ T cells also differentially partitions the T-box transcription factors eomesodermin and T-bet between the daughter cells. T-bet protein is primarily partitioned in the proximal, effector-like daughter cell, while eomesodermin is localized to the distal, memory-like daughter cell (Chang et al., 2011). The $eomesodermin^{Hi}$ daughter cells produce less IFN-γ than the $T\text{-}bet^{Hi}$ daughter cells, but survive much better in the long term, and can give rise to a memory population (Banerjee et al., 2010; Intlekofer et al., 2005). Subsequent divisions enhance the effect of this partitioning, and the relative amounts of eomesodermin and T-bet have been shown to have a predictive

value as to which daughter cells will survive as memory cells (Banerjee et al., 2010; Intlekofer et al., 2005; Rao et al., 2010).

In the case of $CD4^+$ T cells, the source of memory cells may depend on the effector type. For Th2-derived memory cells, the linear differentiation model is supported by experimental evidence. IL-4 producing Th2 effectors generated *in vitro* and transferred to recipient mice have been shown to give rise to a long-lived post-effector population *in vivo*, demonstrating that Th2 cells progress from an effector state to a memory state (Adeeku et al., 2008; Löhning et al., 2008). The source of Th1 memory has been a point of contention, with some data supporting the direct model (Swain, 1994) and other data supporting a model of partial differentiation similar to that found in CTL memory (Ahmed and Gray, 1996; Kaech et al., 2002; Moulton and Farber, 2006). In particular, Th1 cells expressing less Ly6c and T-bet have been found to persist longer and respond better to secondary antigen exposure (Marshall et al., 2011). The similarities between findings in Th1 cells and $CD8^+$ cells suggests that partial differentiation may be a result of IFN-γ production (Stockinger et al., 2006).

A.



B.

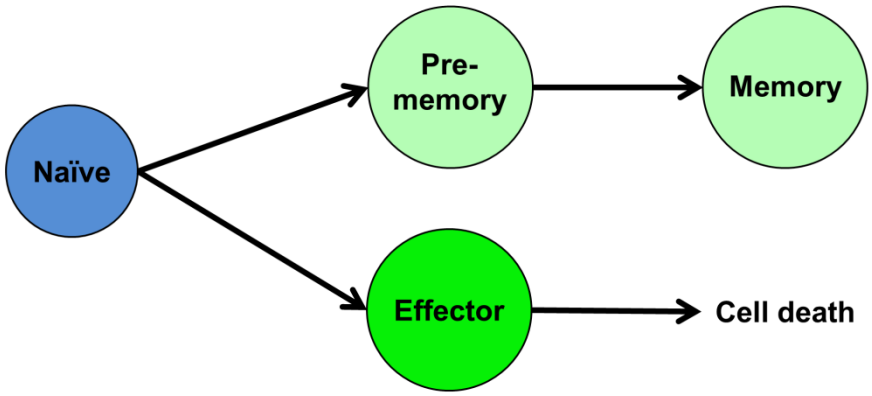


Figure 1.4 Alternative models of T cell memory. Shown are the linear (A) and partial differentiation (B) models of T cell memory formation.

Regulation of cytokine genes in the memory phase

Memory cells are able to rapidly produce cytokines a secondary exposure to antigen. Whether this is due to differences in the regulation of cytokine genes or the maintenance of a 'primed' chromatin state has been the subject of study. In the case of $CD8^+$ cells, studies on the regulation of the *Ifng* promoter have revealed a degree of dynamism of CpG methylation. A naïve CTL has an *Ifng* promoter that is predominantly CpG methylated and is unable to produce IFN-γ (Fitzpatrick et al., 1998). During the course of cell divisions undertaken after activation, methylation of the *Ifng* promoter is lost from the population and the ability to produce IFN-γ is acquired (Fitzpatrick et al., 1998, 1999). Importantly, DNA methylation of the *Ifng* promoter is re-established in $CD8^+$ memory populations, but rapidly lost upon restimulation (Kersh et al., 2006). This loss of CpG methylation, occurring in about five hours, correlates with the renewed production of IFN-γ (Kersh et al., 2006). This suggests that methylation of the *Ifng* may not be the stable repressor that it is commonly portrayed as being.

For memory cells derived from Th2 effectors, the transcriptional readiness of the Th2 cytokine locus is actively maintained (Yamashita et al., 2006), even in the absence of cytokine signaling (Yamashita et al., 2004). The expression of GATA3 at high levels is also actively maintained in Th2 memory cells. The transcription factor MLL has been shown to be essential for the maintenance of GATA3 expression and permissive histone modifications on the Th2 cytokine locus (Yamashita et al., 2006). Reduction of MLL expression in $CD4^+$ cells has also been demonstrated to impair the formation of Th2 memory, but not effector, populations (Yamashita et al., 2006). These data demonstrates that MLL is at least indirectly responsible for the maintenance of Th2 lineage commitment, and suggest a continued requirement for the expression of GATA3.

Regulation of cytokine genes in the memory phase: Flexibility

While commitment to express IL-4 was maintained in the memory phase, these Th2-derived memory cells could also produce significant amounts of IFN-γ simultaneously with IL-4 when restimulated under Th1-polarizing conditions (Adeeku et al., 2008; Krawczyk et al., 2007; Löhning et al., 2008). Further research in mouse models identified IL-12 signaling, T-bet expression, and signaling from type-I interferons as essential to the flexible production of IFN-γ, and reported IFN-γ producing cells as still expressing GATA3 (Hegazy et al., 2010). Work in human cells has shown that IFN-α/β can inhibit GATA3 expression and thus abrogate IL-4 production in Th2 effector cells (Huber et al., 2010). This suggests that there is either a difference in this regard between mice and humans or between memory and effector cells.

The ability of Th2-derived memory cells to produce a cytokine inimical to Th2 function such as IFN-γ raises many questions. It is currently unknown if the methyl-CpG mediated repression of the *Ifng* promoter formed in Th2 differentiation is maintained in Th2 memory cells. Several possible models explaining the reported flexibility of IFN-γ production can be put forth. In the first, the repressive DNA methylation of the *Ifng* promoter is established in polarization, but lost sometime between antigen withdraw and restimulation. The second is that the repression of the *Ifng* promoter is maintained in memory phase but overcome by Th1 signaling, as it has been demonstrated that exogenous T-bet can transactivate a fully methylated *Ifng* promoter (Tong et al., 2005). The third model is that DNA methylation is maintained in memory cells, but lost upon restimulation under Th1 conditions. Identification of the correct model is crucial to furthering our understanding of the molecular underpinnings of flexibility in memory cells and the stability of CpG methylation in T helper cells.

A second unknown is whether STAT4 is required for the expression of *Ifng* by Th2-derived memory cells. While IL-12 has been identified as essential for flexibility (Adeeku et al., 2008; Hegazy et al., 2010), it remains unclear if STAT4 is involved in flexibility. IL-12 signals through multiple pathways, many of which can impact *Ifng* transcription without STAT4 (Rao et al., 2010, 2012; Yoo et al., 2002). The absence of STAT4 activation would imply a mechanism of *Ifng* transcription vastly different from that seen in naïve cells acquiring Th1 effector function. Finally, it remains to be seen whether either T-bet or STAT4 has any bearing on the initiation or maintenance of the DNA methylation at the *Ifng* promoter.

The extra adaptability that flexible cytokine expression brings to the $CD4^+$ component of the adaptive immune system is still poorly understood in terms of both mechanism and significance. The uniqueness of this reprograming of differentiated somatic cells without recourse to the highly artificial methods used to create induced stem cells (Adachi and Schöler, 2012; Takahashi et al., 2007) or to trans-differentiate committed cells into a new lineage (Sundrud et al., 2003; Zhou et al., 2008) makes understanding the mechanisms underlying plasticity of cytokine expression important. Likewise, an understanding of the significance of plastic cytokine expression in T helper cells will allow us to better grasp the nature of an adaptive immune response from initiation to memory to recall. This work aims to identify mechanisms and requirements for the flexible expression of *Ifng* by memory cells derived from fully differentiated Th2 effectors. Understanding the mechanisms underlying this natural reprograming will add new depth to our understanding of cell fate commitment, gene regulation, and the function of T helper memory.

II. HEMIMETHYLATION OF THE *Ifng* PROMOTER IN TH2 EFFECTOR CELLS

Strand-biased acquisition of *Ifng* promoter methylation in Th2 effector cells

The flexibility of cytokine expression demonstrated by Th2-derived memory cells acquiring the ability to produce IFN-γ (Adeeku et al., 2008; Löhning et al., 2008) raised several questions central to the understanding of lineage commitment in the post-effector phase. The first of these which we addressed was whether the inhibitory CpG methylation of the *Ifng* promoter reported in effector Th2 cells (Jones and Chen, 2006; Melvin et al., 1995; Yano et al., 2003; Young et al., 1994) was maintained in the memory phase. At the outset, we formulated two hypothetical models. In the first model, methylation remained, but the repressive effects were overcome during the course of the recall response. This model was supported by the observation that expression of T-bet could transactivate a methylated *Ifng* promoter (Tong et al., 2005). The second model was that, in the time between the initial response and the recall response, methylation of the *Ifng* promoter was lost. In $CD8^+$ cells, loss of *Ifng* promoter methylation has been reported (Kersh et al., 2006), but no studies had investigated *Ifng* promoter methylation in $CD4^+$ memory cells. Also supporting this model was research showing that the global DNA methylation in memory cells was decreased, as was the expression of DNA methyltransferases (Li et al., 2012). To investigate this, we turned to bisulfite DNA modification followed by strand-specific PCRs (Fig. 2.1A) as a means to detect and quantify CpG methylation.

We began by analyzing DNA from naïve $CD4^+$ T cells, Th1 effectors (13 days post stimulation), and Th2 effectors for methylation of the *Ifng* promoter (Fig. 2.1B). Naïve $CD4^+$ and Th1 effector cells showed little methylation of either strand of DNA upstream from the transcription start site (Fig. 2.2), whereas high methylation densities

were found at two dinucleotides in exon 1, independent of T cell differentiation, as expected (Deaton et al., 2011; Maunakea et al., 2010). In effector Th2 cells (Fig. 2.2), we found increased methylation of the coding strand of the *Ifng* promoter, with a majority of samples exhibiting modification of the -53 CpG whose modification was reported to abrogate promoter activity (Jones and Chen, 2006). Surprisingly, however, the noncoding strand was reproducibly and significantly less methylated in Th2 effector cells relative to the coding strand (Fig. 2.3), particularly at the -53 CpG (Fig. 2.3D). In light of this unexpected result, we tested samples including DNA from a mouse brain and 3T3 cells, both of which would be expected to have symmetrical hypermethylation of the *Ifng* promoter, along with thymocytes, which are reported to have symmetrical hypomethylation. Brain and 3T3 cell DNA demonstrated a high density of methylation symmetrically across the surveyed region and in particular at the crucial -53 CpG. Thymocytes, like naïve CD4 T cells, exhibited little CpG methylation (Table 2.1). The frequency of non-coding strand DNA methylation in Th2 cells was too low simply to represent a lack of modification on one chromosome, e.g., from mono-allelism (Bix and Locksley, 1998). Separate analyses (Table 2.1 and later results) exclude a strand bias in the detection method as the basis for the observation. Accordingly, we infer from these data that *Ifng* promoters were hemimethylated in these Th2 effector cells.

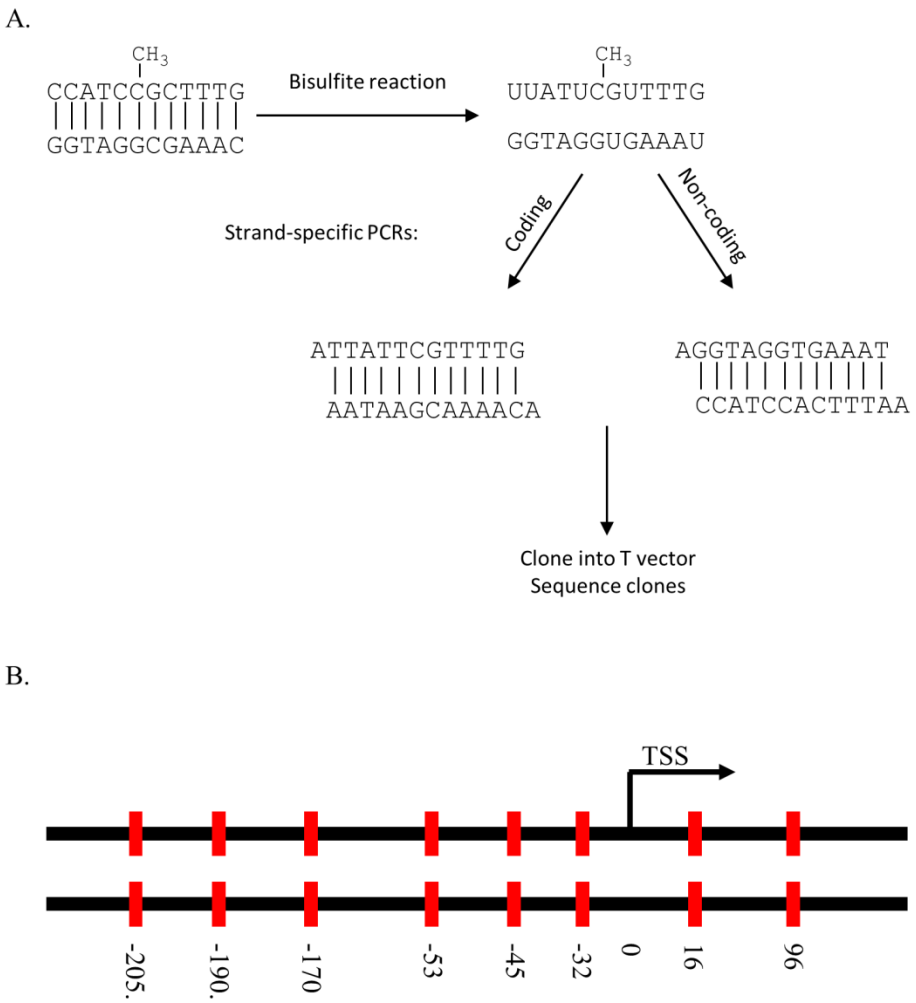


Figure 2.1. Bisulfite modification for the detection of CpG methylation. A. Schematic of bisulfite sequencing work-flow. Isolated DNA is reacted with sodium bisulfite, resulting in all unmethylated cytosine bases being converted to uracils. The C to U base mutation results in the two DNA strands no longer being reverse complements to each other. This in turn allows for PCR analysis of each strand individually. B. Map of the *Ifng* promoter region assayed by bisulfite sequencing. CpG dinucleotide positions are given relative to the transcription start site (TSS).

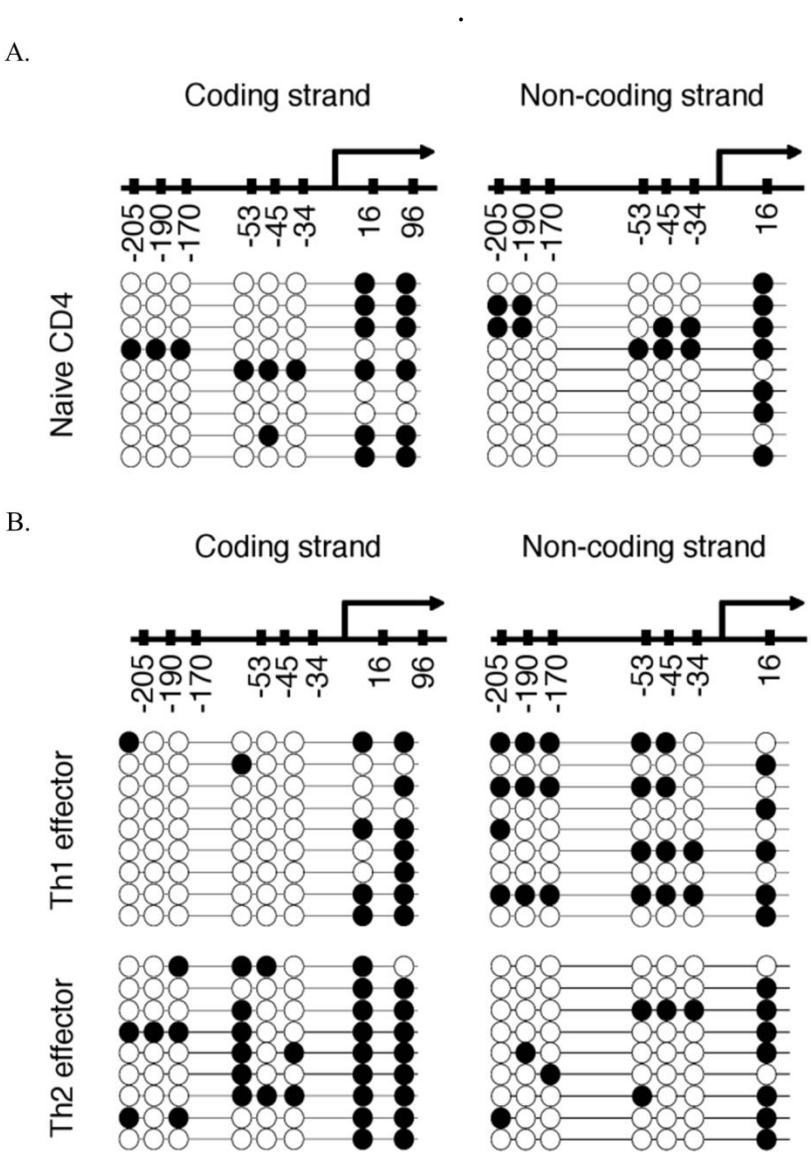


Figure 2.2. Methylation of the *Ifng* promoter is lineage-specific. The results of bisulfite sequencing analysis of DNA from naïve $CD4^+$ (A), Th1, and Th2 (B) effectors demonstrate lineage specific methylation. Each row of the above plots represents one sequenced clone, with filled dots representing a methyl-CpG. Results are representative of two (A) or three (B) biological replicates.

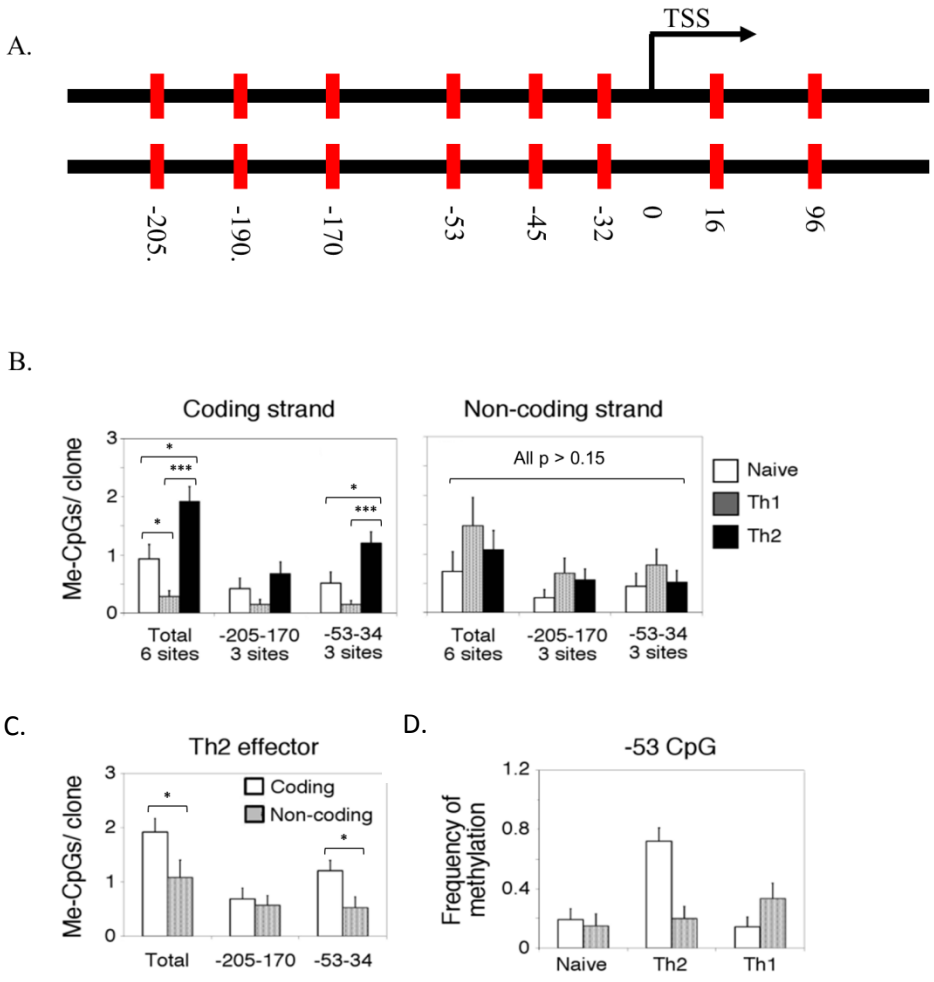


Figure 2.3. Asymmetric methylation of the *Ifng* promoter in Th2 effectors. A. Schematic of the *Ifng* promoter. B. Quantification of the methylation of all CpG dinucelotides upstream of the TSS, CpGs -205 to -170, and -53 to -32 across two (naïve) or three (Th1 and Th2) biologically independent replicates. Methylation frequencies are compared for Th1, Th2, and naïve cells for the coding (upper left) and non-coding strands (upper right). C. Methylation of the coding strand is significantly higher than the non-coding strand in Th2 effectors (lower left). D. Methylation of the -53CpG is highly strand and lineage specific. Shown are means ±S.E.M. *p<0.05, ** p<0.01, ***p<0.001.

Table 2.1. CpG methylation in naïve CD4$^+$ T cells, thymus, brain, and NIH 3T3 cells

A.

		CpG position[a]						
Sample	**Strand**	-205	-190	-170	-53	-45	-34	16
Naïve CD4	Coding	11.4 (3.6)[b]	11.4 (3.6)	14.4 (3.6)	17.4 (3.9)	14.4 (3.6)	14.4 (3.6)	59.8 (6.1)
	Non-coding	17 (3.6)	10.8 (1.6)	6.25 (2.3)	17 (3.4)	15.3 (2)	15.3 (2)	90.9 (3.6)
Thymus	Coding	0	0	0	0	0	0	72.2 (3.5)
	Non-coding	0	0	0	0	0	0	71.3 (3.5)
Brain	Coding	100	86.6 (2)	86.6 (2)	100	100	100	100
	Non-coding	95 (2.7)	76.7 (3)	100	95 (2.7)	100	91.7 (3.3)	83.3 (4.9)
NIH 3T3	Coding	78.9 (3.2)	63.1 (3.8)	52.6 (1.8)	63.1 (0.9)	63.1 (0.9)	52.6 (3.5)	68.4 (2.8)
	Non-coding	62.5 (4.2)	36.3 (6.1)	43.8 (3.0)	56.3 (3.0)	50 (0.0)	50 (0.0)	87.5 (4.2)

B.

Sample	Strand	Total[c]	-205-170	-53-34
Naïve CD4	Coding	0.9 (0.25)[d]	0.4 (0.18)	0.52 (0.2)
	Non-coding	0.7 (0.34)	0.25 (0.14)	0.45 (0.22)
Thymus	Coding	0.11 (0.07)	0 (0)	0.11 (0.07)
	Non-coding	0 (0)	0 (0)	0 (0)
Brain	Coding	5.7 (0.12)	2.7 (0.12)	3 (0)
	Non-coding	5.6 (0.16)	2.6 (0.15)	2.9 (0.06)
3T3	Coding	3.8 (0.45)	2.1 (0.27)	1.8 (0.31)
	Non-coding	2.9 (0.53)	1.4 (0.26)	1.5 (0.35)

a: position is presented as the distance in bases from the transcription start site

b: data are presented as the mean (± SEM) percentage of samples methylated at a given position.

c: Cluster of CpG dinucleotides being assayed.

d. Mean (± SEM) number of methyl-CpGs per clone in a given cluster.

Hemimethylation occurs naturally during the course of DNA replication (Bhutani et al., 2011; Ehrlich and Wang, 1981; Tatematsu et al., 2000), and we wished to verify that the hemimethylation we were detecting was not due to DNA synthesis and cell division. Therefore, we measured the amount of DNA synthesis at the time of DNA extraction via BrdU uptake and cell division activity via CFSE partitioning (Fig. 2.4). We detected little to no cell division or BrdU incorporation at the time of DNA extraction, and so we conclude that the hemimethylation of the *Ifng* promoter is not due to DNA replication, but rather is a unique feature of gene transcriptional regulation in Th2 cells.

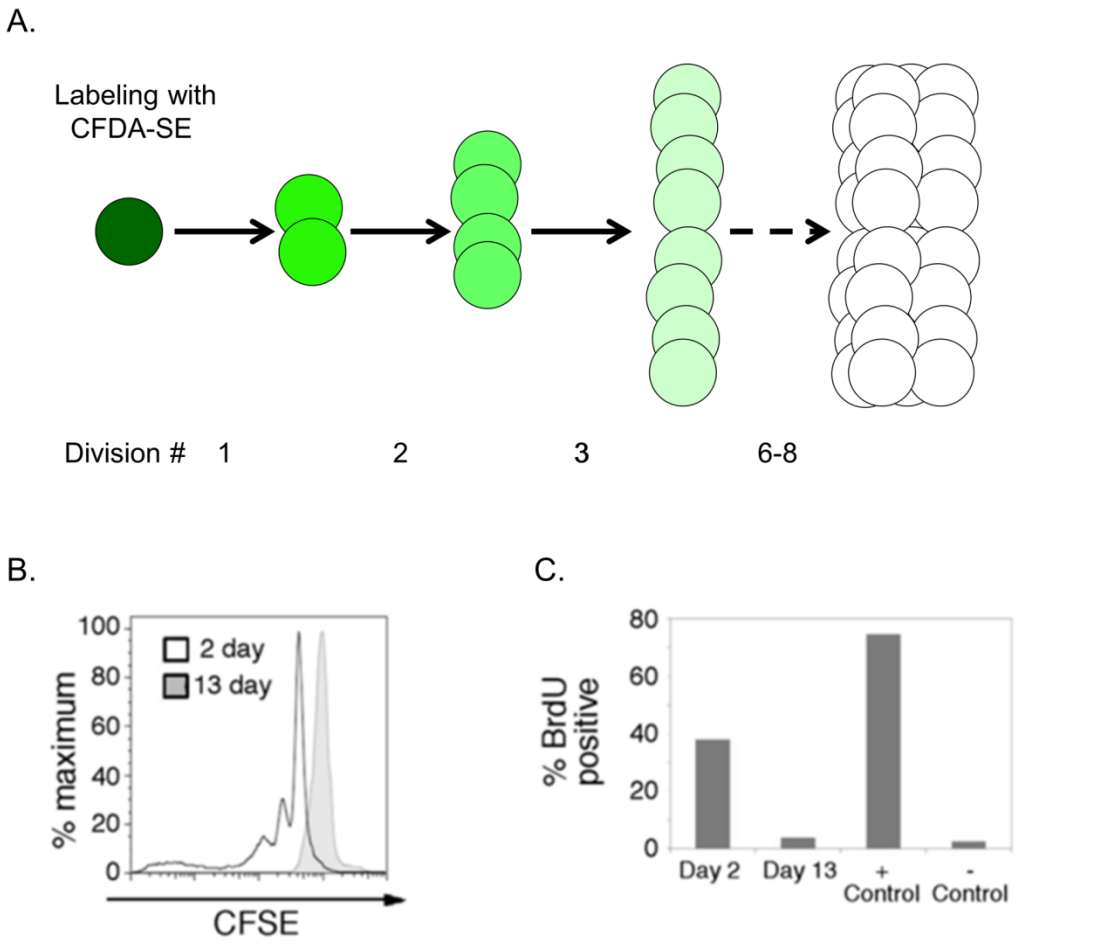


Figure 2.4. Th2 effectors have exited cell cycle at the time of DNA methylation analysis. Th2 cultures were analyzed for CFSE partitioning (B) and BrdU uptake (C). (A) Schematic showing the partitioning of CFSE over the course of multiple cell cycles. (B) CFSE labeling occurred either at the time of stimulation (day 2 samples) or on day 11 (day 13 samples). After 48 hours the CFSE staining profiles of the samples were compared. (C). Th2 cultures either 2 or 13 days after initial stimulation were pulsed with BrdU for four hours and then stained to determine BrdU incorporation into DNA. Cells grown continuously in the presence of BrdU serve as a positive control for BrdU staining, while cells left untreated serve as a negative control.

SnaBI digestion is sensitive to hemimethylation

Previous studies have used the methylation sensitive restriction enzyme SnaBI to detect CpG methylation at the *Ifng* promoter (Melvin et al., 1995; Winders et al., 2004; Young et al., 1994). The -53 CpG is within a SnaBI cleavage site, meaning that methylation at this dinucleotide would prevent SnaBI from digesting DNA. While this has been verified with symmetrically methylated DNA, the effects of hemimethylation are unknown. If SnaBI is hemimethylation sensitive, then an enzymatic digest would not be able to discriminate between symmetric and asymmetric methylation. This would explain in part why the strand-specific methylation we describe has not been previously reported. To determine if SnaBI digestion is inhibited by hemimethylation, we used probes consisting of the region of the *Ifng* promoter encompassing the proximal three CpG dinucleotides (C-53, C-45, C-34) (Fig. 2.6A, Table 5.1). Probes were either unmethylated or methylated at C-53 on the coding strand. These probes were digested with SnaBI, and the extent of cleavage was assayed via autoradiograph. We found that while SnaBI is able to cut the unmethylated probe, the methylation of C-53 on the coding strand significantly inhibits digestion (Fig. 2.5). This finding suggests that any assays of DNA methylation in the *Ifng* promoter using SnaBI would be unable to distinguish between symmetrical and asymmetric methylation and possibly explains why hemimethylation of the *Ifng* has not been previously reported.

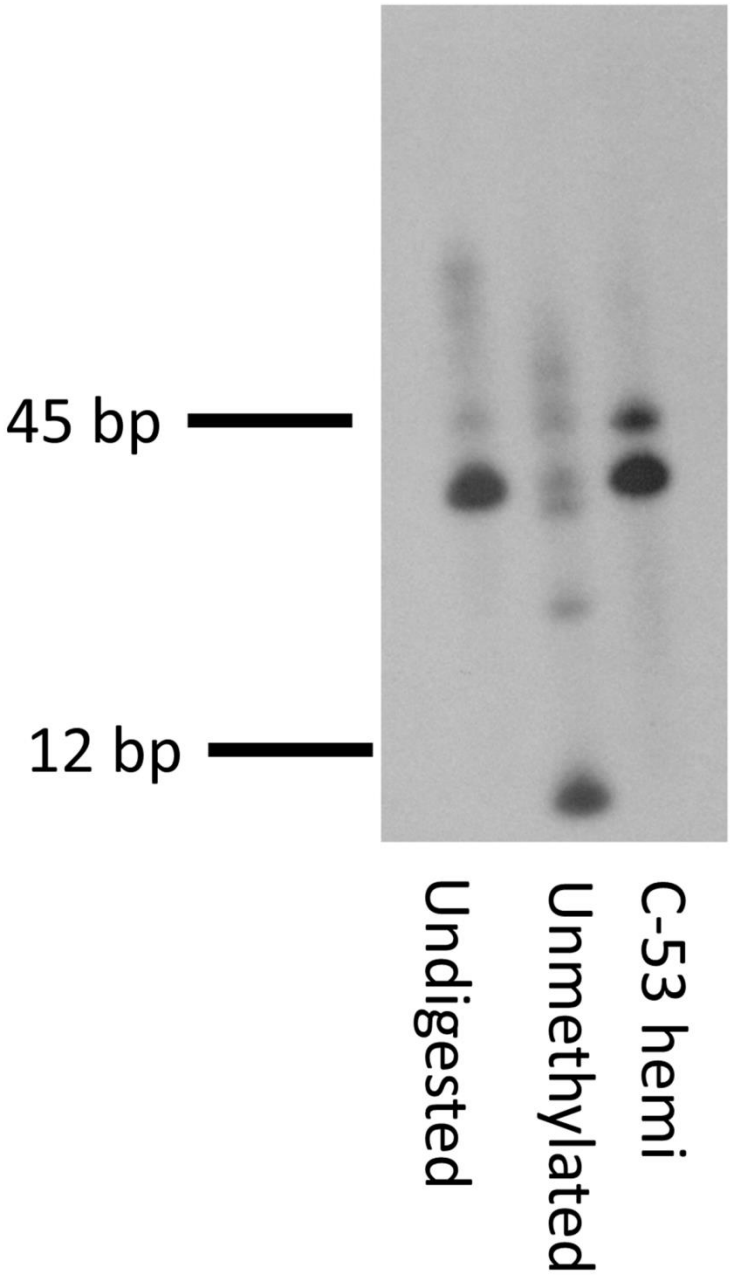


Figure 2.5. SnaBI digestion is inhibited by hemimethylation at C-53. Unmethylated (center lane) or C-53 probes (right lane) (Fig. 2.6A, Table 5.1) were labeled with ^{32}P and then digested to completion with SnaBI. The reactions were resolved via electrophoresis on a 20% acrylamide gel and visualized via autoradiography. Undigested, radiolabeled probe (left lane) was included as a negative control for digestion.

Asymmetrical methylation impacts transcription factor binding to the *Ifng* promoter

The -53 CpG dinucleotide is situated in a CREB/ATF binding site (Jones and Chen, 2006) and adjacent to a known T-bet binding site (Tong et al., 2005). Previous work had demonstrated that the binding of members of the CREB/ATF family of transcription factors could be inhibited by symmetric DNA methylation (Jones and Chen, 2006; Sunahori et al., 2009), but little was known about the effects of hemimethylation on transcriptional regulation. Therefore, we investigated whether hemimethylation could impact the ability of transcription factors to bind the *Ifng* promoter.

To determine the effects of coding-strand specific methylation of the *Ifng* promoter on the formation of protein-DNA complexes, we performed electrophoretic mobility shift assays (EMSA) using the hemimethylated and non-methylated probes based on the *Ifng* promoter as described above, along with a probe having all three proximal CpGs methylated on the coding strand (Fig. 2.6A, Table 5.1). Hemimethylation of the -53 CpG or of all three CpG dinucleotides in the probe impaired the formation of the slower migrating complex (Figure 2.6B, filled arrow). To determine the effects of hemimethylation on the relative affinity of the slower migrating complex, we turned to a competition assay using labeled unmethylated probe (Fig. 2.7). Competition assays using unlabeled competitor DNA and labeled unmethylated probed confirmed that the mobility shift bands represented sequence-specific binding. Moreover, 10-fold more hemimethylated cold competitor (relative to unmethylated probe) was needed to attenuate probe binding to the slower complex (Fig. 2.7). Quantification of the results of three such experiments combined with regression modeling verifies that hemimethylation intereferes with th binding of proteins to the *Ifng* promoter (Fig. 2.8).

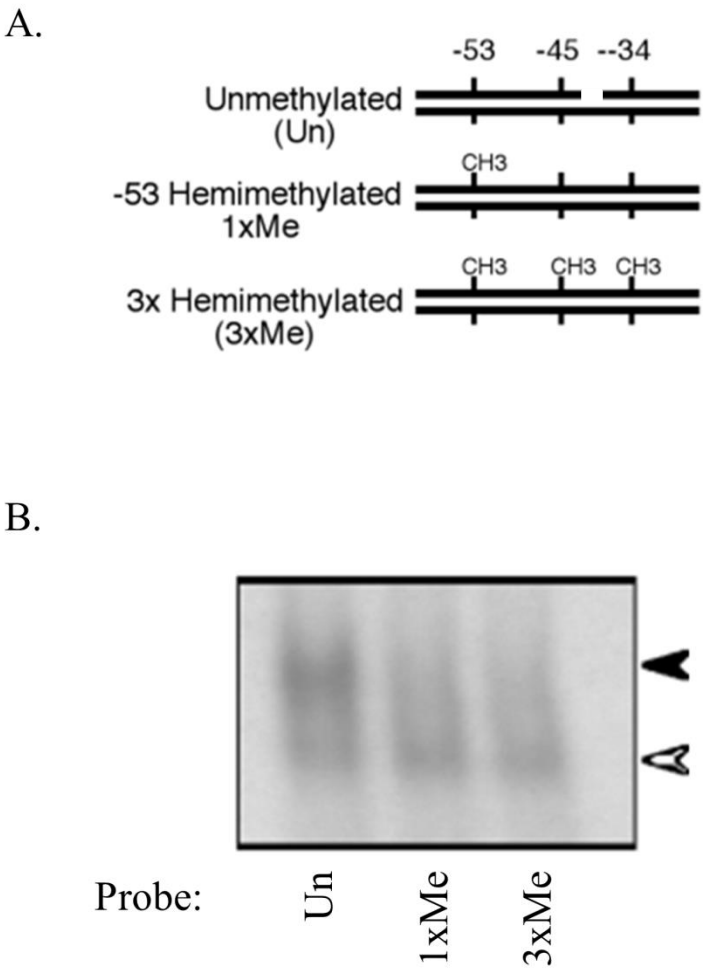


Figure 2.6: Inhibition of protein-DNA interaction by hemimethylation. A. Probes based on the *Ifng* promoter were generated with no methylation (top), methylation at the -53 C nucleotide of the coding strand, or with methylation of the -53, -45, and -34 C nucleotides of the coding strand. B) Electrophoretic mobility shift assay (EMSA) using nuclear extract from primary Th1 cells shows two bands (upper and lower arrowheads) with the unmethylated probe, and altered complex formation with the hemimethylated probes.

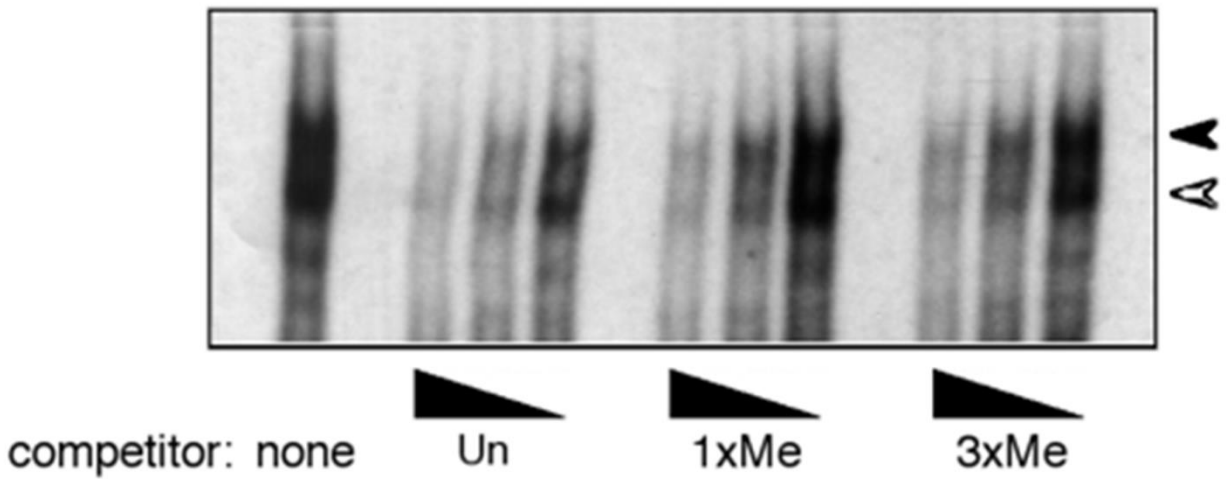


Figure 2.7. Effects of hemimethylation on protein-binding affinity. Competition assays were carried out with radiolabeled unmethylated probe, and either an equimolar, 10-fold excess, or 100-fold excess of competitor. Results are representative of three experiments.

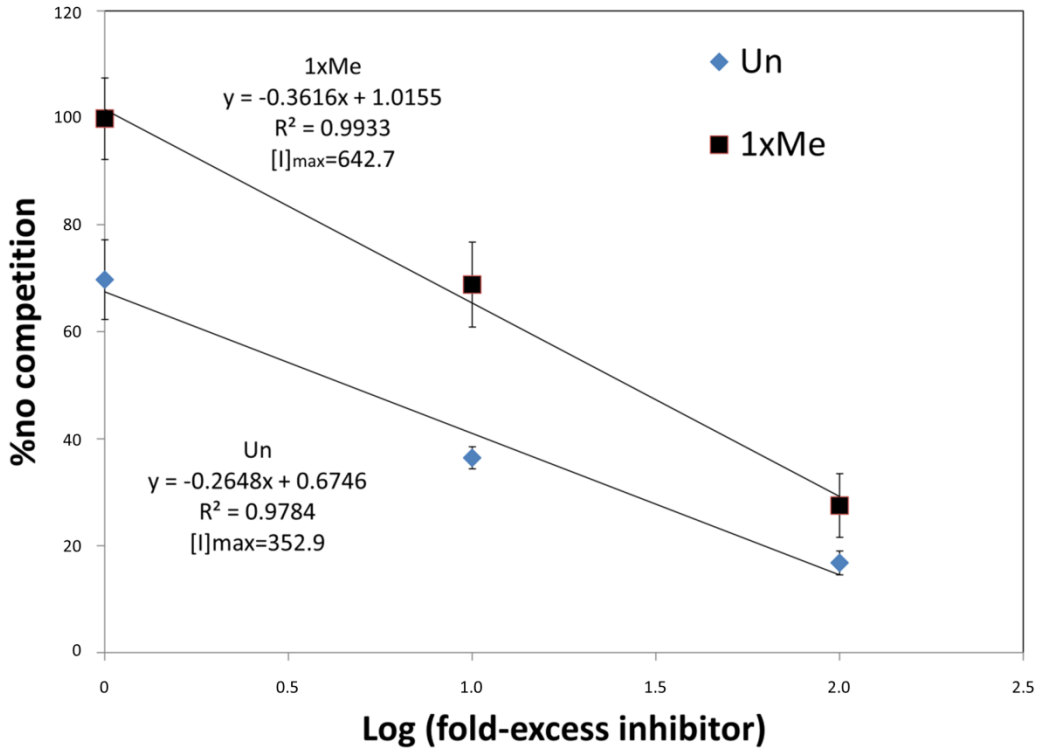


Figure 2.8. Calculation of relative affinities of unmethylated and C-53 hemimethylated (1xMe) probe. Results from three independent experiments were quantified, and regression equations were used to extrapolate the concentration which would yield complete inhibition ([I]max).

In order to identify the slower migrating complex, we then employed antibody blocking/supershift assays using antibodies against CREB1, ATF2, and c-Jun (Fig. 2.9). Only the CREB1 antibody affected the formation and position of the slower migrating complex, from which we inferred that the hemimethylation-sensitive transcription factor was CREB1.

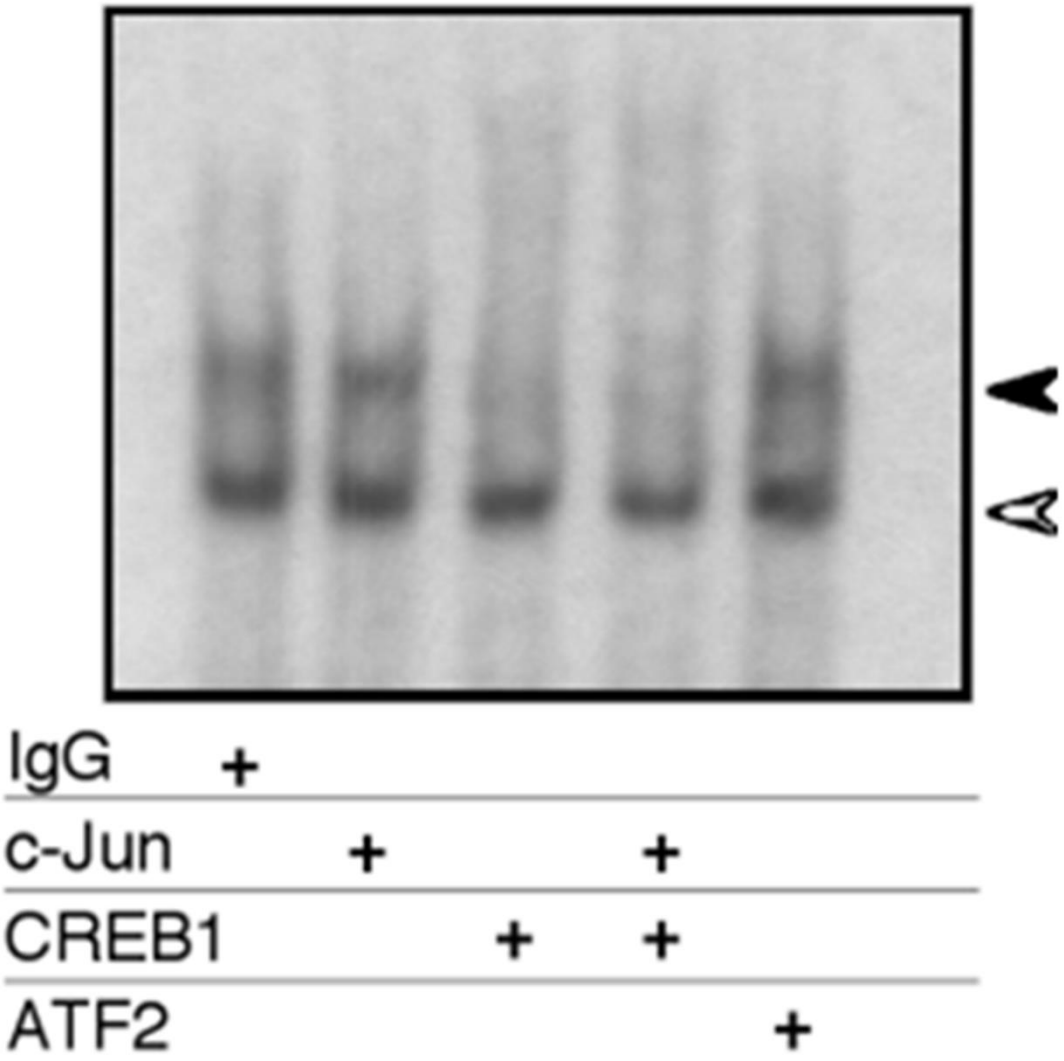


Figure 2.9. Identification of the slower migrating band as a CREB1 containing complex. Electrophoretic super-shift assays were carried out using normal IgG, anti-c-Jun, anti-CREB1, and anti-ATF2.

CREB1 and the *Ifng* promoter

After identifying CREB1 as a transcription factor capable of binding unmethylated, but not hemimethylated probes derived from the *Ifng* promoter, we then set out to determine the functional significance of CREB1 in the regulation of IFN-γ production. If the recruitment of CREB1 to the *Ifng* promoter is regulated by hemimethylation, we would expect it to be present at the *Ifng* promoter in Th1 but not Th2. To test this hypothesis, we preformed chromatin immunoprecipitations (ChIPs, see Fig. 5.1 for workflow) with an anti-CREB1 antibody and an anti-acetyl histone H3 antibody as a control for precipitation (Fig. 2.10). We were able to detect CREB1 occupancy at the *Ifng* promoter in Th1 cells, but not Th2 cells, demonstrating a lineage specific recruitment of CREB1.

As CREB1 is recruited to the *Ifng* promoter in a Th1-specific, methylation-sensitive manner, we then wished to determine what effects CREB1 exerted on promoter activity. To this end, we nucleofected primary Th1 cells with a minimal *Ifng* promoter reporter luciferase construct alongside either a CREB1 expression vector or an empty vector control (Fig. 2.11). We found a striking increase in reporter activity in CREB1 nucleofected samples relative to empty vector controls, demonstrating that CREB1 is capable of transactivating a minimal *Ifng* promoter. One signaling pathway known to activate CREB/ATF family members is the cAMP responsive protein kinase-A (PKA). Interestingly, activation of PKA via dibutyrl-cAMP (db-cAMP) has been shown to inhibit the production of IFN-γ (Bartik et al., 1994; Shin et al., 1998). We therefore set out to test what effects exogenous expression of CREB1 would have in cells treated with db-cAMP. We found that db-cAMP was unable to repress reporter activity in CREB1 nucleofected samples (Fig. 2.12). This result suggests that the repressive effects of PKA activity are mediated by other transcription factors than CREB1, although more work is

needed to determine the relationship between CREB1 and PKA signaling in the regulation of IFN-γ production.

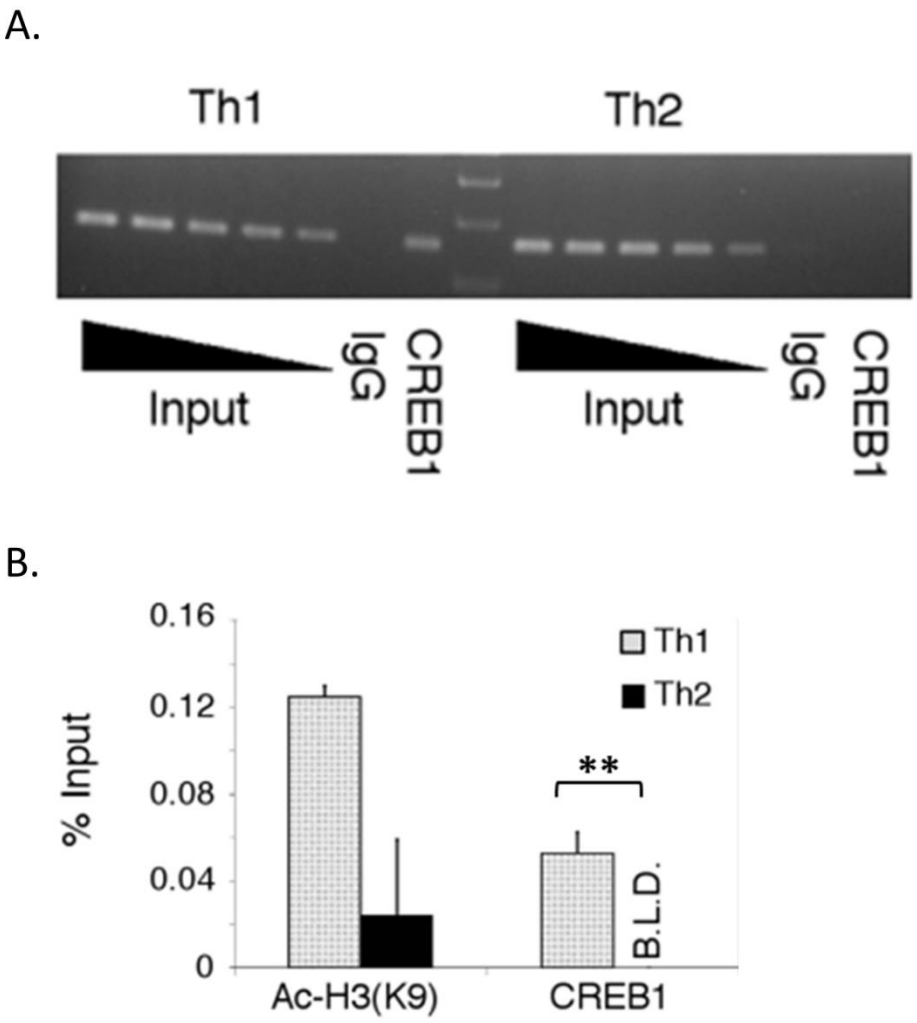


Figure 2.10. CREB1 is recruited to the *Ifng* promoter in Th1, but not Th2 cells. ChIPs for CREB1 and Ac-H3(K9) were carried out on chromatin from primary Th1 and Th2 effectors. Results are shown as a representative image (A.), and were quantified via phosphor imaging to determine the precipitated DNA as a percent of input (B). Results are from three independent experiments. **p<0.01

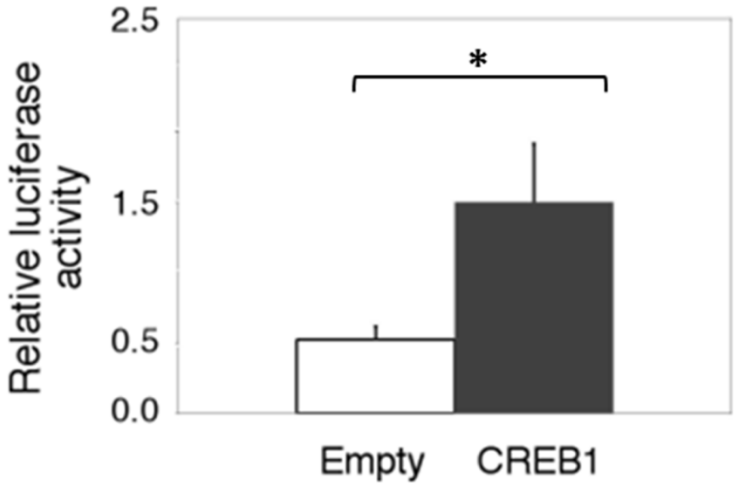


Figure 2.11. CREB1 can transactivate a minimal *Ifng* promoter. Primary Th1 cells were nucleofected with a minimal *Ifng* promoter-luciferase construct and pSport6 or pSport6-CREB1, luciferase activities were normalized to GFP expression. Shown are mean (± SEM) data from three independent experiments. (* $p \leq 0.05$)

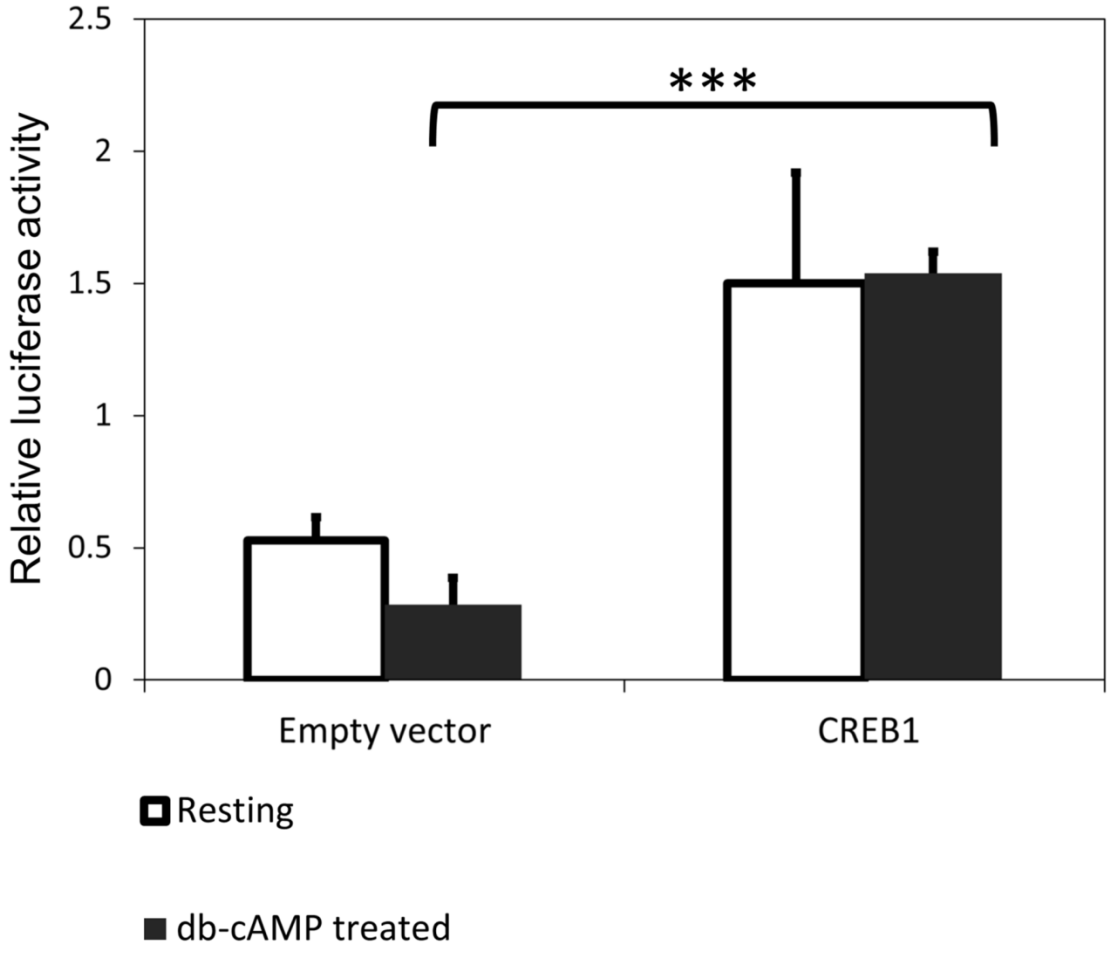


Figure 2.12: Dibutyrl-cAMP driven inhibition of *Ifng* promoter activity is blocked by exogenous CREB1 expression. Th1 effectors were nucleofected as in Fig. 2.11, and then treated with 5μM db-cAMP for four hours. Luciferase activity was then measured and normalized to GFP expression. The resting samples were reproduced from Fig. 2.11. Results shown are from three independent experiments. (*** $p<0.001$)

Conclusions

In this chapter we have described a novel and functional epigenetic modification of the *Ifng* promoter that is reproducibly found in Th2, but not Th1, effectors. We have shown that the DNA methylation of the *Ifng* promoter occurs preferentially on the coding strand as naïve cells develop towards a Th2 effector fate. We have also shown that this asymmetric methylation is sufficient to inhibit the binding of transcription factors. We have identified CREB1 as a hemimethylation-sensitive transactivator of the *Ifng* promoter that is present at the promoter in Th1, but not Th2 cells. Furthermore, while we have reproduced reported data in which the PKA-agonist db-cAMP decreased transcription from an *Ifng* promoter-based construct (Shin et al., 1998), we have also shown that exogenous expression of CREB1 blocks the inhibitory action of db-cAMP. From this, we infer that activation of CREB1 during Th1 responses is accomplished by PKA-independent mechanisms.

III. REGULATION OF IFN-γ PRODUCTION IN Th2 MEMORY CELLS

Absence of promoter methylation in Th2-derived memory cells

Given our findings of asymmetric methylation at the *Ifng* promoter in Th2 effectors, we then wished to determine if the methylation status of the *Ifng* promoter in memory Th2 cells. Th2-derived memory cells were generated as per our previous work (Adeeku et al., 2008), and the process is summarized in Figure 3.1. DNA was extracted from cells at the time of transfer (Th2 effector samples) and upon recovery 4-8 weeks after transfer into either athymic (BALB/c nude) or wildtype BALB/c recipients. As expected, cells transferred into both recipient types underwent homeostatic expansion (Fig. 3.2) and acquired the ability to produce IFN-γ after restimulation and growth under Th1 conditions (Figure 3.3).

Strand-specific analysis of DNA methylation in DNA recovered from purified memory cells showed a decrease in CpG methylation across the coding strand of *Ifng* promoter (Fig. 3.4 and 3.5), and little change in the methylation density of the non-coding strand. Most strikingly, coding strand methylation of the crucial -53CpG was almost entirely absent in all analyzed sequences (Fig. 3.5B). Given that cells have divided post-transfer as previously discussed, we have developed a model in which the hemimethylation in Th2 effectors is diluted out over the course of multiple cell divisions, allowing for a passive, promoter specific mechanism of demethylation (Fig. 3.6).

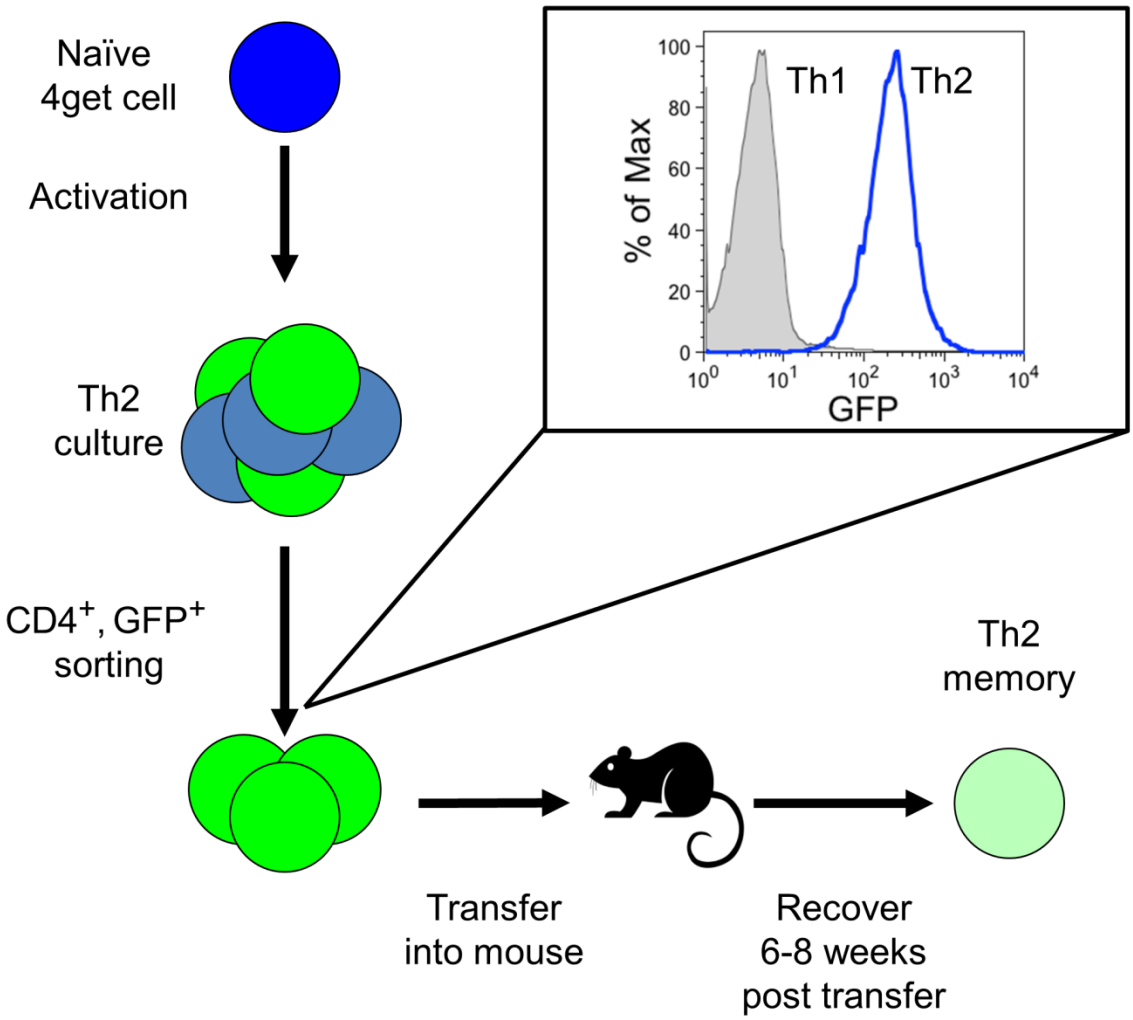


Figure 3.1. Generation of memory cells from IL-4 producing Th2 effectors. Schematic of memory cell generation. Splenocytes expressing the transgenic DO11.10 receptor and the 4get bicistronic IL-4 reporter allele are raised under Th2 conditions for four days. The cells are then sorted for $CD4^+$, $DO11.10^+$, GFP^+ cells. These are expanded for a week, and then transferred into a recipient (DO11.10 negative) mouse. After 6-8 weeks, recipient mice are harvested, and memory cells are identified via the DO11.10 TCR. Inset: Flow cytometric plot for GFP in expressed in Th1 (solid) and Th2 (outline) effectors.

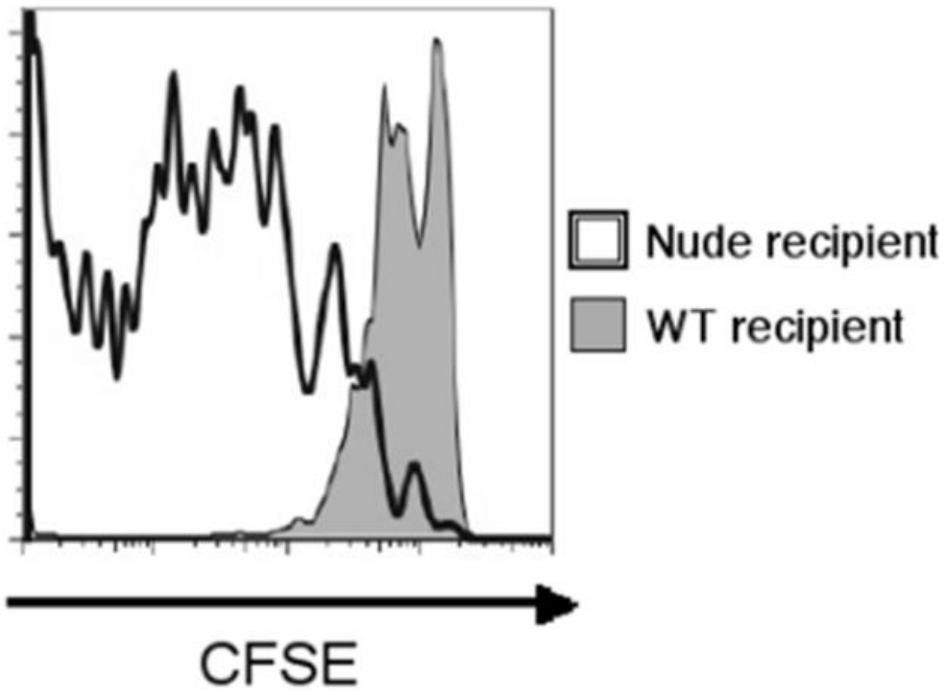


Figure 3.2: Homeostatic expansion in BALB/c and BALB/c Nude recipients. A. Schematic of CFSE partitioning as a means to track division history in a cell population. Th2 cells were labeled with CFDA-SE and transferred into recipient mice. Shown are the CFSE staining profiles of donor-derived cells recovered from wildtype (shaded) and Nude (outline) recipients twelve days post-transfer. Results are representative of two independent experiments.

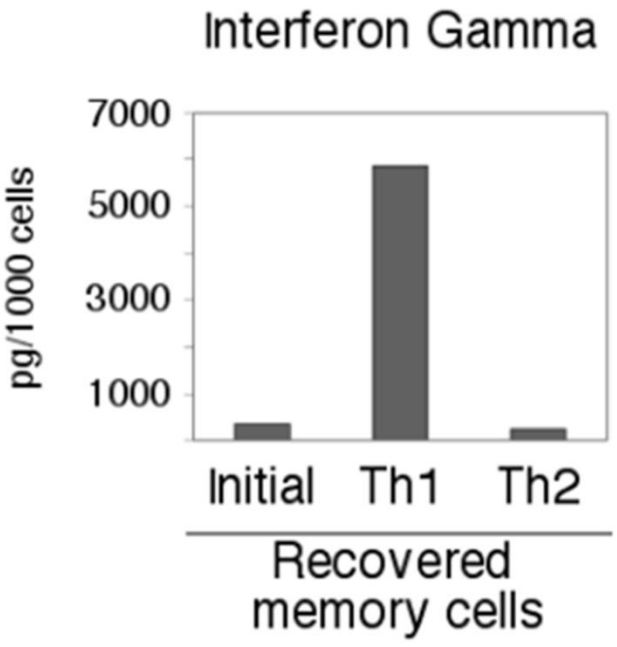


Figure 3.3. IFN-γ production in Th2-derived memory cells. Th2-derived memory cells were restimulated for 36 hours with peptide+APCs at the time of recovery (initial) and after one week of restimulation under Th1 or Th2 conditions, and the concentration of IFN-γ in the supernatant was measured. Results are shown as picograms of IFN-γ produced per 1,000 donor-derived (DO11.10 TCR^+ $CD4^+$) cells, and are representative of four independent experiments consistent with previous studies (Adeeku et al., 2008; Löhning et al., 2008).

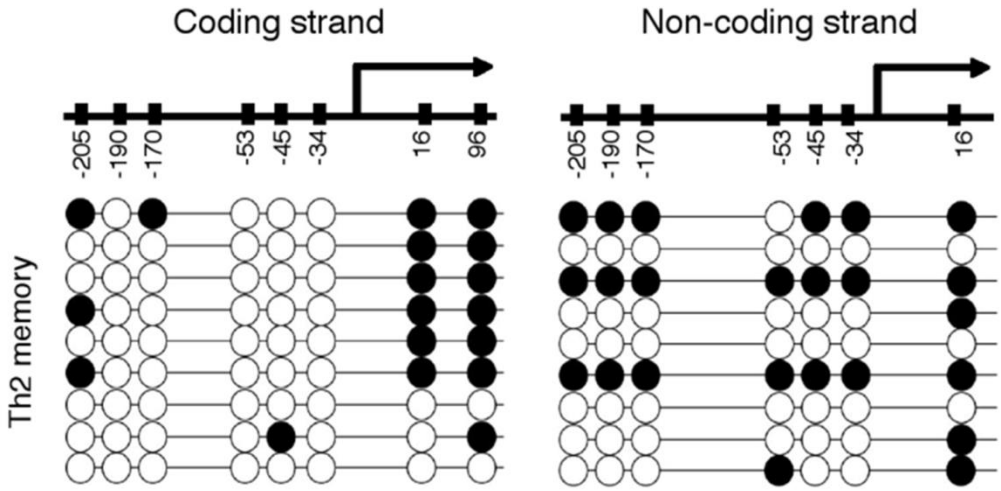


Figure 3.4. Absence of coding strand methylation in memory Th2 cells. Shown are representative sequences of bisulfite modified DNA as in Figure 2.2. Results are from four independent experiments.

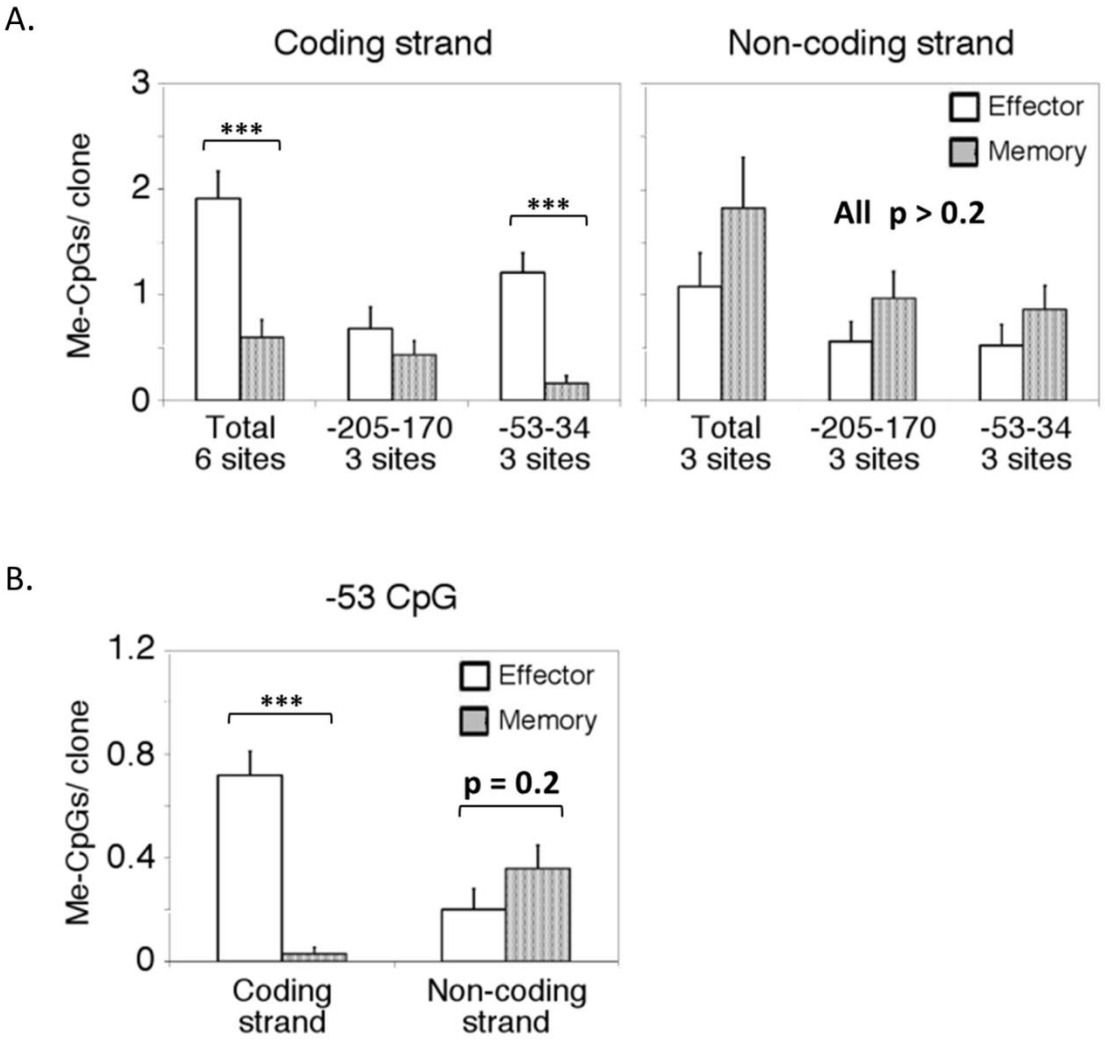


Figure 3.5. Quantification of methylation of the *Ifng* promoter in Th2-derived memory cells. A. . Quantification of the methylation of all CpG dinucelotides upstream of the TSS, CpGs -205-170, and -53-34. Shown are the mean of four independent experiments (±SEM). B. The mean (±SEM) frequency of methylation at the -53CpG dinucleotide in Th2 memory cells is shown, with effector data from Fig. 2.2 shown for comparison *p<0.05, ** p<0.01, ***p<0.001.

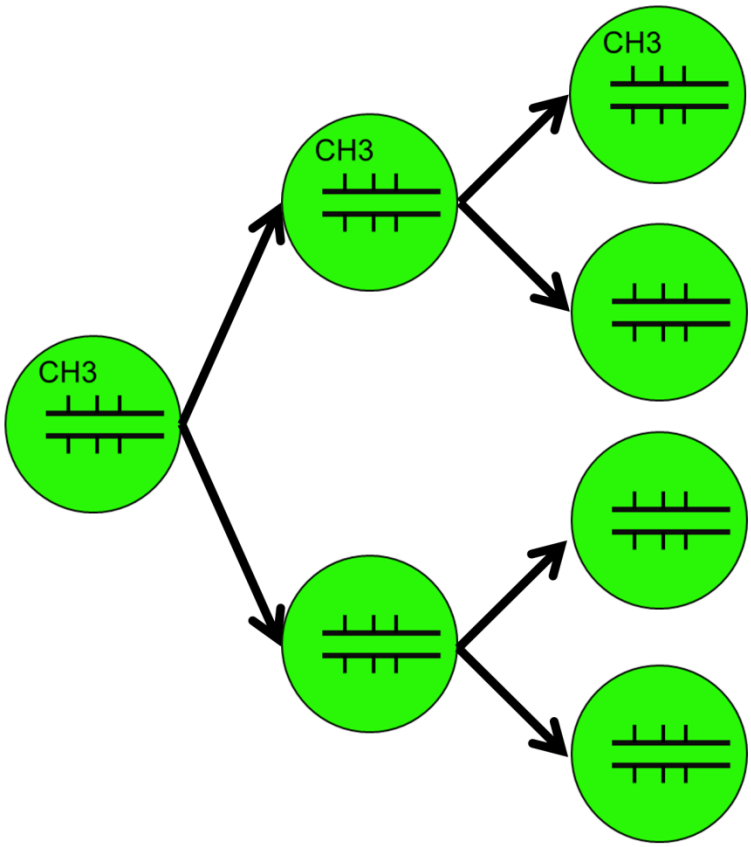


Figure 3.6. A model of cell division dependent passive demethylation. Shown is a model of demethylation in which hemimethylation is diluted out over the course of successive cell divisions.

Requirement for STAT4 in flexible production of IFN-γ

The development of Th1 effector cells from naïve $CD4^+$ T cell precursors is highly dependent on IL-12-induced STAT4 and, in most settings, on T-bet (Jacobson et al., 1995; Szabo et al., 2000, 2002). IL-12 is required for the facultative induction of IFN-γ production by memory Th2 cells after recall stimulation in vitro and in vivo (Adeeku et al., 2008; Hegazy et al., 2010; Löhning et al., 2008). However, the IL-12 receptor elicits multiple intracellular signals (Fig. 1.3) (Rao et al., 2012; Yoo et al., 2002), and which of these pathways are essential for the plasticity of gene expression is not known.

To investigate whether STAT4 is required for the production of IFN-γ by Th2-derived memory cells we generated memory cells from STAT4 deficient or T-bet deficient Th2 effectors and compared the amount of IFN-γ produced upon initial restimulation with antigen, along with that produced after one week of expansion under Th1 or Th2 recall conditions. After one week of Th1 culture, neither STAT4-deficient nor T-bet deficient Th2 memory cells secreted as much IFN-γ upon restimulation with peptide antigen and APC as the matched wildtype control. However, the amount of IFN-γ detected in the supernatants of the transcription factor deficient cells still was above the background of the assay (Fig. 3.7). Comparison of the relative amounts of IFN-γ produced in each experiment revealed that cultures of T-bet deficient or STAT4-deficient memory cells consistently produced about 30% of the IFN-γ secreted by the matched wildtype control (Fig. 3.8). This finding was consistent between cells recovered from both athymic nude mice and from wildtype recipients (Figure 3.8). Intracellular cytokine staining showed that upon recovery, all three genotypes maintain commitment to produce IL-4 upon recall stimulation (Fig. 3.9A), but approximately 31% of wildtype donor-derived cells have become IFN-γ^+, IL-4^+ double producers after 1 week of Th1, but not Th2 recall culture (Fig. 3.9B). Furthermore, no IFN-γ production

could be detected via ICCS in T-bet or STAT4-deficient Th2 memory cells, even after 1 week of Th1 culture (Fig. 3.9B). These findings demonstrate that STAT4 is required for the flexible expression of the *Ifng* gene and confirm previous reports that T-bet is also required (Hegazy et al., 2010).

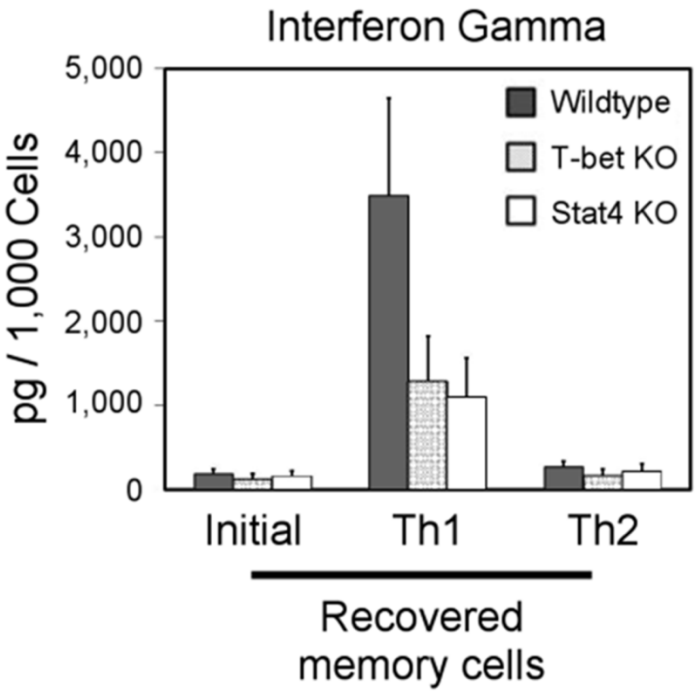


Figure 3.7. IFN-γ secretion is impaired in T-bet or STAT4 deficient Th2-derived memory cells. The mean (±SEM) amount of IFN-γ (in pg) secreted by 1,000 cells in 36 hours is shown at the time of harvest (initial), and after one week of Th1 or Th2 recall conditions. Shown are the averages for at least 6 independent experiments.

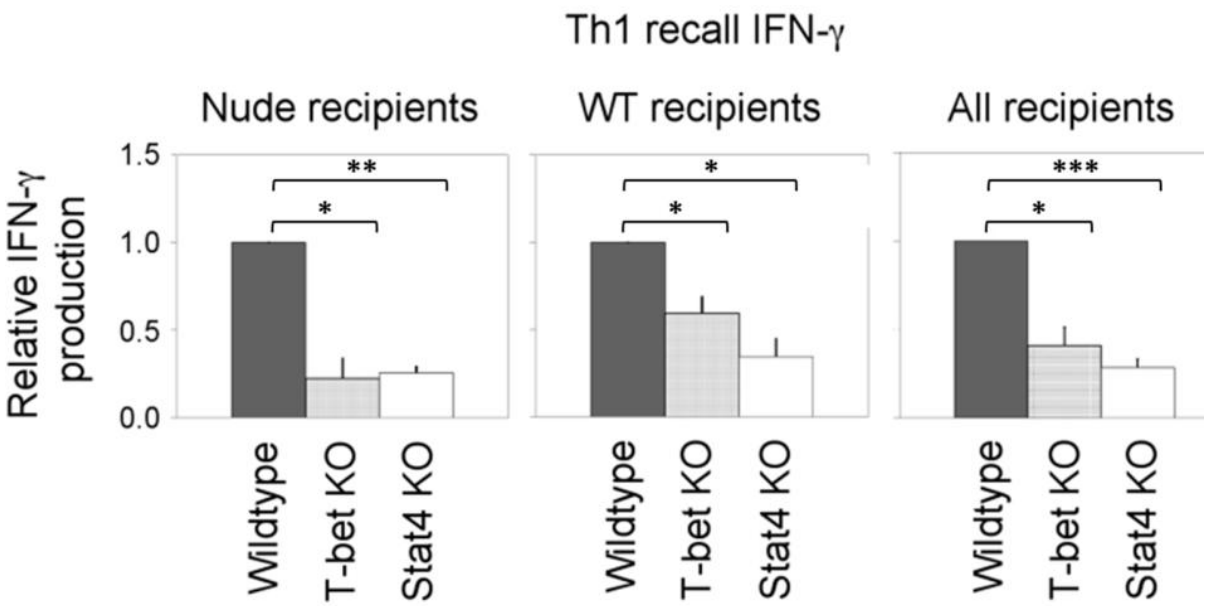


Figure 3.8. IFN-γ production of T-bet or STAT4-deficient Th2 memory cells relative to wildtype Th2 memory cells. The amount of IFN-γ produced by Th2 memory cells after one week of Th1-recall culture was calculated as a ration of mutant to wildtype. Shown are the average ratios (±SEM) for cells recovered from nude recipients (left panel, four independent experiments), wildtype recipients (center panel, four independent experiments), or all recipients (right panel, 8 independent experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

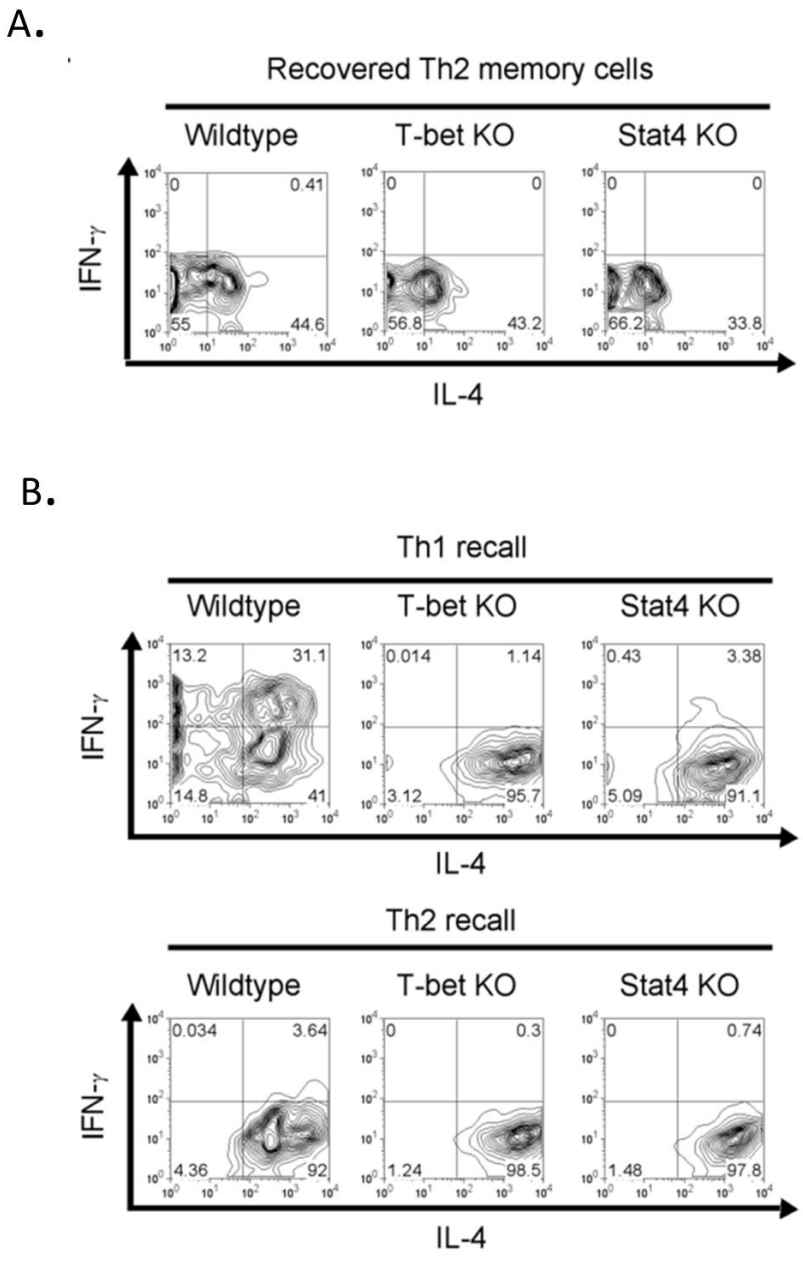


Figure 3.9. Intracellular cytokine staining of T-bet-deficient, STAT4-deficient, and wildtype Th2-derived memory cells. Intracellular staining for cytokines was carried out at the time of harvest (Panel A) or after one week of Th1 or Th2 recall stimulation (B.) FACS plots shown are gated for $CD4^+$, DO11.10 TCR^+ cells.

Stat4-deficient cells have normal T-bet induction and homeostatic expansion

We then investigated potential mechanisms by which STAT4 could facilitate flexible IFN-γ production in memory Th2 cells undergoing a Th1 recall response. Previous studies have shown that STAT4 is required for the production of IFN-γ in a primary response by $CD4^+$ T cells (Jacobson et al., 1995; Kaplan et al., 1996b; Zhang and Boothby, 2006). Beyond this direct transactivation some studies have also shown that STAT4 is required for the maintenance of T-bet expression (Schulz et al., 2009; White et al., 2001), suggesting that any requirement for STAT4 may be due to its role in maintaining T-bet expression. To test this, we carried out FACS staining for T-bet on Th1 effectors, and Th2 effectors under Th1 and Th2 recall responses from both wildtype and STAT4-deficinet backgrounds. While we were able to reproduce the reported results that STAT4-deficiency results in an inability to maintain T-bet expression in effector Th1 cells (White et al., 2001) (Fig. 3.10A, top panel), we found that at least half of donor-derived cells expressed T-bet at a level comparable to Th1 effectors when cultured for one week under Th1 conditions (Fig. 3.10). We therefore concluded that the requirement of STAT4 in the production of IFN-γ by memory Th2 cells is not due to an ability to regulate T-bet expression.

Finally, since we are proposing a model in which homeostatic expansion dilutes out repressive CpG methylation, it is possible that the requirements for STAT4 and/or T-bet could arise from reduced cell division activity after transfer, resulting in a higher degree of methylation being retained. Accordingly, we tested if the rates of division were slower for *Tbx21* -/- or *Stat4* -/- Th2 cells in recipient mice. *In vivo* CFSE partitioning assays showed that there was no defect in rates of division for DO11.10 Th2 cells that were T-bet- or STAT4-deficient cells compared to controls that were wildtype with respect to the transcription factors (Figure 3.10B). To assay for possible defects longer after transfer, recipients of wildtype, *Tbx21* -/-, or *Stat4* -/- Th2 effectors were

pulsed with BrdU prior to harvest, and the incorporation of BrdU into genomic DNA was measured. We found that the transferred cells had almost completely exited cell cycle and that the low rates of DNA synthesis were similar for all genotypes (Fig. 3.10C). Therefore, alterations in the number or frequency of cell divisions post transfer could not account for differences in plastic *Ifng* expression between wildtype and T-bet-deficient or STAT4-deficient samples.

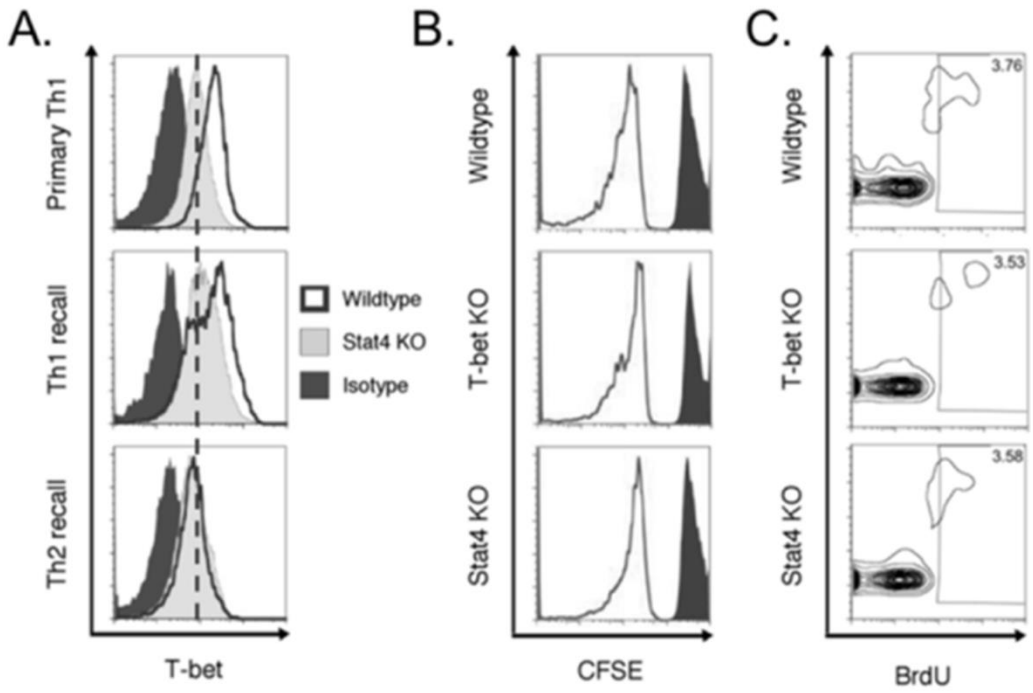


Figure 3.10. T-bet induction and homeostatic expansion in STAT4-deficient Th2 memory cells. (A) T-bet expression was measured via FACS in WT and *Stat4-/-* Th1 effector (top panel), recovered Th2 memory cells after one week of Th1 recall stimulation (middle panel), and recovered Th2 memory cells after one week of restimulation under Th2 conditions (bottom panel). (B) Th2 effector cells from Wildtype, *Tbx21*-/-, and *Stat4*-/- DO11.10 mice were labeled with CFDA-SE and transferred into BALB/c nude recipients. After two weeks, donor derived cells were analyzed for CFSE partitioning. The initial level of CFSE staining (dark outline) are shown for each cell type. (C) In vivo BrdU incorporation into Th2 effector cells from Wildtype, *Tbx21*-/-, and *Stat4*-/- DO11.10 mice five weeks post-transfer. Recipient animals were injected with BrdU prior to harvest, and BrdU incorporation in donor derived cells was determined via FACS with an anti-BrdU antibody. All events shown are gated on $CD4^+$, DO11.10 TCR^+ viable cells.

Alterations in CpG methylation in *Tbx21-/-* and *Stat4 -/-* Th2 effector and memory cells

Another possible mechanism that would account for the need for both STAT4 and T-bet in plastic *Ifng* expression would be that in the absence of these transcription factors, conditions in the effector phase of the T cell response would not allow for flexible cytokine production in the memory phase. This could be in part due to increased maintenance of CpG methylation or from other epigenetic mechanisms. To test this, DNA was extracted from *Tbx21-/-* and *Stat4-/-* effector Th2 cells after 11-12 days of Th2 culture and CpG methylation of the *Ifng* promoter was analyzed as before (Figs. 2.3, 3.5). We found that there was little change in the methylation densities on the coding strands of T-bet or STAT4 deficient Th2 effectors (Fig. 3.11A). Surprisingly, the non-coding strands of the transcription factor Th2 cells demonstrated a significantly higher degree of methylation across the assayed region of *Ifng* promoter (Fig. 311A). As opposed to the wildtype effectors, *Tbx21-/-* and *Stat4-/-* Th2 effectors displayed symmetric CpG methylation of the *Ifng* promoter (Fig. 3.11B).

According to the model of cell-division driven passive demethylation we proposed, this symmetric methylation would be better maintained into the memory phase. To test this, we carried out methylation analysis of DNA from *Tbx21-/-* and *Stat4-/-* Th2 memory cells. As predicted by our model, we find that CpG methylation of both strands has been maintained, especially at the crucial -53CpG (Fig. 3.12). This maintenance of repressive DNA methylation likely forms one of the several barriers to *Ifng* expression in T-bet or STAT4-deficient memory cells.

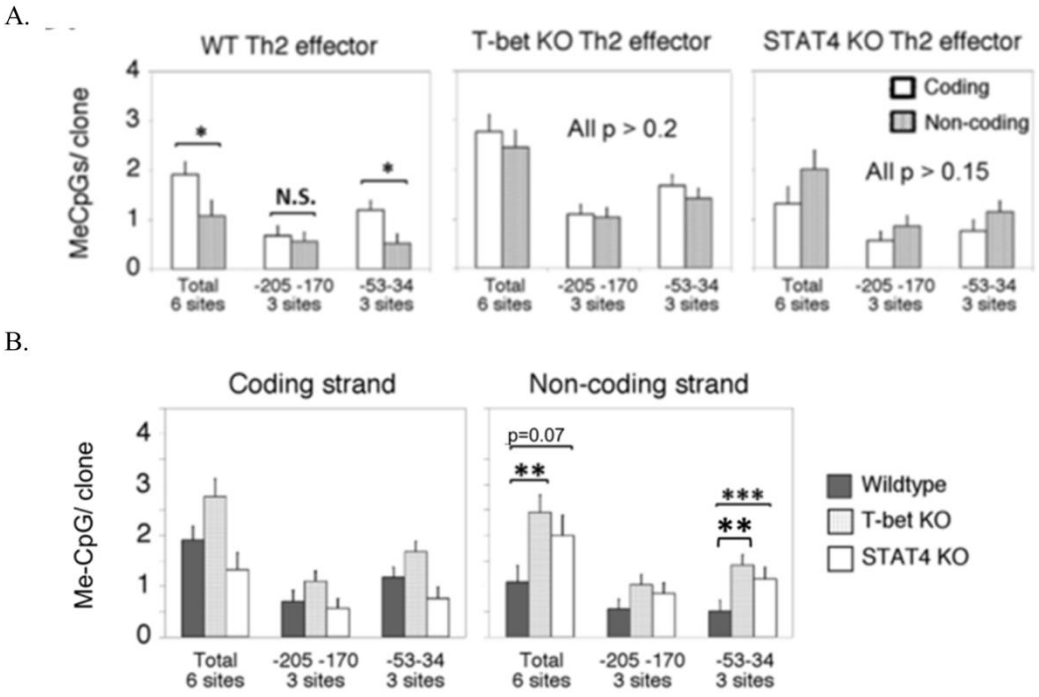


Figure 3.11. Symmetry of methylation in *Tbx21*-/- and *Stat4*-/- Th2 effector cells.

Mean (±SEM) meCpG dinucleotides per clone are shown for *Tbx21*-/-, and *Stat4*-/- Th2 effector cells as in previous figures. WT Th2 effector results from Fig. 2.3 are shown for comparison. Clones from three independent experiments were analyzed for both coding and non-coding strands. (A) Comparison of symmetry of methylation in which the frequency of methylation on coding and non-coding strands from each sample are compared. (B) Comparison of frequencies of methylation between WT, *Tbx21*-/-, and *Stat4*-/-. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

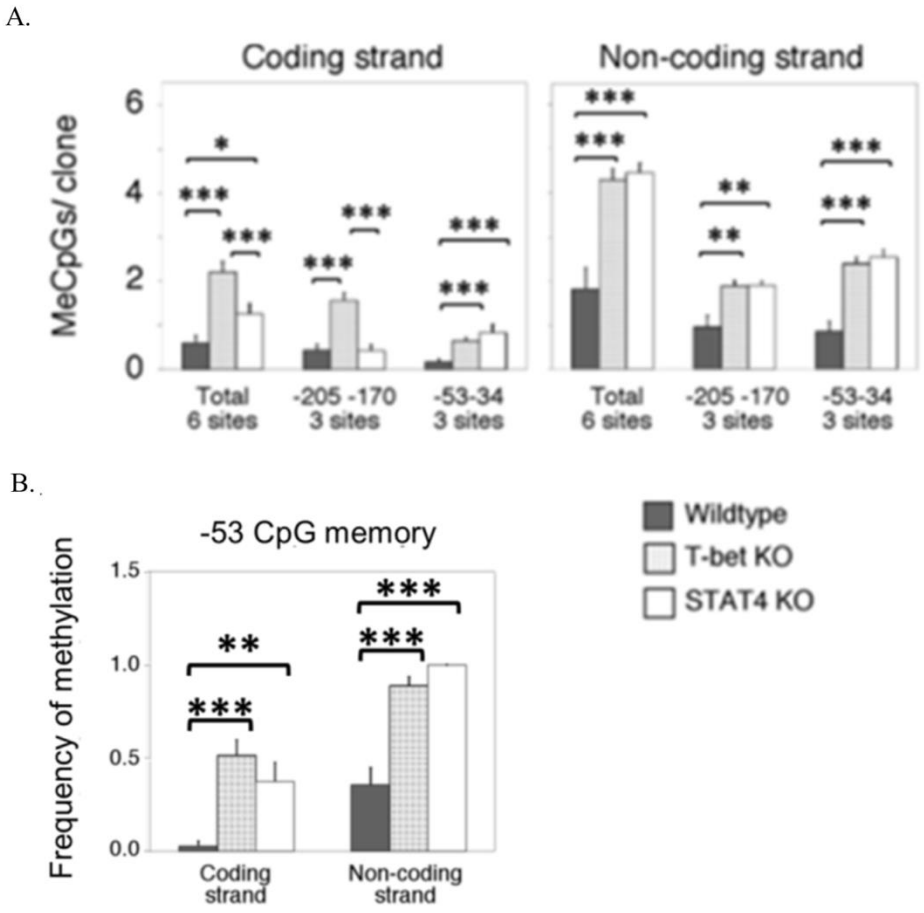


Figure 3.12. Alterations in DNA methylation in *Tbx21l-/-* or *Stat4 -/-* Th2 memory cells. (A) Mean (±SEM) meCpG dinucleotides per clone are shown for *Tbx21*-/-, and *Stat4*-/- Th2 effector cells as in previous figures. WT Th2 memory cell results from Fig. 3.5 are shown for comparison. Clones from three independent experiments were analyzed for both coding and non-coding strands. (B) The frequency of methylation at the -53CpG dinucleotide in memory Th2 cells is shown for coding and non-coding strands. Results are from three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

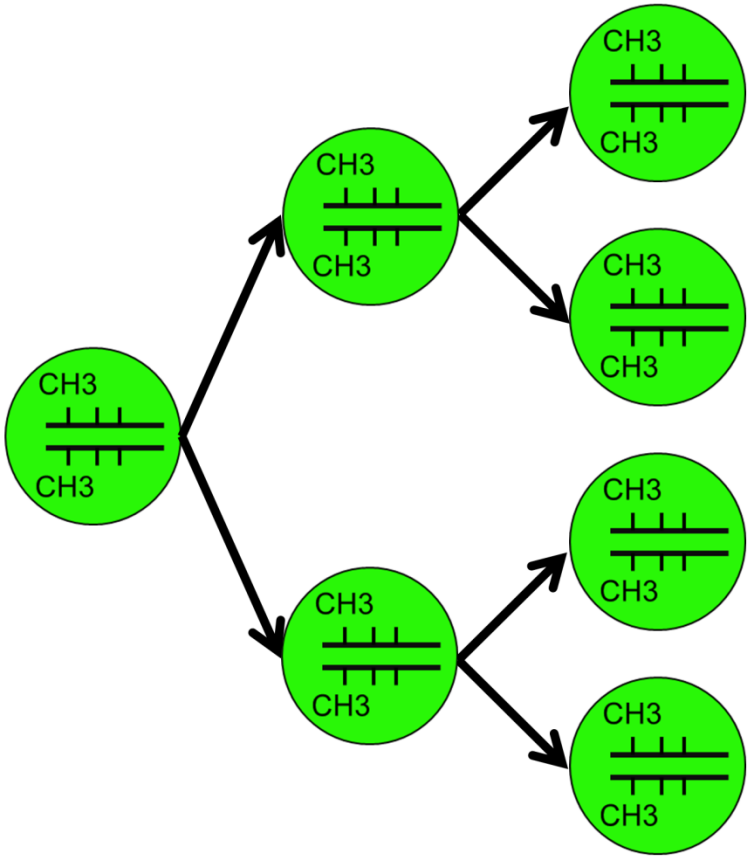


Figure 3.13. Symmetrical methylation inhibits passive demethylation. Shown is a model of methylation status over the course of two cell divisions as per Fig. 3.6. In the case of symmetrical methylation, dilution of CpG methylation would not occur.

Exogenous expression of T-bet in *Tbx21-/-* Th2 effector and Th2 memory cells

Having observed that deficiency of T-bet or STAT4 leads to an epigenetic environment antagonistic towards *Ifng* expression, we then wished to determine if restoring the expression of T-bet in polarized Th2 cells would allow for the production of IFN-γ by Th2-derived memory cells. Previous studies have shown that ectopic expression of T-bet in fully polarized Th2 cells can lead to substantial IFN-γ production (Szabo et al., 2000, 2002) and that over-expression of T-bet can transactivate a fully methylated *Ifng* promoter (Tong et al., 2005). A parallel study in human $CD4^+$ T cells found that many Th2 cells would, under Th1 conditions, switch on their *IFNG* gene. A subset of Th2 cells that could not be switched to IFN-γ producers was found, and this subset was unable to upregulate T-bet under Th1 conditions (Messi et al., 2002). Further studies showed that human $CD4^+$ T cells lose the ability to express *IFNG* after several cycles of polarization, even when T-bet expression is driven by a retroviral vector (Sundrud et al., 2003). However, no studies have touched on the effects of forcing T-bet expression in *Tbx21* deficient Th2-derived memory cells.

To rescue T-bet expression in T-bet deficient Th2 effectors we used retroviral transduction with an MSCV-based retrovector with Thy1.1 as a bicistronic reporter (MSCV-IRES-Thy1.1 or MiT) carrying T-bet cDNA (MiT-T-bet). After one cycle of Th2 differentiation, effector cells were restimulated and transduced with MiT-T-bet or an MiT vector lacking T-bet cDNA (MiT empty vector), and cultured under Th1 and Th2 conditions for another week. After a subsequent restimulation with APCs and antigen, intracellular staining for IL-4 and IFN-γ revealed that T-bet expression forced IFN-γ expression in *Tbx21* -/- Th2 cells (Figure 3.14) This is consistent with previous work carried out in Th2 effector cells (Szabo et al., 2000, 2002; Tong et al., 2005). Thus we were able to reproduce previous results on the effects of exogenous T-bet expression in committed Th2 cells.

In parallel with the intracellular staining, MIT-T-bet transduced cells kept under Th2 conditions were transferred to recipient mice. After three weeks, mice were harvested and cells were restimulated under Th1 and Th2 conditions (as per Fig. 3.3) followed by restimulation with antigen and APCs and intracellular staining (Fig.3.15). We found that donor-derived cells expressing Thy1.1 on their surface also were typically able to produce IFN-γ. In contrast, non-transduced Thy1.1$^-$ cells did not stain for IFN-γ, but did have a high frequency of IL-4 positive cells. Interestingly, culture under Th2 or neutral conditions did decrease the proportion of IFN-γ$^+$ cells, suggesting that the forced expression of T-bet still benefits from IL-12 signaling found under Th1 conditions. We therefore conclude that the expression of T-bet at a sufficient level can overcome the block to IFN-γ production seen in *Tbx21-/-* Th2-derived memory cells. Based on these findings, we propose that multiple barriers to *Ifng* transcription are enacted during Th2 differentiation, and that these barriers are reversed in wildtype Th2 cells as they become memory cells.

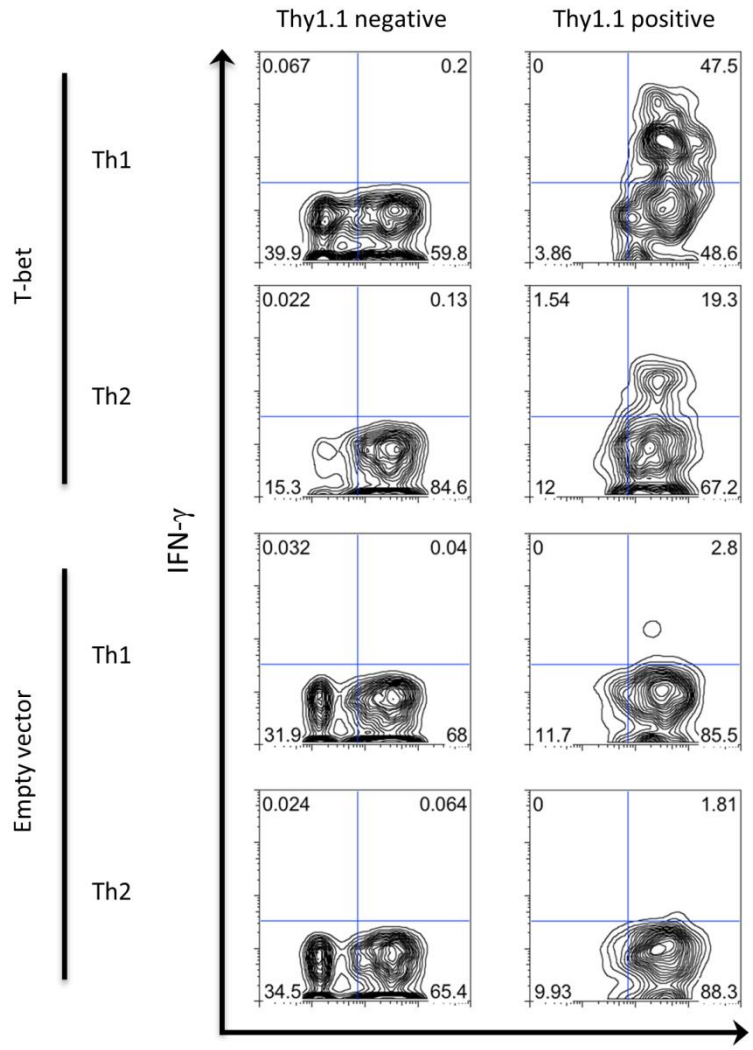


Figure 3.14. T-bet transduced *Tbx21-/-* Th2 effectors produce IFN-γ. *Tbx21-/-* 4get DO11.10 cells were cultured under Th2 conditions. After four days, GFP^+ cells were collected and restimulated via antigen and APCs. Two days after restimulation cells were underwent retroviral transduction with viruses derived from MiT-T-bet or an empty vector control. Two days post-transduction cells were restimulated under Th1 or Th2 conditions and cultured for one week. ICCS was then carried out. Events shown were gated on the viable (as determined by forward and side scatter) $CD4^+$ cells. Data are representative of two experiments.

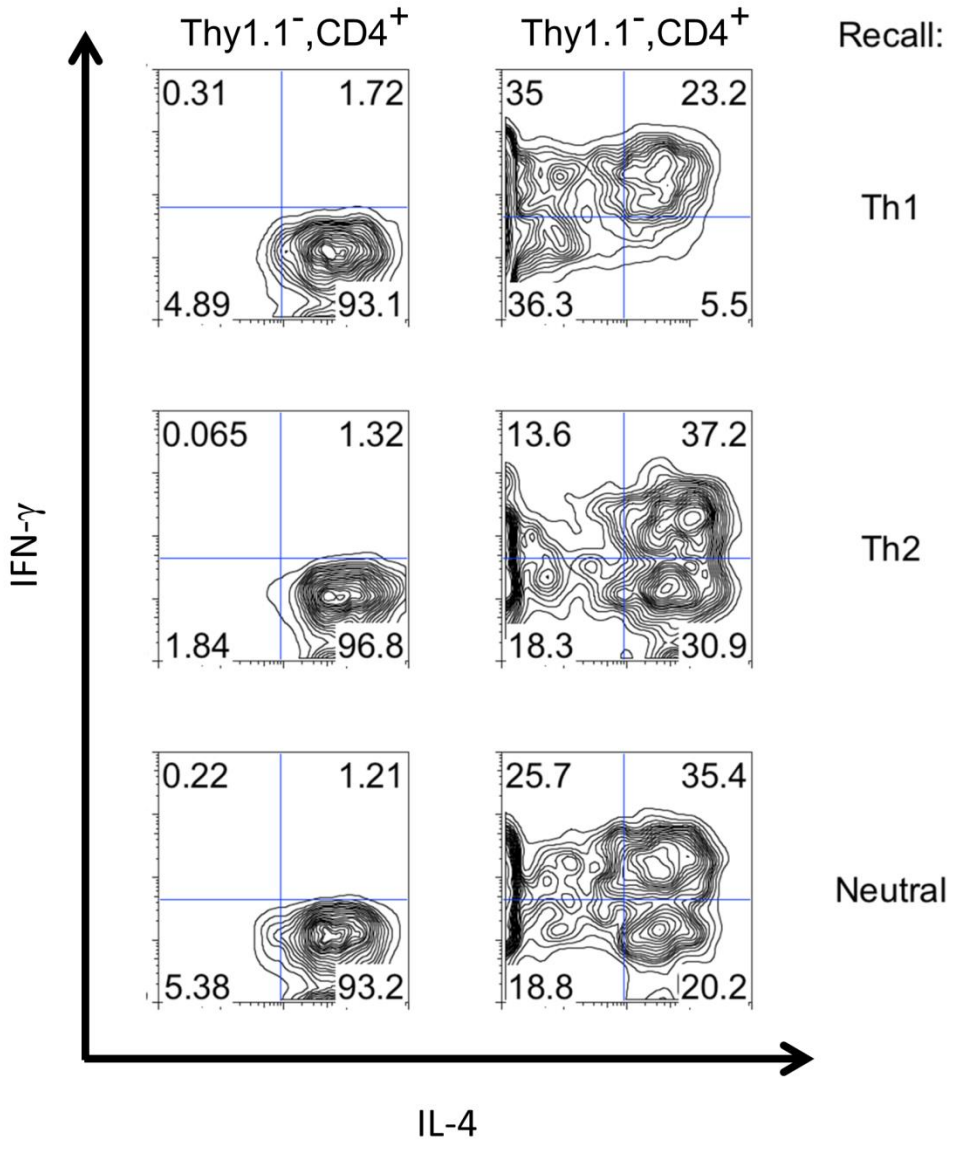


Figure 3.15. Production of IFN-γ by memory cells derived from T-bet transduced *Tbx21*-/- Th2 effector cells. *Tbx21*-/- were transduced as in Fig. 3.14, and then restimulated under Th2 conditions. After one week of culture, Th2 cells were transferred to nude mice. Cells were recovered after four weeks and cultured under Th1, Th2, or neutral conditions for one week. ICCS was then carried out. Events shown are gated as per Fig. 3.14. Results are representative of two independent experiments.

Conclusions

We have found that the CpG methylation of the *Ifng* promoter established during Th2 effector development is not maintained into the memory phase in wildtype cells. We have also demonstrated that STAT4 is required for the flexible production of IFN-γ. Furthermore we have provided evidence that the requirement for STAT4 is not due to a failure to induce T-bet during Th1 recall, and that neither T-bet nor STAT4 alter the rate of homeostatic cell division occurring after the effector phase. Intriguingly, we have found that Th2 effectors deficient in STAT4 or T-bet have symmetrically methylated *Ifng* promoters, and that this methylation is retained in memory cells deficient for either transcription factor. Taken together, these results suggest that T-bet and STAT4 serve to inhibit symmetrical methylation of the *Ifng* promoter, resulting in a hemimethylated state. Promoter hemimethylation is sufficient to inhibit CREB1 binding, but is not maintained through divisions occurring after the end of the effector phase. The absence of repressive methylation alongside the induction of T-bet and activation of STAT4 in a Th1-biased recall response then allows for the flexible production of IFN-γ.

In our work, we have also confirmed previous reports that STAT4 is required to maintain T-bet expression in effector Th1 cells, and that forced expression of T-bet can cause IFN-γ production, even from a fully committed Th2 cell. We have expanded this observation to show that forced T-bet expression in Th2-derived memory cells will also result in indiscriminate IFN-γ production.

IV. Discussion and future directions

The capacity of Th2-derived memory cells to produce significant amounts of IFN-γ when carrying out a recall response under Th1 conditions is an example of natural cell reprogramming. Previous studies into lineage plasticity in $CD4^+$ T cells demonstrated that IL-12, type I interferons, and T-bet are all required for transcription of *Ifng* by memory Th2 cells (Adeeku et al., 2008; Hegazy et al., 2010; Löhning et al., 2008), but little was understood about the molecular mechanisms that underlie this flexible cytokine expression. Foundational work revealed that the *Ifng* promoter undergoes repressive CpG methylation (Fitzpatrick et al., 1998, 1999; Melvin et al., 1995; Winders et al., 2004; Young et al., 1994), but it was unknown whether inhibitory DNA methylation is maintained in Th2-derived memory cells. At the outset of this study, it was also unclear whether STAT4, crucial for IFN-γ expression in Th1 effectors (Jacobson et al., 1995; Morinobu et al., 2002; Zhang and Boothby, 2006), was required for flexible IFN-γ production. In this work, we explored molecular mechanisms underlying cytokine production plasticity. In undertaking this investigation, we have discovered a novel and functional epigenetic state of the *Ifng* promoter and further defined the transcription factor requirements for flexible expression of IFN-γ.

In Th2 effector cells, we made the novel observation that the *Ifng* promoter exhibits asymmetric methylation in committed Th2 effectors. The coding strand DNA preferentially acquires significantly increased methylation relative to the low frequency of meCpG in naïve CD4+ T cells and on the non-coding strand. Furthermore, we have demonstrated that hemimethylation of the *Ifng* promoter was sufficient to alter protein-DNA interactions and inhibit CREB1 binding at a highly conserved sequence required for promoter activity. Consistent with this data, forced expression of CREB1 was able to transactivate a minimal *Ifng* promoter. Using chromatin immunoprecipitation, we demonstrated that CREB1 preferentially bound to the *Ifng* promoter in Th1, but not Th2

effector cells. This was the first report of the presence and inhibitory function of hemimethylation in lymphoid cells. In Th2-derived memory cells we found a striking loss of methylation on the coding strand with overall CpG methylation densities similar to naïve $CD4^+$ cells. As methylation of the *Ifng* has been shown to have a strong repressive effect, these findings led us to infer that the loss of CpG methylation contributes to the flexibility of IFN-γ production. In investigating the transcription factor requirements for the facultative production of IFN-γ, we found that the IL-12-induced factor STAT4 is required along with T-bet. Surprisingly, Th2 effector cells deficient in T-bet or STAT4 had largely symmetric methylation of the *Ifng* promoter and memory cells derived from these effectors maintained promoter methylation. Taken together, these data suggest that changes in the frequency of CpG methylation at the *Ifng* promoter is one part of the molecular mechanism underlying the reprogramming of gene expression in Th2-derived memory cells during a recall response.

While we have shown that the loss of methylation at the *Ifng* promoter is likely to be one mechanism underlying flexible gene expression, we do not contend that it is the only repressive mechanism. We have shown that about 30% of *Ifng* promoter alleles from Th2 cells are unmethylated, and yet we see no IFN-γ production from this population. Furthermore, in memory cells prior to recall stimulation, the *Ifng* promoter is unmethylated, but it requires several days of recall culture under Th1 conditions before cells begin to produce IFN-γ. We therefore propose that other barriers to flexible expression of the *Ifng* gene exist, and that among these are the ability to up-regulate T-bet and achieve signaling through the IL-12 receptor.

Cells are flexible, genes are plastic

The ability of memory Th2 cells to produce IFN-γ in a Th1 recall response is a rare example of fully differentiated somatic cells undergoing reprogramming without genetic or pharmacological manipulation. This behavior has been termed both "plasticity" (Ahmadzadeh and Farber, 2002; Krawczyk et al., 2007; Sundrud et al., 2003) and "flexibility" (Adeeku et al., 2008; Messi et al., 2002) within the literature, and both of these terms convey the needed information: a mature somatic cell takes on new functions when face with a new stimulus. Plasticity however, carries meanings beyond what is truly happening in memory Th2 cells in that it often refers to the loss of previous cellular characteristics (Brockes and Kumar, 2002; Zhou and Melton, 2008a, 2008b). While this difference in terminology may seem subtle, the differences between the two terms are very significant in terms of ontogeny and functionality. Plasticity carries with it the implication, not just of reprogramming, but also of a loss of the original function (Cobaleda and Busslinger, 2008). We use the term flexibility to describe a lineage committed cell acquiring new programming without abandoning previous programing. Comparisons of the behavior of memory Th2 cells with instances of truly plastic behavior highlight the differences in the terms.

Phenotypic plasticity in mammalian cells is most often seen in cell developmental processes where cells transition from a pluripotent state such as hematopoietic stem cells to a less plastic, more differentiated cell type (Askenasy et al., 2006; Rovó and Gratwohl, 2008; Williams and Klinken, 1999). In the absence of experimental manipulation, this is typically an irreversible process (Briggs and King, 1952; Waddington, 1957; Weismann, 1893) involving alterations in multiple signaling pathways and gene expression (Abraham et al., 2013; Hu et al., 2012; Takahashi, 2012; Weissman et al., 2001; Zhou and Melton, 2008). These changes during the process of differentiation also involve substantial modifications in the chromatin structures of target genes (Hawkins et al.,

2010; Hu et al., 2012; Meissner, 2010; Zardo et al., 2008). The stability of these epigenetic modifications allows for transient activity of lineage-determining transcription factors to have a lasting impact on gene expression in the absence of continued stimulus (Fletcher et al., 2002; Niller et al., 2011; Paschos and Allday, 2010). The brevity of the stimulus which leads to the epigenetic changes has led many to describe this mechanism as a "hit and run" (Fletcher et al., 2002; Niller et al., 2011). The establishment of these epigenetic landscapes serves to continually reinforce a cell's identity and provides a substantial obstacle to true plasticity in mature somatic cells (Boheler, 2009; Conrad Hal Waddington, 1957; Papp and Plath, 2011; Takahashi, 2012; Zhou and Melton, 2008).

Phenotypic plasticity and cancer

One regretfully common instance of a non-stem cell attaining plasticity is in the pathogenesis of cancer. While cancer cells often retain some features of their original lineage such as surface molecules, the functionality of the cells has been lost in the transition to a neoplastic state. A very striking example of plasticity underlies the process of cancer metastasis. During metastasis, epithelial-like tumor cells transition to a mesenchymal state to leave the primary tumor (Thiery, 2002; Thiery et al., 2009). The epithelial to mesenchymal transition (ETM) in cancer is thought to occur stochastically as genetic aberrations in cancer cells lead to the loss of epithelial cell traits (Craene and Berx, 2013; Rangel et al., 2012; Thiery, 2002). Unlike differentiation in non-cancerous cells, this transition can be reversed, allowing mesenchymal cancer cells to transition back to epithelium and establish new solid tumors (Craene and Berx, 2013; Marjanovic et al., 2013; Strauss et al., 2012).

Stem cell populations share several other characteristics with cancer cells beyond phenotypic plasticity (Strauss et al., 2012). One important cancer trait shared by stem

cells is a rapid cell cycle lacking checkpoints in the G1 phase (Boheler, 2009; Kapinas et al., 2013). One reason for this shared trait may be the centrality of the c-Myc pathway to both cancer and embryonic stem cell programing (Kim et al., 2010; Takahashi et al., 2007). While central to many cell processes, c-Myc is a potent oncogene (Ischenko et al., 2013; Kim et al., 2010; Poe et al., 2012), and murine recipients of stem cells generated by using transgenic Myc have an increased incidence of tumor formation (Okita et al., 2007). Both embryonic stem cells and cancer cells also show wide variations in epigenetic modifications across populations, suggesting a state of epigenetic instability (Humpherys et al., 2001; Marjanovic et al., 2013; Meshorer et al., 2006). The similarities between cancer cells and stem cells do, in part, arise from shared pathways and possibly similar epigenetic states.

Induction of plasticity: Transdifferentiation and dedifferentiation

While there is little evidence for healthy somatic cells to display plasticity in nature, much work has centered on experimental manipulations that reprogram differentiated cells (reviewed in Gurdon and Melton, 2008). Early work in amphibians found that the transfer of a nucleus from a more developed cell to a enucleated oocyte would allow the fused oocyte to develop into an embryo, albeit at a relatively low frequency (Briggs and King, 1952; Gurdon, 1962; Gurdon et al., 1975). From these experiments, it was learned that differentiated cells retain the necessary genetic information, but usually not the developmental potential of embryonic stem cell. This incomplete plasticity is due in large part to epigenetic conditions established as a cell progresses towards a less pluripotent state. Often referred to as "Waddington's epigenetic landscape," the chromatin modifications enacted upon differentiation serve to prevent lineage plasticity under normal conditions, and any reprogramming strategy must overcome this barrier (Davis and Eddy, 2013; Ladewig et al., 2013; Takahashi, 2012; Zhou and Melton, 2008).

One way to achieve phenotypic plasticity is to prevent other fates from being foreclosed upon. A seminal example of plasticity of this variety is the loss of B cell identity in pro-B cells lacking the *Pax5* gene (Nutt et al., 1999). These *Pax5-/-* pro-B cells express some B cell lineage markers and undergo some rearrangement of the Ig-heavy chain locus, but their development does not progress to mature B cells (Nutt et al., 1998). After *in vitro* culture with cytokines other than IL-7, *Pax5-/-* cells lost characteristics of the B lineage and gained the phenotype and function of one of several other cell populations such as NK cells, Macrophages, and T cells (Cobaleda and Busslinger, 2008; Nutt et al., 1999). When transferred into *Rag-/-* deficient mice, these *Pax5-/-* pro-B cells were able to generate a long-term T cell population (Rolink et al., 1999). This finding demonstrates that, in the absence of *Pax5*, pro-B cells maintain the plasticity and self-renewing capabilities of hematopoietic stem cells even after appearing to commit to the B cell fate.

Alongside the removal of transcription factors which promote a distinct lineage, plasticity can also be induced by the exogenous expression of transcription factors linked to a cell population in a process termed transdifferentiation (Cobaleda and Busslinger, 2008). Pancreatic exocrine cells have been reprogrammed into insulin-producing cells almost entirely identical to pancreatic β cells via the forced expression of three transcription factors that drive β cell development: *Mafa*, *Ngn3*, and *Pdx1* (Zhou et al., 2008). This method has been shown to directly reprogram cells to the endocrine cell fate without regression to a pluripotent progenitor state. Likewise, the introduction of lineage-specifying transcription factors has been able to convert fibroblast to neurons (Vierbuchen et al., 2010) and cardiomyocytes (Ieda et al., 2010). In all of these settings the gain of a new lineage-defined function is coupled to the loss of the characteristics of the original lineage.

Perhaps the most striking instance of induced plasticity is in the generation of pluripotent cells from somatic cells in a process termed dedifferentiation (Cobaleda and Busslinger, 2008). These iPSs (induced pluripotent stem cells) were first created by retroviral introduction of the transcription factors Oct3/4, Sox2, Klf4, and c-Myc into a fibroblast (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Later, the process was altered to exclude c-Myc to avoid tumor formation (Nakagawa et al., 2008; Okita et al., 2007). Further experiments have used progenitor cells derived from iPS cells to correct genetic conditions in mouse models of sickle cell anemia (Hanna et al., 2007) and hemophilia (Xu et al., 2009). The ability to dedifferentiate somatic cells and subsequently redifferentiate them along a desired line is a prime example of plasticity.

While Th2-derived memory cells do lose certain aspects of the Th2 gene-expression program, they do not fully revert to a plastic state. That the commitment to produce IL-4 is maintained even in a Th1 recall response (Adeeku et al., 2008; Hegazy et al., 2010; Krawczyk et al., 2007; Löhning et al., 2008) suggest not a complete loss of effector programming (as would be entailed by plasticity), but rather a modification of the existing effector program. Therefore it is our contention that these cells are exhibit a functional and transcriptional flexibility rather than true plasticity. That being said, a case can be made that the *Ifng* gene does exhibit plasticity, at least pertaining to CpG methylation, as DNA from memory Th2 cells has almost identical *Ifng* promoter methylation to that found in naïve $CD4^+$ T cells. In summary, genes associated with an effector program may be plastic, but the memory cells themselves adapt existing programing in response to stimuli during recall responses.

An extra layer of adaptability: Why might this reprograming exist?

The exact effector program a $CD4^+$ T cell executes upon recognizing its cognate antigen is mostly determined by signals other than the peptide antigen (Abbas et al., 1996; Kaplan and Grusby, 1998). This in turn entails that the effector program adopted is not so much dependent on the nature of the pathogen, but rather the local cytokine milieu at the time of TCR-pMHC engagement. While the local cytokine milieu typically reflects the response needed to clear a given pathogen, there are exceptions. In foundational immunologic research in mice, it was found that some mice strains readily cleared an infection of *Leishmania major* while other strains developed chronic infections (Heinzel, 1989; Liew, 1989; Scott et al., 1988). Resolution of leishmaniasis requires IFN-γ secretion by Th1 effector cells (Carl et al., 1993). In susceptible mice, an initial burst of IL-4 in the local environment occurs at the time of exposure. This in turn prevents Th1 polarization and effective clearance of *Leishmania* (Aseffa et al., 2002; Himmelrich et al., 1998; Launois et al., 1995). Given that exposure to IL-4 during initial T cell activation can preclude the formation of a protective immune response, a mechanism by which Th2-derived memory cells could acquire the capacity to produce IFN-γ provides an additional layer of adaptability in the T helper response.

In terms of human health, consideration of the developing world also can lead to an appreciation for the need for flexible IFN-γ production. Infections by parasitic worms have been common throughout much of our evolutionary history and remain endemic developing regions of the world (Crump et al., 2012; Dold and Holland, 2011; Gryseels, 2012; Neghina et al., 2012). As discussed previously, helminthes drive robust Th2 responses leading to the production of IL-4 (Abbas et al., 1996; Svetic et al., 1993). Many pathogens common in the developing world such as mycobacteria or plasmodia also require a Th1 response for effective control (Green et al., 2013; Orme et al., 1993), and the presence of IL-4 from a Th2 response to parasitic worms could theoretically

prevent the development of an appropriate Th1 response. Studies have found significant rates of co-infection between pathogenic worms and intracellular parasites occurring in multiple countries (Abate et al., 2012; Borkow et al., 2001; Rafi et al., 2012; Tristão-Sá et al., 2002) In some cases, parasitic worm infections have been found to exacerbate TB in human patients (Borkow et al., 2001; Elias et al., 2001, 2006; Potian et al., 2011; Resende Co et al., 2007; Stewart et al., 1999; Tristão-Sá et al., 2002) and decrease Th1 responses in cases of lepromatous leprosy (Diniz et al., 2010; Prost et al., 1979). While there is strong correlative data spanning decades and multiple human populations, co-infection experiments using cotton rats as a model showed little to no effect of either nematode or helminth infection on the progression of tuberculosis (Hübner et al., 2012). While these data appear contradictory, it may indicate that variables such as parasite load, order of infection, host species, and exact species of parasite have a bearing on the relationship between worm infection and the response to mycobacterium (Rafi et al., 2012). The ability of a mycobacterium-reactive Th2-derived memory cell to produce IFN-γ during a recall response in the absence of Th2-skewing parasites could provide some measure of prophylaxis against reinfection.

Another reason why Th2 memory cells might have developed plastic *Ifng* expression is the difference in the magnitude between loss of IFN-γ signaling and the loss of IL-4 signaling. Loss of IFN-γ signaling or production in humans results in many clinically significant and life threatening issues (Glosli et al., 2008; Vinh et al., 2009). The loss of IL-4 production by Th2 cells is less likely to cause significant medical issues aside from a higher predisposition to inflammation and difficulty clearing infections of parasitic worms and is less likely to affect long-term survival (Brewer et al., 1996; Hultgren et al., 1998; Jankovic et al., 1999; Kopf et al., 1993; Pearce et al., 1996; Tissi et al., 2009). Therefore, having a mechanism for Th2 memory cells to acquire IFN-γ production capabilities could allow these cells to provide a response to acutely lethal pathogens.

A final reason for flexibility might be found in the nature of antigen recognition. T cells recognize peptide antigens which could be derived from any number of exogenous sources. A clone which recognizes a peptide that is highly conserved between different pathogens could experience stimulation under a variety of polarizing conditions.

DNA methylation, cytokine expression, and memory

Early work in IFN-γ and IL-4 producing $CD4^+$ T cell clones identified differential DNA methylation of the *Ifng* promoter as a possible mechanism for regulation of IFN-γ production (Melvin et al., 1995; White et al., 2006; Winders et al., 2004; Young et al., 1994). Expression of the primary *de novo* DNA methyltransferase in T cells: DNMT3a, is upregulated following stimulation via the TCR (Gamper et al., 2009) , and it has been shown to be vital in the prevention of inappropriate cytokine expression in T helper responses (Gamper et al., 2009; Thomas et al., 2012). The symmetry enforcing methyltransferase DNMT1 has also been shown to be vital in repressing the expression of Th2 cytokines during a Th1 effector response (Lee et al., 2001; Makar and Wilson, 2004). These past studies have indicated that the DNA methylation of cytokine genes is a crucial feature of the transition from a naive $CD4^+$ T cell to an effector.

Typically, CpG methylation is highly symmetrical, with CpGs on both the coding and non-coding strand having the same methylation frequency (Ehrlich and Wang, 1981). These symmetrically methylated dinucleotide pairs would, upon cell division, give rise to hemimethylated daughter strands. In late S phase, DNMT1 is recruited to sites of hemimethylation where it then enforces symmetry (Bashtrykov et al., 2012; Tatematsu et al., 2000), allowing CpG methylation to be a heritable, and potentially permanent, repressive epigenetic modification (Bender et al., 1999; Deaton and Bird, 2011).

The inhibitory effects of symmetrical CpG methylation on gene expression both directly in a site-specific manner (Jones and Chen, 2006; Sunahori et al., 2009) and indirectly via the recruitment of transcription factors with a methyl-binding domain (Bird and Wolffe, 1999; Dhasarathy and Wade, 2008; Hendrich and Bird, 1998) have been studied in great detail, but little was known about the functionality of asymmetric methylation. In DNA synthesis, hemimethylated daughter chromosomes are created, but the action of DNMT1 quickly reinforces symmetry (Tatematsu et al., 2000). Strand-biased asymmetric methylation has previously been described in cancer cells (Ehrlich and Lacey, 2013; Jain et al., 2011; Shao et al., 2009) but has not been reported in normal, non-cycling somatic cells prior to this work.

While losses of DNA methylation have been reported in T cells (Kersh et al., 2006; Lee et al., 2002; Makar and Wilson, 2004; Makar et al., 2003), direct evidence for DNA demthylases has only recently been published (Kangaspeska et al., 2008; Métivier et al., 2008). Prior to these findings the existence of active DNA demethylases was suspected but unverified. The exact mechanisms underlying DNA demethylation are still an active field of research, and are known to involve members of the ten-eleven translocation (TET) and AID/APOBEC enzyme families (Arioka et al., 2012; Bhutani et al., 2010; Guo et al., 2011; Ito et al., 2010; Tahiliani et al., 2009) as well as base excision repair glycosylases (Cortellino et al., 2011; Hashimoto et al., 2012a; He et al., 2011). The process of active demethylation mediated by TET enzymes starts with the conversion of 5-methyl cytosine (5mC) into 5-hydroxymethyl cytosine (5hmC) (Bhutani et al., 2011; Guo et al., 2011; Ito et al., 2010) eventually leading to base excision repair enzymes swapping out the modified methyl-cytosine for an unmethylated cytosine (Bhutani et al., 2011; He et al., 2011). AID/APOBEC has been shown to deaminate me-C to thymidine, which is then replaced via DNA repair mechanisms (Cortellino et al., 2011; Hashimoto et al., 2012a).

The functional difference between hydroxymethyl cytosine and methyl cytosine extends beyond active demethylation by base pair excision. The preferential demethylation of the paternal genome in zygotes has been found to be preceded by a conversion of methyl cytosine to hydroxymethy cytosine on paternal, but not maternal genetic information (Inoue and Zhang, 2011). Furthermore, loss of 5hmc was found to be replication dependent, suggesting a passive demethylation process (Inoue and Zhang, 2011) similar to the one we are proposing. Evidence that 5hmC might favor passive demethylation via promoting asymmetry has also been reported. Hydroxymethylated cytosine has been shown to inhibit DNMT1's activity leading to an unmethylated daughter strand (Chen et al., 2012; Hashimoto et al., 2012b; Valinluck and Sowers, 2007). Therefore, the TET enzymes may be central both to some mechanisms of active and passive demethylation.

Interestingly, recent work has shown that there is a global decrease in methylation in $CD4^+$ memory cells, as well as expression of DNA methyltransferases (Hashimoto et al., 2013; Li et al., 2012). The decrease both in methylation and the enzymatic machinery needed to maintain it across generations has implications for the plasticity of cytokine genes in T helper memory. In short, less DNMT1 in the cell could translate to decreased maintenance of methylation during cell division, which could in turn hasten the passive loss of CpG methylation from cytokine genes.

Future directions: flexible IFN-γ production and the antibody response

While the production of IFN-γ by Th2-derrived memory cells is well established (Adeeku et al., 2008; Hegazy et al., 2010; Krawczyk et al., 2007; Löhning et al., 2008), the extent to which it can influence an actually immune response has never truly been investigated. One major effect of the Th1 effector program is to guide B cells into class

switch recombination to the IgG2a antibody subtype (Brewer et al., 1996; Snapper and Paul, 1987). Individuals lacking the ability to make IgG2 subtypes are significantly more susceptible to certain bacterial infections, especially in the respiratory tract (Jefferis and Kumararatne, 1990; Oxelius, 2008; Preud'homme and Hanson, 1990). Research on the functional significance of IgG2a have revealed that it is the most efficient IgG subtype at opsonization in response to capsular antigens (Oishi et al., 2013; Schlageter and Kozel, 1990). It has also been shown to be the most effective subtype in conferring protection to lymphocytic choriomeningitis virus whereas IgG1 antibodies against the same epitopes failed to provide protection (Baldridge and Buchmeier, 1992). The increased activity of IgG2a is thought to stem from its ability to bind its phagocytes-expressed Fc receptor, FcγRIV, with a 40-fold higher affinity than IgG1 can bind FcγRIII, its activating Fc receptor (Bolland, 2005; Nimmerjahn and Ravetch, 2006; Nimmerjahn et al., 2005). This increased binding affinity to the Fc receptor allows IgG2a to effectively opsonize antigens at a lower concentration. In order to test the physiological significance of IFN-γ productions, we have designed experiments to test the ability of a memory Th2 population to induce an IgG2 response when given a recall challenge *in vivo* under Th1-skewing conditions.

The experimental methodology (summarized in Fig. 4.1) was in short to immunize mice with KLH (keyhole limpet hemocyanin) coupled with nitrophenol (NP) as a hapten. The initial immunization was carried out in the Th2-response promoting adjuvant alum (Grun and Maurer, 1989). After two weeks the recipients were boosted with NP-KLH. After two weeks, wildtype or *Tbx21*-/- GFP^+ 4get DO11.10 Th2 effectors were transferred into immunized mice, with some immunized mice not receiving Th2 cells as a control. After six weeks, mice were given a recall challenge of NP-Ovalbumin in complete Freund's adjuvant (CFA). The carrier switch to ovalbumin allows us to introduce an antigen to which the endogenous T cells will have had no exposure to while

providing the donor-derived DO11.10 cells with their cognate antigen. We bled mice before the recall challenge, one week post-recall, and two weeks post recall. Serum was analyzed for NP-specific IgG2a content and the effect of recall stimulation was calculated by subtracting the pre-recall value from the post-recall values. In the pilot experiment, we did see in increase in NP-specific IgG2a in the recipients of WT Th2, but not T-bet deficient Th2 cells or in the immunized mice that did not receive Th2 effectors (Fig. 4.2). This preliminary result suggests that Th2 memory cells can influence B cells to class switch to IgG2a producers. If this result is reproducible, it will be the first confirmation of the physiological relevance of flexible IFN-γ production.

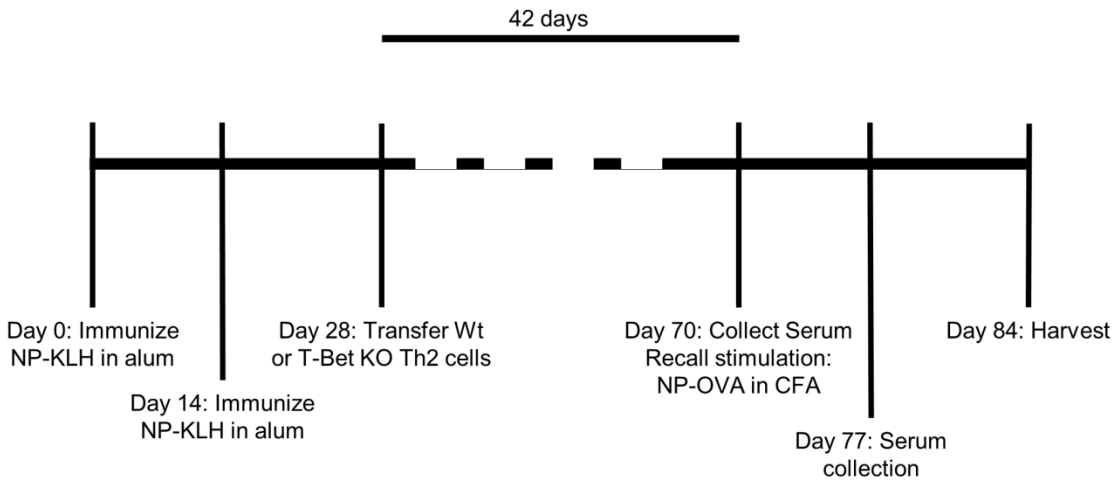


Figure 4.1. Timelineof antibody class switching experiment. Shown is a schematic for the experiment designed to test whether IFN-γ produced by Th2-derived memory cells can generate an IgG2a antibody response

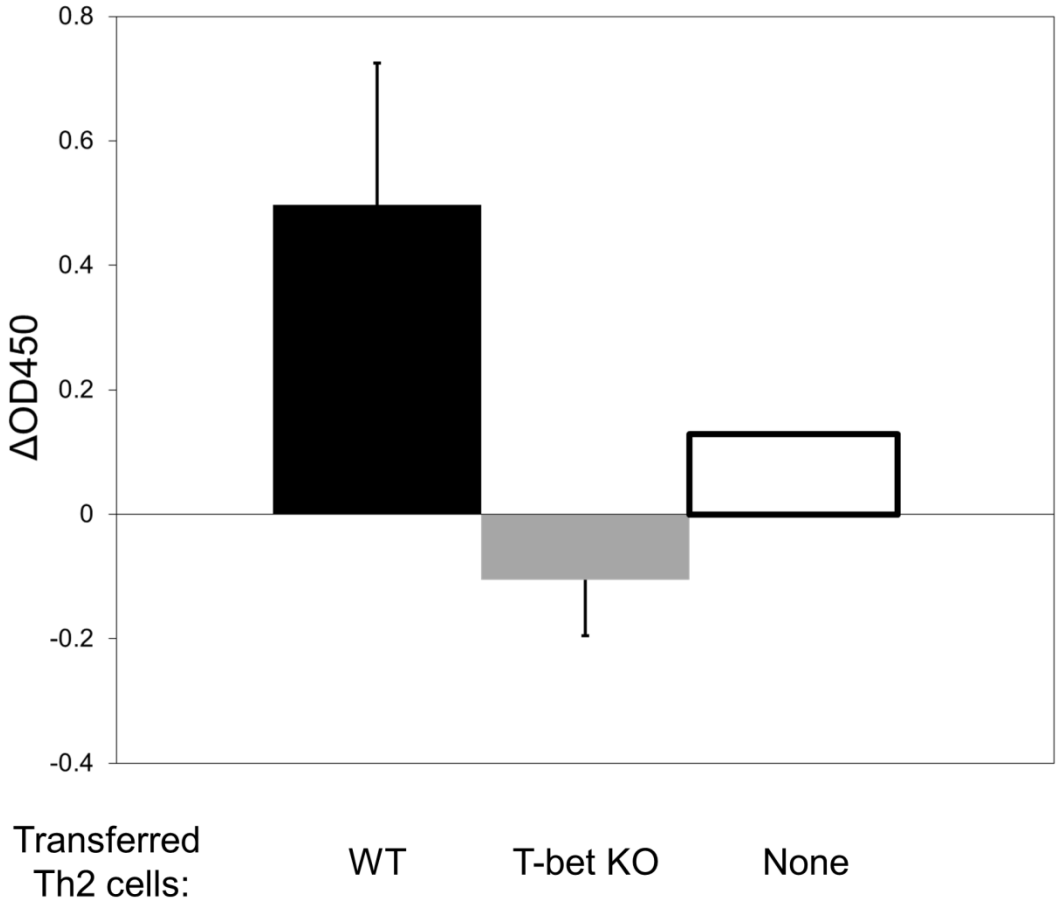


Figure 4.2. Changes in NP-specific IgG2a one week after recall. Serum samples acquired one week after recall stimulation and samples immediately prior to recall stimulation were analyzed via ELISA for NP-specific IgG2a. Shown is the mean difference in IgG2a concentration as determined by subtracting the pre-recall OD450 from the one week post recall OD450. Error bars represent one standard deviation. For recipients of WT or T-bet KO Th2 effectors, N=2. The sample which did not receive Th2 effectors is N=1.

Future directions: methylation Vs. hydroxymethylation

A second exiting direction that this project could take is to determine if the methylation at the *Ifng* promoter is actually hydroxymethylation. As discussed above, hydroxymethylation does not activate DNMT1, making it a likely candidate for a mechanism underlying hemimethylation. An alternative hypothesis is that the recruitment of DNMT1 to the *Ifng* is blocked during Th2 polarization, resulting in a true hemimethylated state.

Along this vein, if hydroxylmethylation of the *Ifng* promoter is detected in Th2 cells, two further questions suggest themselves: By what mechanism is hydroxymethylation established, and when is the methyl-cytosine (mC) converted to a hydroxymethly-cytosine (hmC)? The resolution of the first question would likely center on the TET family of enzymes, as they have been shown to carry out this conversion in several systems. Detection of TET proteins at the *Ifng* promoter in Th2 cells would suggest their involvement in establishing the asymmetrical methylation in Th2 effectors. The second question centers more on what stimuli would promote hydroxymethylation. I have considered two models which would fit with our current understanding of T helper cell differentiation and the regulation of *Ifng* expression.

In the first model, enzymes to modify mC into hmC would be recruited to the *Ifng* promoter early in Th2 differentiation, and possibly at the same time as DNMT3a, meaning that there would be little time in which actual mC would be present at the *Ifng* promoter. In the second model, enzymes catalyzing the mC to hmC conversion would arrive at the *Ifng* promoter as signaling from the TCR and the IL-4 receptor was lessened later in the Th2 response, meaning that hmC would only be detected at later timepoints.

A third possibility is that hydroxymethyl is directly added to CpGs at the *Ifng* promoter. As no enzyme has been shown to catalyze this reaction, and evidence suggests

that DNMT3s would not be capable to carrying this reaction out (Chen et al., 2012), I find this to be the least likely of the explanations.

The determination of if, when, and how methyl-cytosines on the *Ifng* promoter are converted to hydroxymethy-cytosines would increase our understanding of the mechinisms facilitating the creation of a unstable epigenetic modification.

V. MATERIALS AND METHODS

Mice BALB/c *Il4-IRES-Gfp* ("4get"), DO11.10 mice were bred with BALB/c *Tbx21* (T-bet) -/- (KO) or BALB/c *Stat4* KO mice (Jackson labs). BALB/c-ByJ (Jackson labs) and athymic BALB/c nude (*Foxn1/Foxn1*) mice were used as recipients for transfer experiments. Recipients were 4-6 weeks old at the time of transfer. Mice were maintained in micro-isolator cages at a Vanderbilt University facility in accordance with Institutional Animal Care and Use Committee guidelines and an approved protocol.

Reagents Fluorophore-conjugated and purified mAb were obtained from BD Pharmingen (San Jose, CA) and purified recombinant cytokines from Leinco (St. Louis, MO) unless otherwise indicated. Purified 11B11 anti-IL-4 and recombinant huIL-2 were obtained from the Biological Response Modifiers Program (NCI, Frederick MD). Anti-T-bet-eFluor 660 was obtained from eBioscience (San Diego, CA). Oligonucleotides were synthesized by Invitrogen (Grand Island, NY) unless otherwise stated.

Cell culture and purification of GFP^+ IL-4 producing effectors and adoptive transfers DO11.10 cells were activated with OVA323-339 peptide, and all cells were cultured, as described (Adeeku et al., 2008) with the following modifications. For Th1 culture conditions, cells were plated at 7×10^6 cells/ml and received 1 µg/ml OVA peptide, 5 ng/ml IL-12, and 3 µg/ml anti-IL-4 antibody 11B11. For Th2 cell cultures, cells were plated at 3.5×10^6 cells/ml and received 0.5 µg/ml OVA peptide, 7.5 ng/ml IL-4, 3 µg/ml anti-IFN-γ antibody, and 2 µg/ml anti-IL-12 antibody. Both Th1 and Th2 cell cultures were supplemented with IL-2 (50 units/ml 24 and 72 hours after Ag stimulation).

GFP^+ Th2 effector cells were purified for transfer as described (Adeeku et al., 2008). After four days of culture in Th2 conditions, 4get, DO11.10 cells (*Tbx21* -/-, *Stat4* -/-, or transcriptionally WT) were stained with APC-conjugated anti-CD4 and PE-conjugated KJ1-26 (anti-DO11.10 TCR) antibodies and flow sorted on a FACS Aria (BD, Franklin Lakes, NJ) to purify (> 98.5%) viable DO11.10 KJ1-26^+ $CD4^+$, GFP^+ cells. Prior to transfer or DNA isolation for methyl-CpG analyses, these cells were cultured in Th2 conditions for 9-10 d after re-stimulation with APCs (4:1 with T cells) and 0.25 µg/ml OVA323-339 (13-14 d total).

Quantitation of cytokine production T helper cell cultures plated with APCs at a 1:4 ratio, and single-cell suspensions of recipient spleens, were stimulated with 1 µg/ml $OVA_{323-339}$. Cytokine concentrations in supernatant collected after 36 hr were quantified using a flow cytometric kit (Th1/Th2/Th17 cytokine bead array; BD Pharmingen). Intracellular cytokines were analyzed by stimulating cells 18 hr with OVA323-339 (1 µg/ml) in the presence of APCs, followed by Golgi-Stop (BD) (2-3 hr), and staining in the presence of anti-CD16/32 (Fc Block, BD Pharmingen) as previously described (Adeeku et al., 2008). Viable cells were selected based on forward and side scatter characteristics, and fluorescence signals representing intracellular cytokines were determined in cells positive for the DO11.10 TCR and CD4.

Bisulfite analysis of DNA methylation DNA isolated from flow-purified naïve, effector (Th1, Th2, 13 d) and memory CD4 T cells, or from tissues, was digested with Bam H1 (New England Biolabs, Ipswich MA), bisulfite-modified using the Imprint bisulfite modifying kit (Sigma-Aldrich, St. Louis, MO), then used as template in quadruplicate PCR performed using primer pairs specific for each modified strand

sequence in the *Ifng* promoter (Table 5.1) (Jones and Chen, 2006). The noncoding strand of the *Ifng* promoter was amplified with a single reaction, while the coding strand was amplified using a nested PCR**.** After pooling four tubes of separate amplification for each sample, specific PCR products were identified on agarose gels, extracted using the Qiaquick gel extraction kit (Qiagen, Valencia, CA), ligated with T-easy vector (Promega, Madison, WI), and then transformed into JM109 cells (Agilent, Santa Clara, CA). For each of three independent biological replicate cell samples and for each strand, eight to ten clones derived from each reaction pool were sequenced and scored for the frequency of unmodified C residues in the CpG dinucleotides; the modification frequency for C residues outside of CpG dyads was verified as > 99% for all sample sets.

EMSA EMSAs were performed as reported (Jones and Chen, 2006; Lee et al., 2010) except that extracts of Th1 cells developing from primary mouse $CD4^+$ T cells were used. Methylated upper strand oligonucleotides were synthesized by Invitrogen (Grand Island, NY). The upper strand oligonucleotides were designated as unmethylated, meC(-53) hemimethylated, and tri-hemimethylated (meC at -53, -45, and -34) (Table 5.1). Each was annealed to an unmethylated lower strand oligonucleotide after radiolabeling with γ-[32P]-ATP (Perkin-Elmer, Waltham MA) and T4 polynucleotide kinase (New England Biolabs, Beverly MA). For competition assays, unlabeled competitor was added simultaneously with the labeled probe at molar ratios (competitor:probe) of 100, 10, and 1:1. Antibodies used for the super shift assays were CREB1 (sc-186), ATF2 (sc-187) and c-Jun (sc-45) (Santa Cruz Biotechnology, Santa Cruz, CA).

Table 5.1. **Sequences of oligonucleotides.**

Oligonucleotide	Sequence	Purpose
Bisulfite coding strand, Outer sense	GAA-ATT-TAT-ATT-ATA-AGG-GTA-AAA-AGG-GGG	PCR primer
Bisulfite coding strand, Outer anti-sense	CAA-ACT-TTC-TAA-ACT-TTC-AAT-AAC-TAT-AC	PCR primer
Bisulfite coding strand, Inner sense	TAG-AGA-ATT-TTA-TAA-GAA-TGG-TAT-AGG-TGG-GTA-T	PCR primer
Bisulfite coding strand Inner anti-sense	CCA-TAA-AAA-AAA-ACT-ACA-AAA-CCA-AAA-TAC-AAT-A	PCR primer
Bisulfite non-coding strand, Outer sense	GTT-ATG-AGG-AAG-AGT-TGT-AAA-GTT-AAG-ATG-TAG	PCR primer
Bisulfite non-coding strand, Outer anti-sense	CCC-AAC-CAC-AAA-CAA-AAA-CTC-CCT-ATA-CTA-TAC	PCR primer
Bisulfite non-coding strand, Inner sense	TAG-GAG-GAG-AAG-TTT-AGA-ATT-TTT-GTT-TTA-AGT-T	PCR primer
Bisulfite non-coding strand, Inner anti-sense	ACA-ATT-TCC-AAC-CCC-CAC-CCC-AAA-TAA-TAT-AAA-A	PCR primer
Ifng promoter sense	CGG-GGC-TGT-CTC-ATC-GTC	PCR primer
Ifng promoter anti-sense	CTC-GGG-ATT-ACG-TAT-TTT-CAC-AA	PCR primer
Coding strand, Un	GTG-AAA-ATA-CGT-AAT-CCC-GAG-GAG-CCT-TCG-A	EMSA probe
Coding strand, 1XME	GTG-AAA-ATA-(Me)CGT-AAT-CCC-GAG-GAG-CCT-TCG-A	EMSA probe
Coding strand, 3xME	GTG-AAA-ATA-(Me)CGT-AAT-CC-(Me)C-GAG-GAG-CCT-T-(Me)CG-A	EMSA probe
Non-coding strand	TCG-AAG-GCT-CCT-CGG-GAT-TAC-GTA-TTT-TCA-C	EMSA probe

Table 5.1. **Sequences of oligonucleotides.** Shown are all PCR primers and EMSA probes used in this work. Methylated cytosines are denoted as (Me)C.

Transient transfection and reporter assays Nucleofection was carried out via the Amaxa T cell kit (Lonza, Basel, Switzerland) using a minimal *Ifng* promoter reporter P1P2-Luc (Soutto et al., 2002) along with pCMV-Sport6-CREB1 or pCMV-Sport6. All results were normalized to GFP expression from the pMAX-GFP plasmid (Lonza) measured via flow cytometry. Luciferase activity was measured using the Dual-glow luciferase assay system (Promega) according to manufacturer's protocols.

Chromatin Immune Precipitation assays Chromatin immunoprecipitation (ChIP) assays were performed essentially as described previously (Zhang and Boothby, 2006). In brief, $10x10^6$ cells were suspended in IMDM supplemented with 10% FBS. Formaldehyde was added to a total concentration of 1%, and crosslinking was carried out on ice for 20 minutes. The crosslinking reaction was quenched by the addition of excess glycine, and the cells were washed in PBS. Cells were then resuspended in lysis buffer (50mM Tris, 10mM EDTA, 1% SDS, pH8) and sonicated in a Bioruptor (Diagenode, Denville, NJ) to produce an average sheered DNA length of 400 bp. Immune precipitation was carried out on one million cell equivalents using 1ug anti-AcH3(K9) (Millipore, #DAM1813175), or CREB1 **(**Santa-Cruz, sc-186X), and the precipitates were analyzed by PCR using primers shown in Supplemental Table I. Quantification of PCR products was carried out via southern blotting and phosphor imaging.

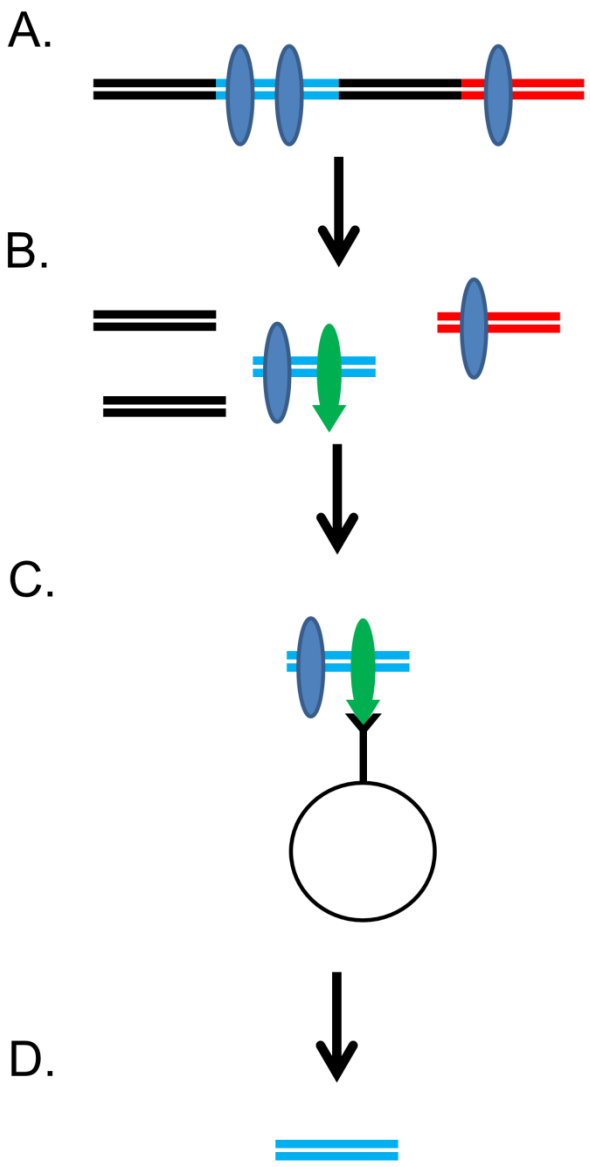


Figure 5.1. Workflow for chromatin immunoprecipitation (ChIP) assay. (A) Chromatin is isolated from formaldehyde cross-linked cells. (B) The chromatin is sonicated or otherwise sheared to approximately 400bp segments. (C). Antibodies against a transcription factor or modified histone and protein-A sepharose beads are used to precipitate a target protein and associated DNA. (D) Protease digestion and ethanol precipitation leaves only purified DNA that was bound to the target protein. Analysis via PCR can now begin.

Proliferation in vitro and in vivo Proliferation studies using CFSE partitioning and BrdU incorporation were carried out as described (Lee et al., 2010). For CFSE partitioning in vivo, Th2 cells were grown for five days, labeled with CFDA-SE (Invitrogen) (2.5μM, 15 min) following manufacturer instructions, and then transferred into BALB/c recipients. Fluorescence was measured on donor-derived cells recovered 12 d after transfer with gating as described above. For CFSE partitioning assays in vitro, DO11.10 splenocytes were either labeled, Ag-stimulated, and cultured 2 d, or activated with Ag, cultured in Th2 conditions (11 d, with one interim Ag stimulation as for cells used in adoptive transfers), labeled with CFDA-SE as above, and then cultured 2 d in IL-2 supplemented medium before analysis by flow cytometry. For assays of BrdU incorporation into donor cells in vivo, recipient mice were injected twice (72, 24 h before harvest; 3 mg i.p. per injection) with BrdU (Sigma Aldrich) in sterile saline. Cells harvested 12 d post-transfer were then processed as described (Lee et al., 2010) to detect Alexa-647 anti-BrdU (Invitrogen) in donor- ($KJ1\text{-}26^+$) and recipient-derived $CD4^+$ T cells by flow cytometry. For in vitro assays, BrdU (1 μM) was added to Th2 cultures (days 2 and 13 after Ag activation) followed 4 h later by processing, direct immunofluorescent anti-BrdU staining, and flow cytometry of $KJ1\text{-}26^+$ $CD4^+$ cells.

Retroviral transduction Retroviral transduction was carried out using as previously described (Zhang and Boothby, 2006). In brief, retrovirus-containing supernatants from ΦNX packaging cells transfected with MSCV-IRES-Thy1.1 (MiT), or MSCV-T-bet-IRES-Thy1.1 (MIT-T-bet) were used to transduce established GFP^+ DO11.10 Th2 cells two days after restimulation. Cells were then cultured under Th1 or Th2 conditions, followed by measurements of Ag-stimulated cytokine production or intracellular cytokines as above.

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