Role of SATB1 in T cell development and differentiation

A thesis

Submitted in partial fulfilment of the requirements

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Doctor of Philosophy

By

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Dedicated to

My grandparents for all their encouragement and support.

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Role of SATB1 in T cell development and differentiation**" Submitted by **Kamalvishnu P Gottimukkala** was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

(Supervisor)

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Declaration

I declare that this written submission represents my ideas in my own words and where others ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Abbreviations

ACF1	ATP-dependent chromatin assembly factor large subunit-1
ATM	Ataxia telangiectasia mutated
Bcl2	B-cell leukemia/lymphoma 2
bp	base-pair
BURs	base unpairing regions
CD-	Clusters of Differentiation
CD	Cut repeat-containing domain
CHD	chromodomain helicase DNA binding protein 1
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP-Sequencing
CHRAC	Chromatin-Accessibility Complex
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CSBS	Consensus SATB1-binding sequence
Ct	threshold cycle
CtBP1	C-terminal binding protein 1
DAPI	4',6-diamidino-2-phenylindole
DP	Double positive
DTT	dithiothreitol
EDTA	Ethylene di-amine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
ERK	extracellular receptor kinases
ETPs	Early thymic progenitors
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FL	Full Length
FOXP3	Forkhead box P3
GATA	Glutamyl-tRNA amidotransferase subunit-A
GDF	growth differentiation factor
GSK-3	glycogen synthase kinase-3
h	hour
H3K14ac	Histone H3 lysine 14 acetylation
H3K27me3	Histone H3 lysine 27 trimethylation
H3K4me3	Histone H3 lysine 4 trimethylation
H3K9ac	Histone H3 lysine 9 acetylation
H3K9me	Histone H3 lysine 9 trimethylation
HAT	histone acetyltransferases
HD	Homeodomain
HDAC	histone deacetylase
HMG-1	High Mobility Group protein-1
Hoxa2	Homeobox protein Hox-A2

HRP	horseradish peroxidase
HS	hypersensitive site
HSCs	Hematopoetic Stem Cells
IB	immunoblot
IFNγ	Interferon γ
IgH	immunoglobin heavy chain
IL	Interleukin
IP	immunoprecipitation
iPSCs	induced pluripotent stem cells
kb	Kilobase
kDa	Kilo Dalton
M	Molar
M2H	Mammalian Two-Hybrid
MACS	Model-based Analysis of ChIP-Seq
MAPK	Mitogen-activated protein kinase
MARs/SARs	Matrix/Scaffold attachment regions
MBP	MAR-binding protein
MPPs	Multipotent progenitors
ESCs	embryonic stem cells
mg	milligram
MHC	Major histocompatibility complex
min	Minutes
miRNAs	microRNAs
ml	millilitre
mM	mill molar
NLS	nuclear localization sequence
NMTS	nuclear matrix targeting sequence
NuRD	Nucleosome remodeling and deacetylase complex
PBS	Phosphate Buffered Saline
PCAF	P300/CBP-associated factor
PcG	polycomb group
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-kinase
PKC	protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PML	Promyelocytic leukemia
PMSF	phenylmethylsulfonyl fluoride
PRC	polycomb repressive complex
RAG	Recombinase activating genes
RPMI-1640	Rosewell Park Memorial Institute-1640
Runx	Runt-related transcription factor
SATB1	Special AT-rich Sequence Binding Protein 1
SBS	SATB binding sequences
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SP	Single positive

STAT	signal transducer and activation of transcription
TCF-1	Transcription factor-1
TCR	T cell receptor
TF	transcription factor
TGF-β	Transforming growth factor-β
Th	T-helper
Thpok	T-helper-inducing POZ/kruppel factor
Treg	T-regulatory cells
TSS	Transcription start site
UTR	Untranslated region
Wnt	wingless type protein
Xist	X-inactive specific transcript
β–ME	β–mercaptoethanol

Contents

Synopsis

Adaptive immune system is a hallmark of all vertebrates (Cooper and Alder, 2006). Adaptive immune system comprises of multitude of differentiated cell types commonly known as lymphocytes. Pertinent feature of lymphocytes is that they exhibit antigen receptor repertoires which are somatically diversified by V(D)J recombination (Schatz, 2004). Based on the site of development and their functions lymphocytes are broadly distinguished into B and T lymphocytes. B lymphocytes in mammals arise in the bone marrow and govern the humoral immune responses (antibody mediated). T lymphocytes carry out the cell mediated immune responses and are generated in a specialized primary lymphoid organ called the thymus.

Common lymphoid progenitors which migrate to thymus microenvironment develop into T lymphocytes. Studies relating to thymectomy in mice and human congenital disorders such as the DiGeorge syndrome have shown the importance of thymus for T lymphocyte development (Miller FJ, 1961). The development of T lymphocytes is delineated into series of chronologically and phenotypically distinct stages based on the expression of surface markers CD4 and CD8. The progenitors are known as DNs (double negatives) which are negative for both CD4 and CD8. DN thymocytes move from the cortical regions of thymus to the medulla where they develop into DPs (double positives). DPs have a complete αβTCR on the cell surface and subjected to selection process. Positively selected thymocytes further develop into either CD4 or CD8 single positive thymocytes. These thymocytes migrate to the periphery were they can respond to antigenic signals and proliferate to mount an effective immune response.

Sequential development of the thymocytes is dependent on T cell TCR signaling and an array of transcription factors. TCR signaling involves many other components that are non-covalently associated with core αβ T cell receptor. CD3 is one of the important components that is associated with the TCR and important in transducing signals via ITAMs (immunoreceptor tyrosine based activation motif) (Smith-Garvin et al., 2009). TCR signaling is important for the thymocyte development as evident from the *RAG* knockout mice, enzymes which are important for the TCR recombination process. The *RAG* KO mice are completely devoid of T and B cells (Mombaerts et al., 1992). TCR signaling is also shown to have a role in the lineage specification of CD4 and CD8 SP thymocytes. Notch signaling and

Notch transcription factors are indispensable for initiating T cell program in the thymus (Hozumi et al, 2008). Transcription factors including *Gata3, Ikaros, Bcl11b* and *Runx3* are shown to play a critical role in T cell development (Rothenberg et al, 2008). The role of various transcription factors such as *T-bet, Gata3, Foxp3, RorϒT* are well studied in the T helper cell differentiation in the periphery.

Special AT-rich binding protein 1(SATB1) is a T-lineage-enriched transcription factor and chromatin organizer. SATB1 was identified by its propensity to bind the base-unpairing regions of IgH locus. SATB1 selectively binds to DNA elements which are rich in AT content. Functionally SATB1 has two domains. The N-terminal PDZ-like domain interacts with various interacting partners like PML, β-Catenin and CTBP-1. Carboxy terminus of the protein is known as MAR-binding domain (MD) which comprises of cut repeat domain (CD) and Homeodomain (HD) which are important for the binding to the DNA (Purbey et al.,2008). SATB1 is known to regulate many genes which are important for T cell development. Post-translational modifications of SATB1 are known to have important functions in regulating gene expression (Kumar et al., 2006). SATB1 directly interacts with chromatin remodeling machinery at genomic loci and has a role in epigenetic gene regulation.

Studies using SATB1 knockout (KO) mice revealed that it plays seminal role in thymocyte development. In Satb1 null mice T cell development is blocked at the DP stage. Ablation of SATB1 has been shown to cause spatial and temporal dysregulation of multiple genes including *Il2-Rα* and *Il7-Rα* (Alvarez et al., 2000). SATB1 is known to organize the chromatin architecture by forming a cage-like structure circumscribing the heterochromatin and traversing through euchroamtic regions of the genome (Cai et al., 2003). SATB1 mediated loop organisation of the *Il4* locus in Th2 cells shows its important role in expression of key genes required for differentiation (Cai et al., 2006). Furthermore, SATB1 interacts with β-catenin in $CD4^+$ T cells and co-ordinately regulates *Gata3* expression which is a master transcription factor of Th2 differentiation (Notani et al., 2010).

In the light of the above findings I proposed to investigate:

1. **Role of SATB1 in thymocyte development and its regulation.**
2. **Regulation of SATB1 during T helper cell differentiation.**

My thesis is accordingly divided into two chapters as follows.

1. Role of SATB1 in thymocyte development and its regulation

Common lymphoid progenitors that migrate to the thymus finally develop into single positive (SP) thymocytes. Developmental stages are distinguished based on surface expression of CD4 and CD8 into DN, DP, CD4 SP and CD8 SP. We wished to monitor the expression of *Satb1* in different subsets of thymocytes during development. We observed that at transcript level SATB1 is predominantly expressed in the DP subset. We further analyzed the protein expression pattern of SATB1 by utilizing flow cytometric analysis. We observed differential expression of SATB1 in developing thymocytes. Interestingly, SATB1 is induced at DN4 stage of thymocyte development which is post TCRβ rearrangement and we speculated that this might be in response to pre-TCR signaling. We also observed different populations of DP thymocytes based on SATB1 expression. DP thymocytes that have received a TCR signal and are positive for early activation marker CD69 ($CD69^{+}$) exhibit higher expression of SATB1 compared to DP thymocytes which are $CD69^{-ve}$. DP thymocytes finally develop into either CD4 or CD8 SP thymocytes. According to Al Singer's model of lineage specification DP thymocytes which have a persistent TCR signaling develop into CD4 SP lineage and those that have a cessation in the TCR signal develop into CD SP thymocytes (Singer et al., 2008). Our data shows that SATB1 expression is regulated in TCR-dependent fashion and therby it might regulate lineage commitment.

CD4 SP thymocytes exhibit a bimodal distribution based on SATB1 expression and we designated them as $SATB1^{Hi}$ and $SATB1^{Lo}$ CD4 thymocytes. Based on CD69 (early TCR activation mark) surface expression we observe CD69 positive cells are $SATB1^{Hi}$ CD4 T cells and CD69 negative cells are $SATB1^{Lo}$ CD4 T cells, indicating SATB1 levels are regulated by TCR signaling. $SATB1^{Hi}$ CD4 T cells are negative for Qa-2 and positive for HSA (CD24) expression, a phenotype of immature thymocytes those that cannot respond to TCR signaling *in vitro*. In contrast, $SATB1^{Lo}$ CD4 thymocytes are positive for Qa-2 expression and have downregulated HSA expression, a phenotype of mature SP thymocytes and those that migrate to the periphery. In β-Catenin transgenic mice which characteristically have high population of CD8 SP thymocytes, has a significant downregulation of SATB1. Taken together, these results support our hypothesis that SATB1 is regulated by TCR stimulation and together these signaling pathways define lineage commitment. To further confirm whether SATB1 expression is dependent upon TCR signaling we activated purified

CD4 T cells with plate-bound anti-CD3 + anti-CD28 and found that SATB1 is upregulated only in response to anti-TCR but not in control T cells. Jurkat T cells are known to have tonic TCR signaling similar to that of the pre-TCR signaling in thymocytes (Roose et al., 2003). We used Jurkat T cells to monitor if chemical inhibitors of T cell activation have any effect on SATB1 expression. Inhibition of MAPK/ERK pathway which are well studied and downstream regulators of TCR signaling have shown significant downregulation of SATB1 expression. Finally, we show that SATB1 is regulated by transcription factors TCF1 and GATA3 in mouse thymocytes. In conclusion, we demonstrate that SATB1 is regulated in a TCR-dependent manner and it regulates thymocyte differentiation.

2. Regulation of SATB1 during T helper cell differentiation

Our studies suggested that CD4 SP thymocytes that have downregulated SATB1 expression migrate to periphery. Peripheral CD4 T cells differentiate into various T helper phenotypes in response to various antigenic stimuli. Microarray based gene expression profiling in Th1/Th2 polarized cells have shown that SATB1 is upregulated during Th2 differentiation. We therefore wished to study the regulation of SATB1 expression during Th2 differentiation. In our *in vitro* model system where we have polarized CD4 T cells to Th2 phenotype in presence of TCR signaling and IL4 cytokine, we observe that SATB1 is upregulated within 48 h under polarizing conditions, and so is the master transcription factor *Gata-3*. Next, we wished to test if SATB1 is required for Th2 polarization. Towards this, we transfected CD4 T cells with siSatb1 and then polarized the cells towards Th2 phenotype. We see that upon knockdown of Satb1 cells express virtually no IL-4, a key effector cytokine for Th2 differentiation. SATB1 is not only required for Th2 cytkoine production but also facilitates downregulation of interferon-γ (IFN-γ), an effector cytokine of Th1 lineage. We demonstrate that SATB1 binds to IFN-γ promoter directly and represses its expression in Th2 cells. Knockdown of SATB1 in Th2 cells causes upregulation of IFN-γ expression. Once we established that SATB1 is necessary for Th2 differentiation, we then wished to study the molecular mechanism of regulation of SATB1 during differentiation of T helper cells. In-silico Promoter analysis of SATB1 promoter revealed that SATB1 has key binding sites for STAT6 and Gata-3. By electrophoretic mobility shift assays and ChIP analysis we show that both the factors which are known to have a key role in Th2 differentiation bind to the

promoter and regulate gene expression. Furthermore, using TCF1 knockout mice CD4 T cells polarised under Th2 conditions, qPCR data show that TCF-1 regulates SATB1 levels by regulating *Gata-3* expression. We also observe that SATB1 is differentially regulated in various CD4 helper T lineages but is downregulated (almost absent) in induced T regulatory cells (i Tregs) showing the important role of SATB1 played in helper lineages but not in suppressor cells.

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Chapter I

Role of SATB1 in Thymocyte Development and its Regulation

1.1 Introduction

The basic function of immune system is to protect the host organisms from diverse foreign agents ranging from microbes to worms to dust allergens. Immune responses are broadly divided into innate and adaptive responses based on the nature of receptors required for antigen recognition. Innate immune system relies on germline encoded receptors on host cells known as pattern recognition receptors (PRRs) and they recognize pathogen associated molecular patterns (PAMPS). Innate immune system is present in both plant and animal kingdoms suggesting that it has evolved before the divergence of both the kingdoms (Hoffmann et al., 1999). Adaptive immune system is a hallmark of all vertebrates and they have an antigen receptor that is obtained by somatic recombination process (Schatz, 2004). Cells of adaptive immune responses are clonal populations i.e. each cell has an antigen receptor that is different from other, they recognize wide range of antigens based on molecular structures and have a property of immune memory (Janeway and Medzhitov, 2002). Key players of the adaptive immune responses are known as lymphocytes. Lymphocytes are developmentally and functionally divided into B lineage and T lineage. Both B and T lymphocytes arise from a common lymphoid progenitor's (CLP's) which in turn are derived from hematopoietic stem cells (HSC's) that arise in the bone marrow.

B lymphopoesis begins in the bone marrow and is completed in different anatomical locations in different organisms. In mammals the final development of B cells takes place in the spleen whereas in birds it is completed in Bursa of Fabricius (Cooper et al., 1965). Contrastingly, T cell development begins in the thymus and is completed in the thymus. Studies relating to thymectomies in mice and congenital genetic disorders such as Digorges syndrome have shown the importance of thymus in T cell development (Miller, 1961). Thymus is located in the pharynx and its epithelium is derived from pharyngeal endoderm. Thymic epithelium has shown to express key transcription factors necessary for the proper development of the thymus organ and thymocytes (Corbeaux et al., 2010). Whn and Foxn1 transcription factors expressed in the thymic epithelia have been shown to have an important role in thymopoesis (Boehm et al., 2012). *Whn* deficient mice have a phenotype similar to that of nude mice, which have congenital athymia (Schuddekopf et al., 1996). Foxn1 is specifically expressed in the thymic epithelia and regulates the expression of chemokine genes in vertebrate embryos which attract progenitors for thymocyte development (Calderon

and Boehm, 2012). Thymus is morphologically distinguished into two regions, outer cortex and inner medulla where distinct stages of the thymocytes development occur.

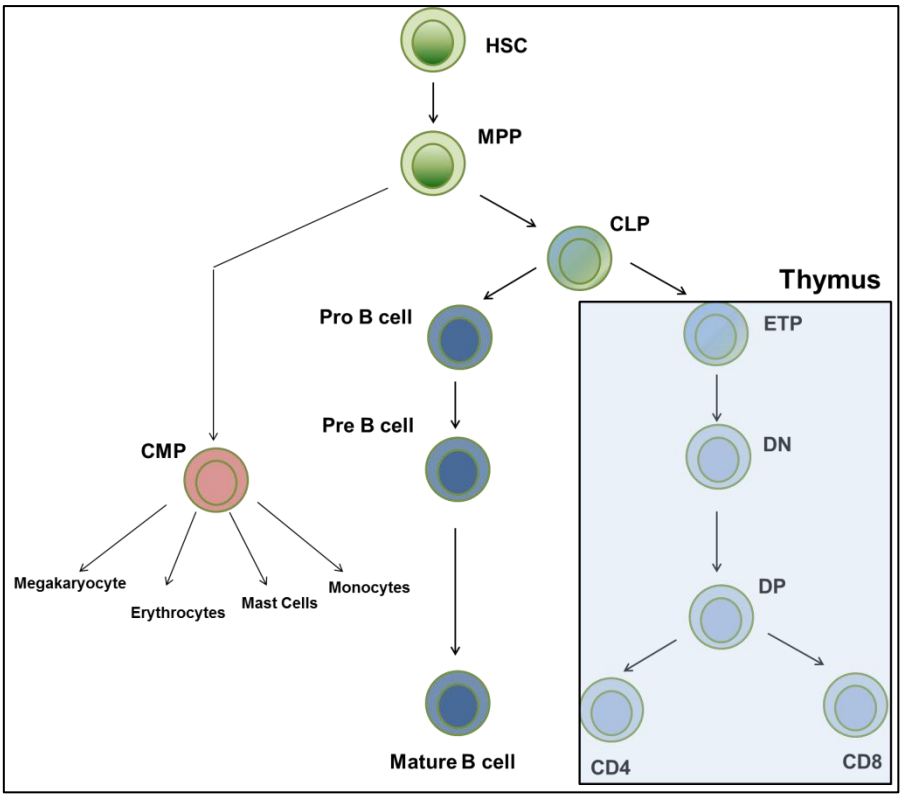

Figure 1.1.1: Development of lymphoid lineages from Hematopoietic stem cells (HSCs). HSCs that arise in the bone marrow differentiate into multipotent progenitors (MPPs) that have the capacity to differentiate into either myeloid or lymphoid precursors. Common lymphoid progenitors (CLPs) in the bone marrow develop into B cell lineage and those that migrate to the thymus gives rise to early thymic precursors (ETPs) which further develop into T lineage. Common myeloid progenitors (CMPs) give rise to various myeloid cells like megakaryocytes, erythrocytes, mast cells and monocytes which also happens in the bone marrow. ETPs in the thymus in response to notch signaling get committed to T lineage, further in response to T cell receptor (TCR) signaling and key transcription factors thymic precursors develop into mature CD4 and CD8 T cells.

Hematopoietic precursors that migrate to the thymus move via a sequential path before becoming mature T cells and migrate to the periphery to perform effector functions. Propagation of the precursors through T lineage program involves various transcription factors and signaling pathways. Notch signaling is one of the well understood signaling pathways in T cell development and differentiation (Radtke et al., 2010). Utilizing loss of function and gain of function studies, inactivation of Notch or RBPJ, a downstream regulator

blocks T cell development and leads to accumulation of B cells in the thymus. Early thymic precursors lose their capacity to differentiate into B lineage, erythroid cells or megakaryocytes even before they migrate to the thymus (Heinzel et al., 2007) however thymic progenitors have a potential to develop into dendritic cells, Natural Killer (NK) cells and macrophages (Masuda et al., 2005; Wu et al., 1996).

Developmental stages of thymocytes development are distinguished based on the expression of surface coreceptors CD4 and CD8. Earliest thymic progenitors lack the expression of CD4 and CD8 are known as double negative (DN) thymocytes. DN thymocytes are further segregated into DN1 to DN4 stages based on the expression of two other surface molecules CD44 and CD25. Cells later than DN2 ($CD44^+$ $CD25^+$) lose their capacity to differentiate to NK cells or dendritic cells. As the cells reach DN3 ($CD44^-$ $CD25^+$) they are committed towards the T lineage and initiate the expression of recombinase activating genes (RAGs) which are important for the somatic recombination of T cell receptor (TCR) β locus. The cells that have efficiently recombined the TCR-β locus start expressing it on their cell surface (Yui et al., 2010) and launch the development of αβ T cells. Other subset of T cells that have a completely different TCR expression on cell surface are the ϒδ T cells whose developmental program begins in the early DN stages prior to αβ T development (Hayes and Love, 2007).

Once the T cells express the TCR β and can initiate pre-TCR signaling they undergo rapid cell division and this process is known as β-selection (Laky and Fowlkes, 2007). Thymocytes post β-selection progress to double positive (DP) stage where thymocytes express both CD4 and CD8 coreceptors on cell surface. All of the thymocyte developmental processes from β-selection onwards are dependent on TCR signaling pathway (Jameson et al., 1995). DP thymocytes that receive TCR signal undergo positive selection and develop into either CD4 single positive (SP) or CD8 SP thymocytes (Singer, 2002). Thymocytes that bear TCRs which cannot respond to intrathymic signals and fail to signal undergo death by neglect (Surh and Sprent, 1994). Positive selection is the rescue of DP thymocytes from programmed cell death as a consequence of interaction between αβ TCR with self-peptide MHC complexes expressed on the thymic epithelia. The requirement of positive selection during thymocyte development is imperative based on fact that TCR generation is random and high diversity of MHC alleles (Carpenter and Bosselut, 2010). A few relatively conserved amino acids were identified in the complementarity determining regions (CDRs) of TCR that interact with

MHC, which acted as an evolutionary pressure to select the αβ thymocytes those that can interact with the MHC molecules (Feng et al., 2007; Marrack et al., 2008).

1.1.1 T cell receptor and TCR signaling

To recognize and neutralize the foreign antigens adaptive immune system has evolved specialized receptors that are generated by somatic recombination process. Antigen recognition molecules of the B lymphocytes are known as immunoglobulins or antibodies commonly. Immunoglobulins on the cell surface serves as receptor for the antigen, and is known as B cell receptor (BCR). A few terminally differentiated B cells known as plasma cells secrete the antibodies which bind to the antigens perform the main effector function of adaptive immune cells. Unlike B cells T cells have only the membrane bound antigen recognition molecules commonly known as the T cell receptor (TCR). The striking difference between the BCR and TCR is that TCR does not recognize the antigen directly, but recognizes small peptide antigens that are bound to MHC molecules present on the membrane of antigen presenting cells. T cells are broadly distinguished into two subsets based on the heterodimeric components of TCR into either αβ T cells or the γδ T cells (Davis and Bjorkman, 1988). As like the immunoglobulin structure each of the TCR chains have N-terminal variable domain and C-terminal constant domains and each of the domains have a characteristic intra domain disulphide linkage (Haskins et al., 1984). Variable domain of TCR is generated by somatic recombination of Variable (V), Diversity (D) and Joining (J) segments (Schatz, 2004). V(D)J recombination is generates antigen recognition molecules than can recognize a wide variety of peptide MHC ligands (Viret and Janeway, 1999). TCR itself does not have intrinsic signaling capacity because of short intracellular domains and depends on accessory molecules which propagate the TCR upon antigen interaction (Call et al., 2002). The TCR α- and β- chains are associated non-covalently associated with transmembrane proteins called the CD3 which initiates the signal transduction after specific TCR recognition of the antigen (Call and Wucherpfennig, 2005). CD3 components have immunoreceptor tyrosine based activation motifs (ITAMs) that become phosphorylated upon TCR engagement and initiates the downstream signaling cascade.

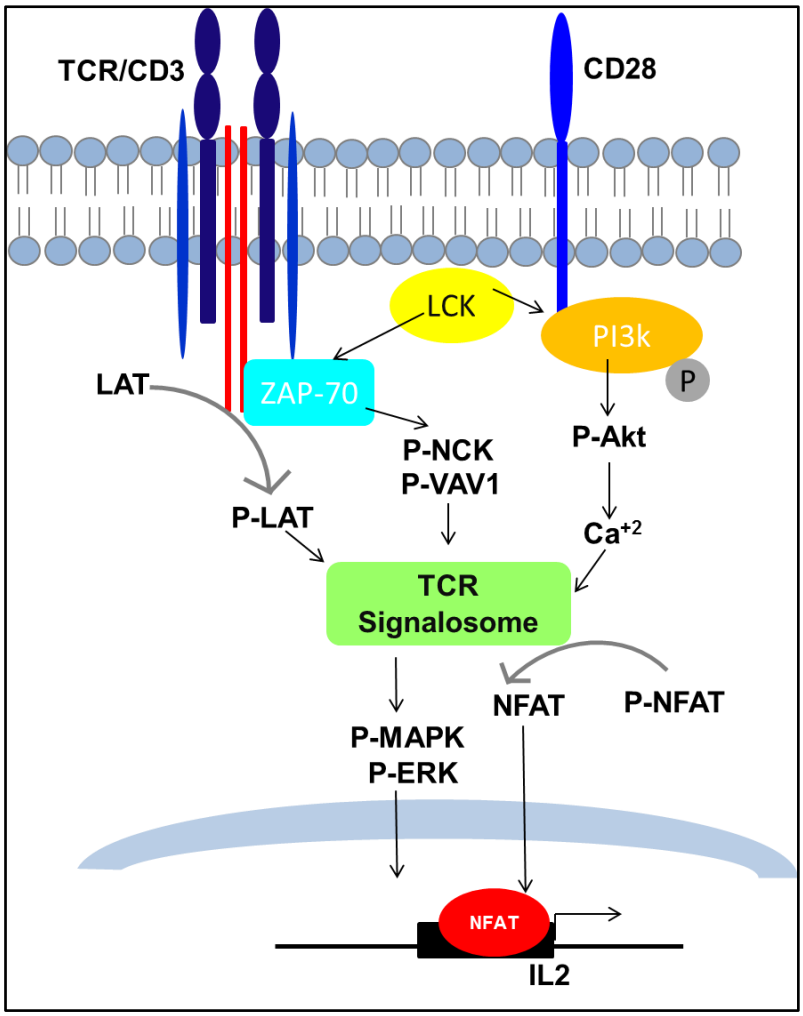

Figure 1.1.2: A simplified version of TCR signal transduction pathway. Ligation of TCR/CD3 results in the activation of receptor protein tyrosine kinases associated with CD3 domains and initiation of the downstream signaling pathway. LcK activates ZAP-70 which in turn phosphorylates tyrosine residues on LAT which further recruits downstream adapter proteins establishing the signalosome. TCR induced IP3 activates IP3 receptors on ER membrane and release Ca^{+2} into cytosol. Calcium signaling pathway activates NFAT which transclocates to the nucleus and regulates the expression of key cytokine genes like IL-2.

Jurkat T cells are a human leukemic cell line that has been of paramount importance in our current understanding of TCR signal transduction (Abraham and Weiss, 2004). Jurkat cells have an aberrant TCR have been shown to transduce TCR signals by pharmacological compounds like phorbol esters and Ca^{+2} ionophores which led to a speculation that signaling via the TCR also could follow a similar pathway (Weiss and Imboden, 1987). Treatment of Jurkat cells with Ca^{+2} ionophore (Ionomycin) and phorbol ester (PMA) induced a strong TCR signal measured by IL-2 cytokine secretion (Truneh et al., 1985). TCR stimulation results in

the activation of protein tyrosine kinases (PTKs) Lck, Fyn and Src which in turn phosphorylate ITAMs present on the cytoplasmic tails of TCRζ and CD3 chains. Multiple phosphorylation's on ITAMs acts as a docking site for various downstream signaling proteins like Phospholipase Cγ1 (PLCγ1), SLP-76 and Phopspoinositide 3-kinase (PI3K) and functions as a TCR signalosome (Smith-Garvin et al., 2009). The importance of LAT signalosome is evident from the studies in Jurkat cells which are deficient in LAT expression displayed impaired PLCγ1 phosphorylation, reduced $Ca^{+}2$ influx and MAPK activation and impaired activation of transcription factors AP-1, NFAT and NF-κB (Finco et al., 1998). Continuous signaling through the TCR results in a hyporesponsive state and the same cells on encounter of antigen at a later stage remains refractory to restimulation and this condition is known as T cell anergy (Schwartz, 2003). To avoid this stage of unresponsiveness or anergy, TCRs depend on other cell surface receptors known as costimulatory receptors to elicit an effective and appropriate T cell response. B7/CD28 pathway of costimulation has shown to be critical for the prevention of clonal anergy. CD28 costimulatory signal has shown to be important for the T cell proliferation, cytokine production, cell survival and metabolism (Acuto and Michel, 2003). PI3k has shown to be one of the key downstream effector of CD28 signaling. Upon binding of CD28 extracellular domain to its cognate ligands on the APCs, regulatory subunit of PI3K associates with the intracellular cytoplasmic tail of CD28 (Pages et al., 1994). PI3K converts Phosphatidylinositol 4, 5 bis phosphate (PIP_2) to Phosphatidylinositiol 3, 4, 5 bis phosphate (PIP_3) on the cell membrane where PIP_3 acts as a docking site for its target Akt. Akt phosphorylates multiple proteins which further affect many other cellular responses. Akt phosphorylates NF-κB which translocates to the nucleus and positively regulates the expression of BCl-xL (Qiao et al., 2008). Akt affects IL-2 expression of activated T cells by controlling the nuclear migration of NFAT proteins. Akt regulates NFAT translocation by phosphorylating GSK-3β and thus deactivating its activity. Unphosphorylated NFAT molecules now translocate to the nucleus and regulate the expression of key genes like IL-2 (Huang et al., 2008). Although the functions attributed to CD28 signaling are also activated by TCR signaling it has been proposed that magnitude of the response is determined by CD28 costimulation. Thus it has been proposed that CD28 signals determine the quantitative changes in the TCR signals as opposed to the qualitative differences as evidenced from CD28 null mice which have dampened immune responses (Acuto and Michel, 2003).

1.1.2 TCR signaling in Thymocyte development

Although the role of TCR signaling in differentiation of DP thymocytes to either CD4 or CD8 single positive (SP) thymocytes is well established , the mechanisms of thymocyte differentiation to either CD4 or CD8 SP thymocytes remains unclear. Different models have been put forward to explain the lineage commitment of DP thymocytes. The classical models suggest that there is a transcriptional termination of either CD4 or CD8 during positive selection. Two models that have been put forward on the lines of classical model are stochastic model and instructive model. Stochastic model suggests that there is termination of either of the coreceptor expression randomly and second step of TCR selection after positive selection SP thymocytes that have a matching TCR and coreceptor survive and develop in to mature T cells (Chan et al., 1993; Davis et al., 1993). The basic argument with the proposed stochastic model is that since the selection is random there is a probability that 50% of the thymocytes which have an effective TCR rearrangement are deleted, which leave the T cell development program as an chance event (knowing that the TCR recombination also a random process). Using transgenic TCR receptors it was shown that T cell repertoire selection attained an efficiency of 90% as opposed to stochastic model which proposed that approximately 50% of thymocytes are deleted because of random choice of either CD4 or CD8 coreceptors (Itano and Robey, 2000). Stochastic model has been replaced by Instructive model which proposes that TCR signals specify DP thymocytes to downregulate either of the mismatching coreceptors, suggesting that MHC class I and class II signals are different from one another (Seong et al., 1992). The instructive model was replaced by 'strength of signal model' which proposes that both TCR and the coreceptor determine the strength of the signal during positive selection and lineage commitment (Itano et al., 1996) where strong signals promote CD4 lineage development and weaker signals drives them to CD8 lineage. Further studies have shown that altering the number of Immune tyrosine–based activation motifs (ITAMS) that are within the TCR signaling complex showed an decrease in the total number of SP cells but the ratio between CD4 and CD8 SPs showed no change, contradicting strength of signal model (Love et al., 2000). Duration of Signal transduction model was proposed to address the loopholes of the previous model. According to this model duration of TCR signals on DP thymocytes determines CD4/CD8 lineage commitment, where longer duration of signals instructs them to CD4 and shorter signals to CD8 lineage (Yasutomo et al., 2000). All TCR signaled DP thymocytes reduce the surface expression of CD8 on cell surface and known as $CD4^{+}$ $CD8^{Lo}$ intermediate thymocytes. As the CD8 levels are downregulated it

disrupts MHC class I signaling but does not affect MHC class II signaling, thus MHC class I restricted TCR signaling is shorter in intermediate DP thymocytes and longer in MHC class II restricted thymocytes (Singer, 2002). The best model to explain CD4/CD8 lineage commitment based on the evidences so far is the kinetic signaling model proposed by Singer and colleagues (Singer et al., 2008). Kinetic signaling model proposes that lineage choice is determined by TCR signal duration and cytokine IL-7. The model proposes that TCR signaled DP thymocytes first terminate *Cd*8 gene transcription irrespective of MHC restriction. If the TCR signaling persists in absence of *cd8* gene transcription then thymocytes differentiates into CD4 thymocytes. Whereas cessation in TCR signaling of intermediate stage thymocytes in absence of *cd8* gene transcription causes their differentiation into CD8 thymocytes. For $CD4^{+}$ $CD8^{Lo}$ intermediate thymocytes to differentiate into CD8 lineage require the repression of *Cd4* gene expression and this process is referred to as 'co receptor reversal' (Brugnera et al., 2000). Thymocytes that no longer receive TCR signals require IL-7 for the survival and importance of IL-7 signaling is well established in the generation of CD8 SP thymocytes (Brugnera et al., 2000; Yu et al., 2003). In short all models including both classical and instructive model emphasize the importance of TCR signaling in the lineage commitment of thymocytes.

1.1.3 Transcriptional regulation of T cell development

Gene knockout studies of various transcription factors have been shown, to address the critical role of each during thymocytes development. Notch signaling has been shown to be indispensable for the T lineage program and inhibition of notch signaling using a dominant negative phenotype has caused the loss of early thymic precursors (ETP) population (Sambandam et al., 2005). A few transcription factors such as GATA-3, TCF-1 and BCL11b are known to play seminal roles in T lineage program (Rothenberg, 2011). Recent studies have shown that TCF1 is a downstream target of Notch signaling (Weber et al., 2011). In adult mice TCF-1 is predominantly expressed in the T lineage and deletion of TCF-1 blocks thymocyte development at DN stages and causes early involution of thymus (Germar et al., 2011; Verbeek et al., 1995). TCF-1 regulates genes that are components of TCR signaling, Gata-3 and BCl11b essential for the T lineage program. Overexpression of TCF1 was shown inhibit B and myeloid lineage differentiation and promote T lineage program (Weber et al., 2011).

Gata-3 is another early gene expressed during T cell development and critical for both early and later phases of thymocyte development (Hattori et al., 1996; Zheng and Flavell, 1997). Expression of *Gata-3* is observed in various adult and embryonic tissues such as kidneys, adrenal gland, brain tissue and mammary glands (Ho and Pai, 2007). Deletion of *Gata-3* has no effect on B cell or NK cell development; however, no T cells are detectable in thymus or spleen indicating its critical role in T lineage specification (Ting et al., 1996). Although Hematopoietic stem cells express low levels of Gata-3, it is required for the development of most primitive T lineage precursors (Hosoya et al., 2009). Gata-3 itself cannot drive the lineage progenitors cells towards T lineage in the absence of Notch signaling and enforced expression of Gata-3 aborts T lineage commitment (Rothenberg and Taghon, 2005). Under B cell promoting conditions overexpression of Gata-3 fails to promote T cell development in lymphoid progenitors (Taghon et al., 2007). Deletion of Gata-3 at DP stage disrupts the development of CD4 SP thymocytes (Pai et al., 2003). Gata-3 regulates the development of CD4 thymocytes by regulating Thpok expression, but overexpression of Thpok in Gata-3 deficient thymocytes failed to rescue the CD4 phenotype in mice (Wang et al., 2008).

Helper deficient (HD) mice lack CD4 lineage but have normal development of $CD8^+$ SP lineage, studies have shown that HD mice have a mutation in the ThPOK locus (He et al., 2005). ThPOK belongs to the POK family of transcription factors that are characterized Kruppel like Zinc finger domain responsible for DNA binding and POZ domain that mediates interactions with other proteins (Kelly and Daniel, 2006). It was also suggested that ThPOK is a component of TCR stimulation since its expression is not observed in $CD69^{-ve}$ subset of DP thymocytes and highly expressed in intermediate $CD4^+$ $CD8^{Lo}$ subset of thymocytes (He et al., 2005; Wang et al., 2008). ThPOK expression is maintained in mature $CD4^+$ T cells and the expression is low in $CD8^+$ cells. Termination of ThPOK expression in mature CD4 T cells causes derepression of CD8 transgene (Wang et al., 2008).

RUNT- related transcription factor (Runx) factor complexes are composed of Runx protein and a non DNA binding Cofactor Cbfβ (Levanon and Groner, 2004). Runx proteins are highly expressed in the hematopoietic cells and Runx knockout animals are devoid of lymphocyte development (Egawa et al., 2007). Runx factors are important in DN cell expansion, b-selection and lineage commitment of $CD8^+$ thymocytes (Telfer and Rothenberg, 2001). Runx3 regulates the development of CD8 lineage commitment by activating *Cd8* locus, silencing *Cd4* locus and repressing ThPOK expression (Collins et al., 2011). In $CD8^+$ T cells, Cd4 silencer and Cd8 loci are brought together by Runx3 and it mediates the

repression of CD4 by binding to CD4 silencer. In $CD4^+$ T cells where Runx3 is not expressed both the *Cd4* and *Cd8* loci are kept a part by ThPOK which inhibits the expression of CD8 and promotes CD4 T cell differentiation (Collins et al., 2011).

Bcl11b is also a T lineage-specific protein predominantly expressed in mouse thymocytes and is expressed at very low levels in NK lineage (Liu et al., 2010). Bcl11b expression is observed in all stages of thymocytes from the late DN stages, DP, CD4 and CD8 thymocytes. Its expression is known to be specifically upregulated after DN stages and was suggested to play a role during repression of *c-Kit*. Bcl11b is known to regulate the expression of *Ccr9* that encodes for the receptor Ccl25 and both of them are shown to be very important for the localization of progenitors to the thymus (Schwarz et al., 2007). In peripheral cells Bcl11b is expressed in both CD4 and CD8 T cells, however, it is downregulated in activated T cells indicating its role in repression of some genes prior to TCR activation (Li et al., 2010). Deletion of Bcl11b in mature T cells causes the expression of NK cell-specific genes (Li et al., 2010).

There are other non-canonical transcription factors which are not lineage specific but are required for T lineage program (Rothenberg et al., 2008; Rothenberg and Taghon, 2005). Ikaros transcription factor is one the first Trans factor shown to have a role in hematopoietic and lymphoid lineage development (Cortes et al., 1999). Ikaros factors are required for the development of both T and B lineages. Studies have shown that Ikaros family members have a role in negative selection in presence of strong TCR signaling (Urban and Winandy, 2004). Hox genes are important in the pattern formation during embryonic development. HOX-A gene cluster is primarily expressed in hematopoietic stem cells. HOX-A9 expression is shown to be important during T cell development and HOX-A9 knockout mice have reduction in DN2, DN3 populations (Izon et al., 1998). c-Myb is a proto oncogene required for both T and B cell development. Its role during T cell development is associated with β-selection stage in DN4 thymocytes (Bender et al., 2004). T-box family of transcription factors are well studied in lymphocyte development. TBX21 (T-bet) transcription factor is induced in activated T cells and is also expressed in CD8 and NK cells (Ho and Glimcher, 2002). In addition to transcriptional activators, transcriptional repressors such as GFi-1, NcoR1, and Hes-1 are also known to play specific roles during different stages T lineage development (Rothenberg and Taghon, 2005; Wendorff et al., 2010).

Transcription Factor	Effect upon deletion or Overexpression	Reference
RBPJ (downstream effector of Notch)	• No T lymphocyte development • Knockouts at later stage have defective β-selection	Maillard et al., 2005
TCF-1	• Block at DN stage • Development is blocked at ISP • Defect in β-selection • Transgenic expression of the factor in HSCs initiates T lineage program	Verbeek et al., 1995; Weber et al., 2011.
Runx3	• Reduced HSC survival • No CD8 SP development	Egawa et al., 2007
Gata-3	• Block at DN stage • Knockout at later stage have defective β-selection and no CD4 SP development. • Overexpression in HSC leads to reduced survival rates.	Hattori et al., 1996; Taghon et al., 2007.
ThPOK	• No CD4 T cell development • Increased percentage of CD8 T cells	He et al., 2005; Fischer et al., 2005.
Bcl11b	• T cell development block at DN2 stage • Deletion at DN and DP stage results in NK cell de-differentiation	Li et al., 2010.

Table 1.1.1: Table enlisting the key transcription factors essential during T lineage commitment and differentiation.

1.1.4 Regulatory T cell development

Regulatory T cells (Tregs) are important for the maintenance of 'tolerance' i.e. unresponsiveness to self-antigens and suppressing excessive immune responses those are harmful to the host (Sakaguchi et al., 2008). Tregs perform their suppressive functions by many ways which include by production of suppressive cytokines, inhibition of cytolysis, metabolic disruption and modulation of dendritic cells (Vignali et al., 2008). Regulatory T cells arise from the thymus and neonatal thymectomy around 3 days after birth would result in severe autoimmune damage (Bonomo et al., 1995). Transplantation and reconstitution studies have identified that $CD4^+$ T cells which have high surface expression of IL-2 receptor α-chain (CD25) into a nude athymic mice would prevent the development of autoimmune disease (Sakaguchi et al., 1995). X-linked immune deficiency syndromes IPEX in humans and Scurfy mouse lead to autoimmune disorders in multiple endocrine organs, inflammatory bowel disorder and other infections (Brunkow et al., 2001). Chromosomal analysis revealed that mutations at the *Foxp3* locus on X chromosome are responsible for the autoimmune disorders (Wildin et al., 2001). Further studies demonstrated that Foxp3 is the master regulator of Treg development, transgenic expression of Foxp3 in $CD4^+CD25^-$ non-Tregs was sufficient to induce suppressive function in these cells (Fontenot et al., 2003; Hori et al., 2003). Foxp3 is known to regulate key genes involved in regulatory T cell function such as CTLA4, GITR. However, most of the Treg cell signatures are not transactivated by Foxp3 but co-regulated along with Foxp3 (Hill et al., 2007). Foxp3 is known to oligomerize and interact with other proteins such as NFAT and regulate gene expression (Wu et al., 2006). Analysis of chromatin accessibility in Tregs and Tconventional (Tconv) cells has shown that Foxp3 binds to the pre-existing enhancer landscape and most of the enhancers become permissive after TCR activation and even before Foxp3 induction (Samstein et al., 2012a). Treg differentiation also depends on the epigenetic modifications like DNA methylation and TCR signaling induces DNA hypo methylation of Treg specific genes independent Foxp3 expression. Both DNA methylation pattern and Foxp3 expression are necessary for the Treg development and important for their suppressive functions (Ohkura et al., 2012).

1.1.5 Chromatin Organizer SATB1

Eukaryotic DNA is packaged into higher order chromatin structure that plays an important role in nuclear functions which are further associated with the proper functioning of the cell (Spector, 2003). In a typical eukaryotic interphase nucleus the chromatin territories are arranged in such a way that they are accessible to the transcriptional regulators which mediate activation or repression of the genes. Organization of the chromatin in the cell nucleus is facilitated by network of proteins that form a framework referred to as nuclear matrix (Kohwi-Shigematsu et al., 1997; Kramer and Krawetz, 1996). Specific DNA regions that have high affinity to the nuclear matrix are known as matrix attachment regions (MARs) or scaffold attachment regions (SARs) (Kohwi-Shigematsu et al., 1997). It has been postulated that MARs/SARs form the base of chromosomal loops and also help in compaction of the DNA, these regions are also have an important role in regulating gene expression. The proteins which bind to the MARs are known as MAR-binding proteins, which include SATB1 (Special AT rich binding protein 1), PARP1, Ku70 and Nucleolin.

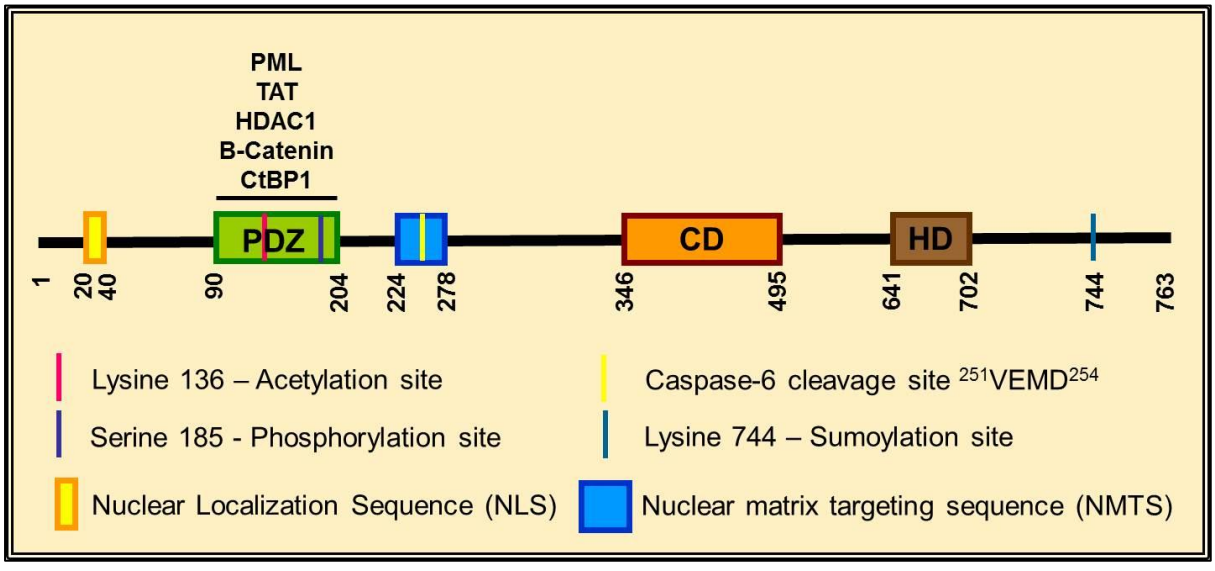

Figure 1.1.3: Schematic representation of SATB1 primary structure. SATB1 is a 763 amino acid nuclear protein. It harbors NH_2-terminal PDZ like domain that is required for homodimerization and heterodimerzation with other PDZ-containing domains. CUT repeats and homeodomain (HD) together confer the MAR-binding activity for the protein. SATB1 undergoes various post-translational modifications in a signal dependent manner and undergoes Caspase-6 cleavage at a particular site in apoptotic T cells.

SATB1 is a T lineage-enriched nuclear protein cloned by its virtue to bind MAR elements located in the 3' enhancer regions of Ig heavy chain enhancer regions (Dickinson et al., 1992). SATB1 protein is composed of three functional domains namely; NH_2-terminal PDZ like domain central CUT domain repeats and the C- terminal homedodomian (HD), the CUT repeats and HD together define the MAR binding feature of SATB1 (Fig. 1.1.3). SATB1 is one of the well characterized MAR-binding proteins in maintenance of chromatin architecture and organization of distinct 'loopscape' by anchoring the loops to base of the MARs to nuclear matrix (Cai et al., 2003; Galande et al., 2007). SATB1 has been shown to regulate distant genes by selectively tethering the MARs to the nuclear matrix resulting in the formation of a characteristic cage like network in mouse thymocytes. Immunostaining of SATB1 has shown that SATB1 specifically binds to the euchromatic regions in the nucleus and completely devoid from the DAPI dense heterochromatic regions in thymocytes (Cai et al., 2003; Notani et al., 2010). Furthermore SATB1 acts as a docking site for various chromatin remodelers such as ACF, ISWI and HDAC1 which are involved in nucleosomal remodeling and regulate transcription (Kumar et al., 2005; Yasui et al., 2002). Exploiting the MHC (Major histo-compatibility) I locus in understanding the chromatin based mechanisms (Kumanovics et al., 2003), it was shown that SATB1 along with PML organizes the loopscape of MHC I locus and regulates the expression of the locus (Galande et al., 2007; Kumar et al., 2007). Using SATB1 RNAi it has been shown that SATB1 is required for the chromatin loopscaspe organization of MHC I locus. It has also been shown that SATB1 is not only important for the organizing of the chromatin but also providing a conducive environment for the coordinated expression of the genes in the MHC I locus (Kumar et al., 2007). Role of SATB1 in chromatin organization is further exemplified by its role in organization of the 200 kb Th2 cytokine locus upon T cell activation and inducing the expression of IL-4, IL-5 and IL-13 in Th2 cells (Cai et al., 2006).

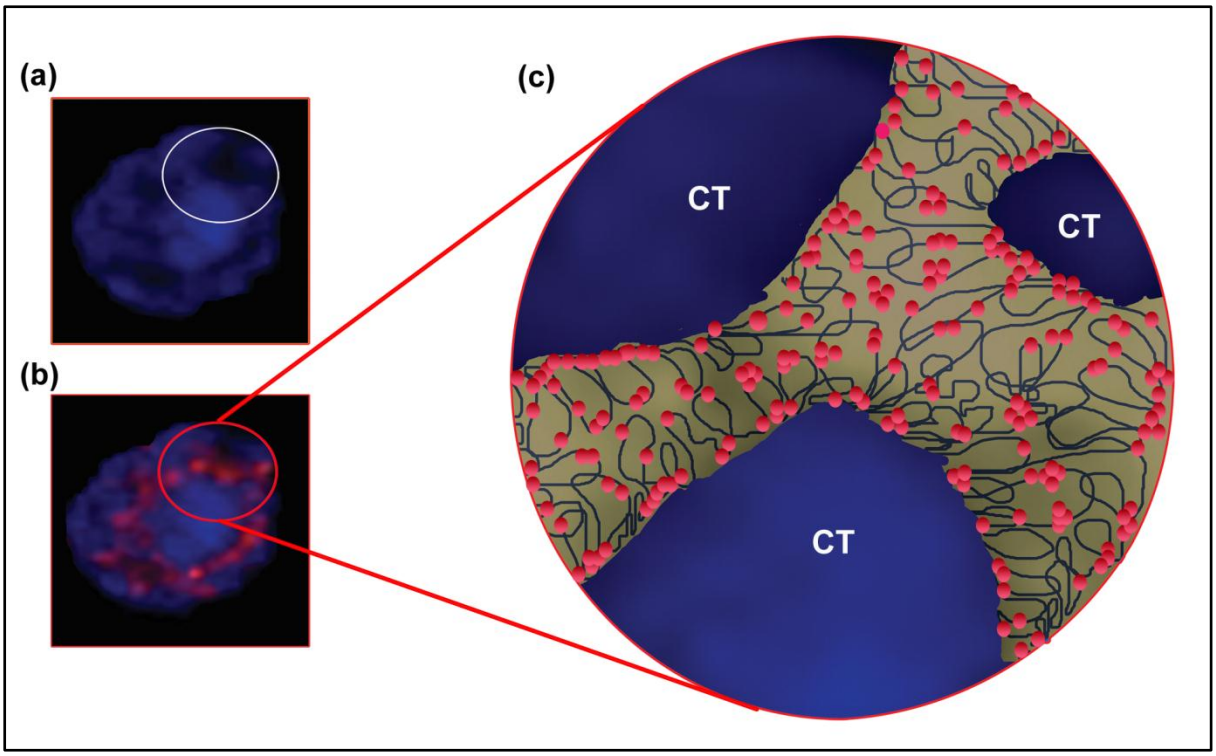

Figure 1.1.4: SATB1 is a global chromatin organizer. A. Mouse thymocytes stained with DAPI reveal densely stained heterochromatic regions otherwise known as chromatin territories. B. Mouse thymocytes stained with stained with SATB1 reveal a characteristic cage like network circumscribing the chromatin territories. C. Enlarged view proposing SATB1 role in organization of the chromatin structure, where SATB1 is shown to bind the MARs present in the transcriptionally poised chromatin territories interspersed within heterochromatic blobs. (Reproduced from Galande et al., 2007).

1.1.6 Transcriptional regulator SATB1

In vivo SATB1 binding sequences (SBS) in Jurkat T cells were isolated by crosslinking, immunoprecipitation and PCR amplification methods (de Belle et al., 1998), and it has been shown that SBS contain typical ATC context and in addition Line1 elements and CpG islands were also identified. To identify a consensus SATB1 binding site (CSBS) a different strategy known as SELEX (Systematic evolution of ligands by exponential enrichment) was utilized in Purbey et al., (Purbey et al., 2008). In this approach a subset of SATB1 binding oligonucleotides were enriched from a library of random oligomers by sequential enrichment of oligonucleotides using gel shift assays using MAR binding domain of SATB1. SELEX approach has identified a consensus binding site "TATTAGTAATAA" which is a reverse palindromic sequence suggesting that SATB1 binds as a heterodimer on genomic sequences. SATB1 binding on genomic loci was shown to provide a tissue-specific organization of DNA

sequences and promote targeted gene expression by regulating histone modifications (Cai et al., 2003). SATB1 has been shown to function as a global gene regulator in T cells which is in turn regulated by the post-translational modifications it undergoes (Pavan Kumar et al., 2006). Phosphorylated form of SATB1 binds to the DNA and has been shown to regulate the reporter activity whereas acetylated form of SATB1 is evicted from the target sites there by promoting the expression of IL-2 in activated T cells (Pavan Kumar et al., 2006). SATB1 is known to interact with other transcription factors such as PML, CtBP1, and β-catenin along with other chromatin remodelers in a signal-dependent manner and regulates target genes (Kumar et al., 2007; Kumar et al., 2005; Notani et al., 2010; Purbey et al., 2009; Yasui et al., 2002). SATB1 is upregulated in breast cancer cells and its expression correlates poor prognosis in breast cancer patients (Han et al., 2008). SATB1 has been shown to promote the expression of genes which facilitate metastasis and downregulates the expression of tumor suppressor genes. SATB1 expression is induced upon activation in Th2 cells and it is involved in the compaction of chromatin loops that induce the expression of cytokine genes on the *Il-4* locus (Cai et al., 2006). SATB1 has been shown to be crucial in establishing X-chromosome inactivation by organizing Xist RNA along SATB1 modeled chromatin and therby repressing gene expression on thr inactive X chromosome. Knockdown of SATB1 abrogates Xist expression and dysregulates the expression of genes (Agrelo et al., 2009). Upon Wnt signaling the SATB1: β-catenin complex activates the expression of SATB1 regulated genes as well as Wnt target genes and governs the differentiation of T cells (Notani et al., 2010).

1.1.7 SATB1 in Thymocyte development

SATB1 knockout mice exhibit a severe defect in T-cell development. SATB1-null mice have disproportionately small thymii and spleens as compared to the wild-type mice. At the cellular level, these mice exhibit multiple defects in T-cell development. The population of immature $CD3^-$, $CD4^-$, $CD8^-$ triple negative (TN) thymocytes is greatly reduced. Most strikingly, the thymocyte development is blocked at the double positive stage and the $CD4^+$ or $CD8^+$ SP thymocytes fail to develop (Alvarez et al., 2000). Ablation of SATB1 also results in spatiotemporal dysregulation of multiple genes such as *Il-2R* and *Il-7R* involved in T cell development and differentiation (Alvarez et al., 2000). Within the thymus majority of the DP thymocytes are eliminated via apoptosis during positive and negative selection process (Surh

and Sprent, 1994). Dexamethasone-induced apoptosis of thymocytes resulted in rapid dissociation of SATB1 from chromatin. Furthermore, SATB1 is specifically cleaved by caspase-6 after the aspartate residue at position 254 which led to the identification of the PDZ-like domain in the N-terminal region of SATB1. In vitro analysis revealed that caspase-6 cleavage also abolished the DNA-binding ability of SATB1 (Galande et al., 2001). The cleavage of SATB1 during T cell apoptosis might be required for the initiation of DNA fragmentation. In SATB1-null mice peripheral $CD4^+$ T cells fail to respond to activation stimulus and undergo apoptosis demonstrating indispensable role of SATB1 during proper T cell development (Alvarez et al., 2000). Comparison of the wild-type mice with the SATB1-/- mice indicated that repression of *Il-2R* gene was caused specifically by recruitment of histone deacetylases by SATB1 (Yasui et al., 2002). SATB1 was shown to be important for the development of CD8 thymocytes and it is required for the coreceptor reversal during the differentiation of DP thymocytes to CD8 SP thymocytes by binding to the CD8 enhancer (Nie et al., 2008).

A recent report has shown that Foxp3 negatively regulates SATB1 expression during Treg cell differentiation. It was shown that SATB1 is specifically downregulated in Tregs and deletion of Foxp3 in these cells resulted in upregulation of SATB1 and loss of suppressive function in Tregs. Further it was shown that in natural Treg cells Foxp3 directly binds to *Satb1* promoter and negatively regulates its gene expression (Beyer et al., 2011). Binding of Foxp3 to *Satb1* promoter enhances the repressive chromatin markers such as H3K27 trimethylation on the locus and also methylate the CpG residues upstream of the *Satb1* promoter and thus silence SATB1 expression. MicroRNAs such as miR-155, miR-21, and miR-7 were previously known to regulate Foxp3 expression (McInnes et al., 2012). In vitro reporter studies revealed that SATB1 is downregulated specifically by miR-155 by binding to the 3' UTR of the transcript (Beyer et al., 2011; McInnes et al., 2012). Thus Foxp3 and Foxp3-regulated microRNAs cause repression of SATB1 expression in mouse $CD4^+$ T cells. It was also shown that SATB1 overexpression in regulatory T cells affects the suppressive function of these cells. In SATB1 knockout mice, *IL2R* is upregulated in the double positive thymocytes (Alvarez et al., 2000) and it was shown that SATB1 is shown to specifically bind to upstream regulatory elements of *IL-2R* locus and represses its expression in mouse thymocytes (Yasui et al., 2002). Therefore, this data indicates that in T cells other than Tregs, SATB1 binds to the *Il-2R* locus and downregulates the expression of IL-2R (CD25). In Tregs,

SATB1 is downregulated in Foxp3-dependent manner and thus downregulation of SATB1 results in the induction of Treg-related genes.

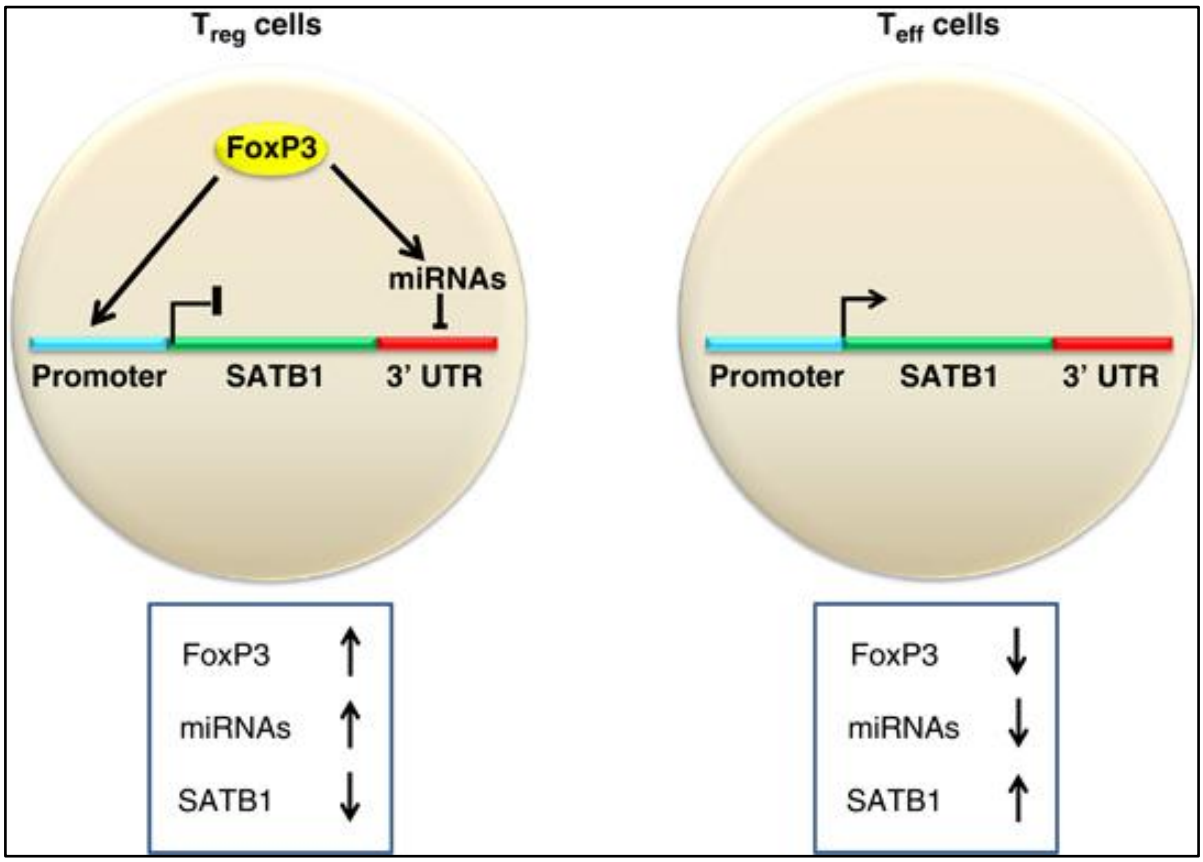

Figure 1.1.5: Schematic for SATB1 regulation in Treg and T effector cells. Foxp3, the master regulator of Treg cells, is induced upon TCR stimulation in anergic cells. Upon induction of Foxp3 and Foxp3 regulated miRNAs Treg-specific genes are upregulated and Teffector genes are repressed. SATB1 is repressed by direct binding of Foxp3 to SATB1 promoter and mir-155 further downregulates SATB1 expression by binding to the 3'UTR of SATB1 transcripts. (Reproduced from Burute et al., 2012).

1.2 Materials and Methods

1.2.1 Mice

The generation of β-cat-Tg mice was described in Mulroy et al., 2003 (Mulroy et al., 2003). TCF-1 KO mice were provided by H. Clevers. Age matched C57BL/6 control mice were used in all experiments. All the mice were bred and maintained according to the regulations and guidelines of National Institute of Aging, Baltimore, USA and National Centre for Cell Science, Pune, India. The animals used in the experiments were in compliance with guidelines from the regulatory bodies of both the institutes.

1.2.2 Antibodies

The following fluorescent conjugated antibodies were obtained from either BD biosciences (San Jose, CA, USA) or eBiosciences (San Diego, CA USA): CD4 (GK1.5), CD8 (53-6.7), CD24, CD69 (H1.2F3), Qa-2, FoxP3, TCR-β (H57-597), CD44 (IM7), and CD25. Anti-SATB1 antibody used for ChIP and immunostaining was raised in rabbit and purified using immunoaffinity chromatography using standard procedures. FITC conjugated anti-Rabbit IgG was obtained from Boehringer Ingelheim (Germany).

1.2.3 Cell sorting

Mouse thymii were surgically removed from 21 day old mice. Mouse thymii were macerated and thymocytes were suspended in 10 ml RPMI (Gibco, CA, USA) with 10% FCS. The cells were pelleted by centrifugation and resuspended in FACS staining buffer (1X PBS with 10% BSA). Thymocytes were stained with CD4 and CD8 antibodies and then sorted on FACS Aria (Becton Dickinson). Cells were analyzed for CD4 and CD8 profile post sort and the purity was confirmed to be 90% for each population.

1.2.4 Immunostaining and FACS analysis

Mouse thymocytes were isolated, stained and analyzed on FACS CantoII (Becton Dickinson, NJ, USA). Dead cells were excluded by forward side scatter. All the data was acquired and presented on Log scale. Staining of intra nuclear antigens such as SATB1 FoxP3 was performed using Foxp3 nuclear staining kit as mentioned (eBiosciences, San Diego, CA, USA). Briefly, mouse thymocytes were isolated and resuspended in FACS staining buffer (1X PBS with 5% BSA) stained with the antibodies corresponding to surface antigens. Then the cells were permeabilized with permeabilization buffer as provided with Foxp3 staining kit (eBiosciences, San Diego, CA, USA) and then stained for nuclear antigens. In case of SATB1 the cells were stained with rabbit anti-SATB1 antibody and then incubated with FITC tagged anti-rabbit secondary antibody.

1.2.5 RNA isolation and cDNA synthesis

Total RNA was isolated using RNAeasy isolation kit (Qiagen, Maryland, USA). Then total RNA (500 ng) is reverse transcribed Dynamo cDNA synthesis Kit (Finnzymes, Cheshire, UK) using oligo dt primer. Quantitative Real-time PCRs are performed using SYBR green (Applied Biosystems,Grand land, NY, USA), with annealing and extension of primers at 60 ^{0}C. Fold changes were calculated using the formula; Fold change = $2^{-(dd\ Ct)}$.

1.2.6 Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (Jayani et al., 2010). Briefly, Mouse thymocytes were crosslinked by addition of formaldehyde to 1% final concentration in media and incubation at room temperature for 10 min, neutralized with 125 mM glycine, and then subjected to sonication using Bioruptor (Diagenode, Belgium) for 15 cycles with 30 sec ‘on’ and 30 sec ‘off ‘to fragment the chromatin to obtain 200–500 base pair fragments. Sonicated chromatin was precleared with a cocktail containing 50% protein A/G beads slurry (Thermo scientific, IL, USA), Salmon sperm DNA, and BSA. Precleared chromatin was incubated with specific antibodies overnight at 4 ^{o}C and respective IgG types were used as isotype controls. Protein A/G bead cocktail was then added to pulldown the antibody-bound chromatin and was subjected to elution using sodium biocarbonate buffer containing SDS and

DTT (Sigma-Aldrich, St. Louis, USA). Eluted chromatin was de-crosslinked and protein was removed by treating with proteinase K. Purified immunoprecipitated chromatin was subjected to PCR amplification using specific primers. Input chromatin was used as a control.

1.2.7 ChIP-seq analysis

Chip-seq analysis was performed using the data available from GEO database (GSE20898). The chipseq reads are aligned to the mm8 genome and peak calling on binding site was performed by MACS (Zhang et al., 2008).

1.2.8 Statistical analysis

All the statistical calculations were performed using Microsoft office Excel (2010) and the graphs were generated using Microsoft office Excel (2010).

1.3 Results

1.3.1 SATB1 expression is developmentally regulated in mouse thymocytes

SATB1 is a chromatin organizer is predominantly expressed in the thymocytes (Dickinson et al., 1992) and we wished to study its role during the ontogeny of T cell development. As a first step towards understanding the role of SATB1 it was necessary to monitor the expression of SATB1 in developmental subsets of thymocytes. Mouse thymocytes were sorted into four distinct developmental stages based on the surface expression of CD4 and CD8 coreceptors (Fig. 1.3.1A). Quantitative RT-PCR analysis in sorted populations showed that there is differential expression of SATB1. DN thymocytes had minimal expression of SATB1 whereas DPs showed the highest expression. CD4 and CD8 thymocytes exhibited intermediate levels of SATB1 expression compared to DN and DP thymocytes (Fig. 1.3.1B), suggesting that SATB1 is induced during T cell development and downregulated in committed mature thymocytes. To ascertain if SATB1 is specifically induced during thymocyte developmental program, we monitored the expression profile of SATB1 by intracellular staining followed by flow cytometric analysis. Mouse thymocytes were stained with a cocktail of lineage (Lin) specific antibodies which included CD4, CD8, TCRβ, TCRγδ. The lineage negative fraction of cells (Lin –ve) represent immature precursors during thymocyte development (Fig. 1.3.2A), were analyzed for CD44, CD25 and SATB1 expression. CD44 and CD25 markers discriminate early thymic precursors into four distinct sub stages - DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{-}$), DN3 ($CD2544^{-}CD25^{+}$) and DN4 ($CD44^{-}CD25^{-}$) (Fig. 1.3.2B). SATB1 expression in early subsets of DN thymocytes (DN1 to DN3) is low, and the expression is induced specifically in DN4 subset of thymocytes (Fig. 1.3.2C, 1.3.2D). Early thymic progenitors (DN1-DN3) have a potential to differentiate into myeloid, NK and B lineage but the cells that have reached the DN4 stage are committed to T lineage (Rothenberg, 2011). During DN3 stage TCR-β locus is rearranged and starts expressing on cell surface. Only DN3 thymocytes that have an efficient TCR-β rearrangement express pre-TCR on the cell surface and pre-TCR signaling and advance further in T lineage program. These results indicate that SATB1 is upregulated in thymocyte precursors that are committed to T lineage and have undergone β-selection.

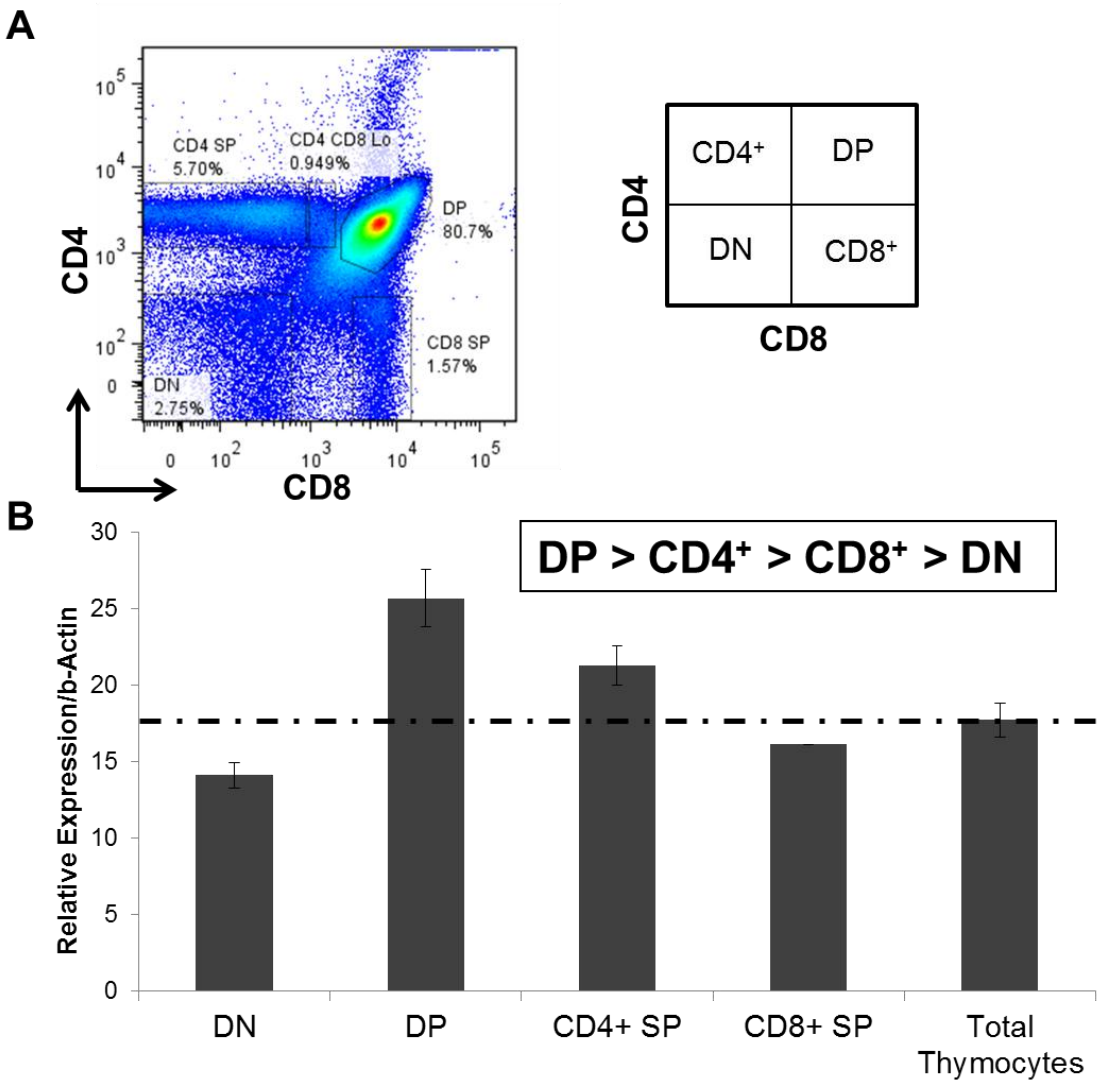

Figure 1.3.1: Differential expression of SATB1 in mouse thymocyte subpopulations. (A) Mouse thymocytes were stained with fluorescently tagged anti-CD4 and anti-CD8 antibodies and sorted into subpopulations as described in 'Materials and Methods' into DN, DP, CD4 SP and CD8 SP. (B) Quantitative RT-PCR analysis was performed using RNA extracted from each subpopulation of thymocytes. Relative gene expression of SATB1 in each population was normalized with β-actin. Bar graph represents the relative expression of SATB1 and error bar denotes standard deviation calculated from triplicates.

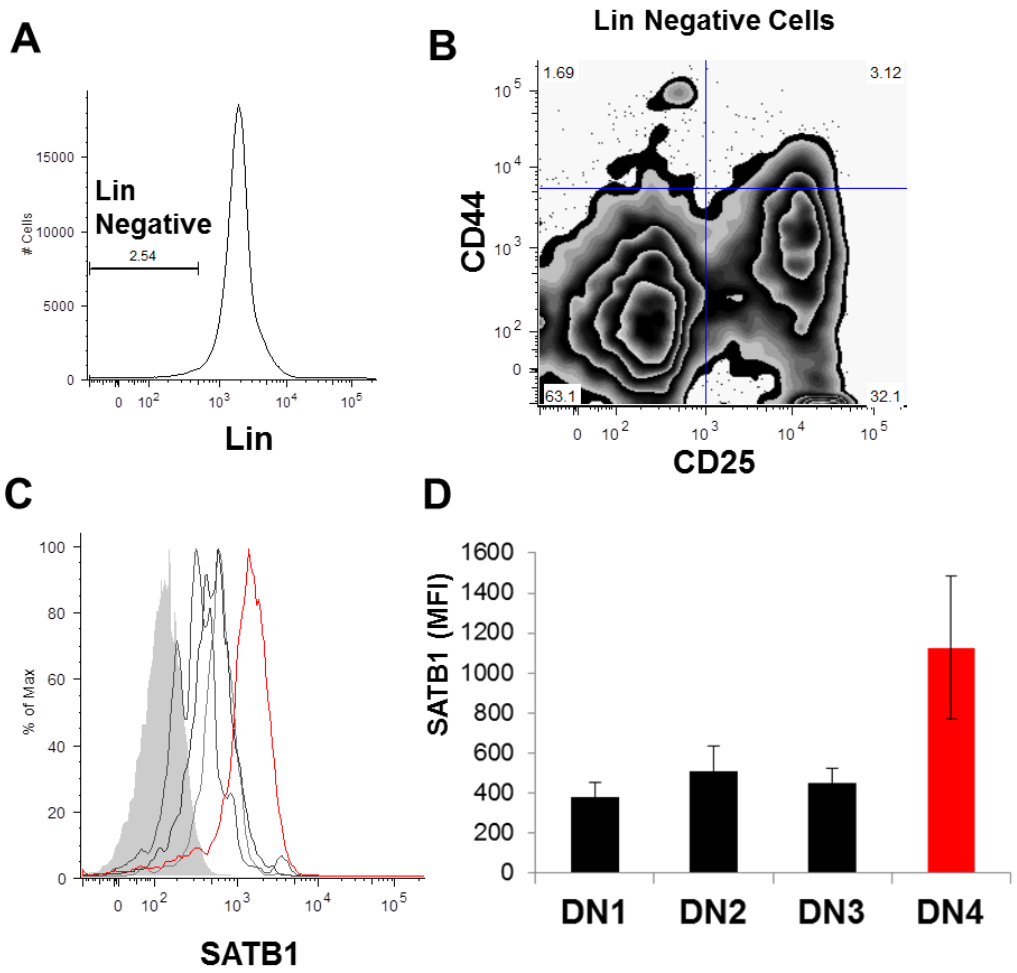

Figure 1.3.2: SATB1 expression is induced during thymocyte development. (A) Mouse thymocytes were stained with a cocktail of fluorescent tagged lineage specific antibodies as described in 'Materials and Methods', thymocytes that are Lineage negative represent the early thymocyte precursors. (B) Lin negative gated population were analyzed for CD44 and CD25 expression which distinguishes thymic precursors into DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+$ $CD25^-$), DN3 ($CD44^-$ $CD25^+$) and DN4 ($CD44^-$ $CD25^-$) populations. (C) Histogram represents the expression of SATB1 in each subset of DN populations. DN1, DN2 and DN3 are represented in black and the histogram in red represents SATB1 expression in DN4 population. (D) Bar graph represents the expression of SATB1 which is based on the mean fluorescent intensities (MFI) in each subset of thymocyte population. Error bars denotes standard deviation calculated from 3 different experiments.

We then determined the expression profile of SATB1 in DP, CD4 SP and CD8 SP thymocytes by intracellular staining followed by flow cytometric analysis (Fig. 1.3.3A). Expression level of SATB1 in DP thymocytes was comparable to that observed in DN4 subset (Fig. 2D and 3B). The profile revealed interesting observations, wherein CD4 thymocytes exhibit bimodal expression pattern of SATB1 which we refer to as $SATB1^{Hi}$ CD4 and $SATB1^{Lo}$ CD4 populations (Fig. 1.3.3A, 1.3.3E). SATB1 expression in CD4-$CD8^{Lo}$

intermediate population is upregulated in comparison to DP thymocytes and CD8 SP thymocytes display an expression pattern similar to that of SATB1^{Lo} CD4 population. These findings together suggest that SATB1 is specifically induced during thymocyte development.

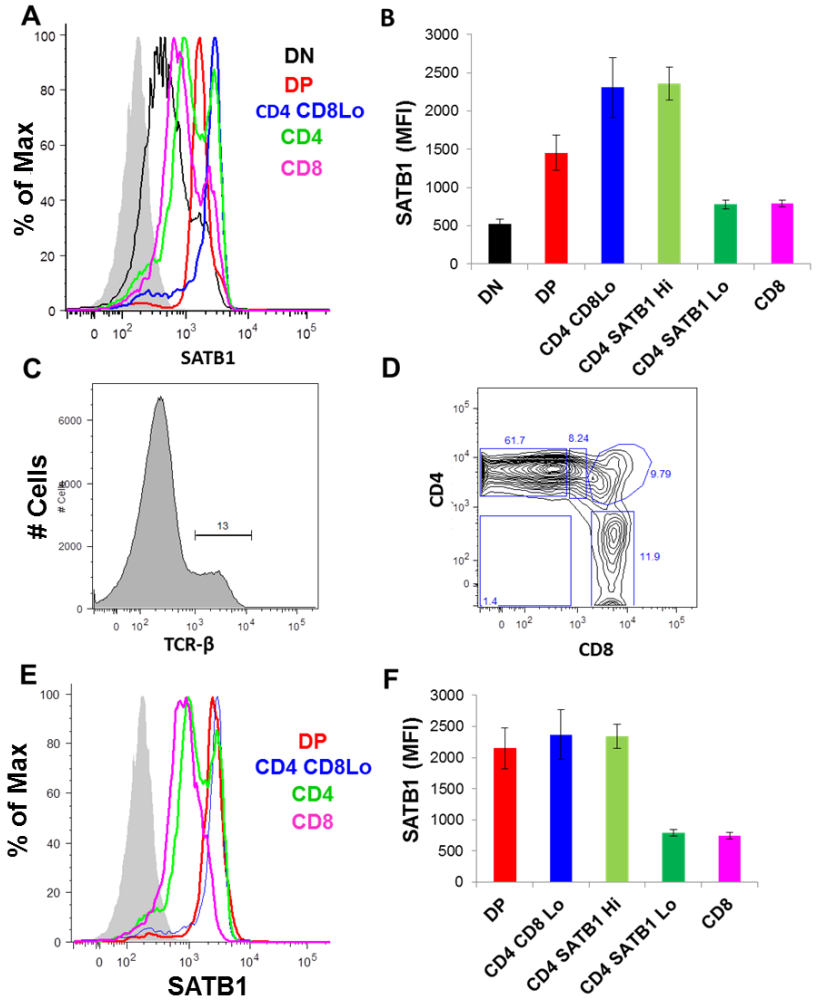

Figure 1.3.3: Expression of SATB1 in mouse thymocytes is dependent on TCR signalling. (A) Mouse thymocytes were stained with CD4, CD8 and FITC SATB1 and histogram represents SATB1 expression levels in distinct subsets of mouse thymocytes based on CD4 CD8 co-receptor expression. (B) Bar graph representing the Mean fluorescence Intensity (MFI) values of SATB1 expression in distinct thymocytes populations based on CD4/CD8 surface expression and error bar represents standard deviation calculated from three experimental replicates. (C) & (D) Thymocytes were gated on TCR-β^{Hi} populations and were analyzed for CD4/CD8 expression. (E)**.** Histogram representing SATB1 expression in each sub population of TCR-β^{Hi} CD4-CD8 gated thymocytes as shown in Fig. 3(D). (F)**.** Bar graph represents SATB1 expression in terms of MFI, in each subpopulation of thymocytes. Error bar represents standard deviation from three independent experiments.

1.3.2 TCR signaling regulates SATB1 expression during thymocyte development

Ontogeny of T cell development is dependent on the expression of TCR and TCR mediated signaling. DP thymocytes are subjected to positive and negative selection in TCR-dependent manner before they differentiate into either CD4 or CD8 single positive thymocytes (Starr et al., 2003). Positively selected thymocytes are those that have had a viable TCR rearrangement, express TCR on cell surface and interact with intermediate affinities to the self-peptide MHC on thymic epithelia. Towards this, we have compared the expression pattern of SATB1 in different subsets of TCRβ^{Hi} thymocytes (Fig. 1.3.3D, 1.3.3E). SATB1 expression in TCRβ^{Hi} DP thymocytes is elevated in comparison to total DP thymocytes (compare Fig. 1.3.3B & 1.3.3F), and expression is comparable to the levels expressed in CD4 $CD8^{Lo}$ intermediate population and $SATB1^{Hi}$ CD4 population. These results suggest that SATB1 is regulated in TCR-dependent manner.

To comprehensively determine if SATB1 expression is regulated in TCR-dependent manner during thymocyte development, we monitored the expression of SATB1 in distinct developmental stages of thymocyte development based on CD69 and TCRβ expression. Thymocytes that have intermediate expression of TCRβ and are negative for CD69 are thymocytes that have not undergone thymic selection (population 1); those that have intermediate expression of TCR and express CD69 represent the population undergoing thymic selection (Population 2). Thymocytes that have higher expression of TCRβ and CD69 on cell surface are those that have successfully undergone positive selection (Population 3). Population 4 that exhibit high TCRβ expression and downregulated CD69 expression are mature thymocytes (Fig. 1.3.4A). We observed that SATB1 is upregulated in population 2 and 3, wherein TCR signaling is on and express CD69 which is an early T cell activation mark. Mature thymocytes that are equipped to migrate to the periphery have abatement of TCR signaling evident from diminished CD69 expression, exhibit downregulation of SATB1 (Fig. 1.3.4B). In positively selected thymocytes (population 3) we determined the expression of SATB1 in each subset of developing thymocytes based on CD4 CD8 expression and observe that SATB1 expression levels are similar in DP and CD4 fractions, whereas in CD8 subset SATB1 expression is lower in comparison to CD4 thymocytes (Fig. 1.3.4C). In mature thymocyte fraction SATB1 is downregulated in both CD4 and CD8 subsets (Fig. 1.3.4D). Together, these data argue that during thymocyte development SATB1 levels could be directly regulated by TCR signaling.

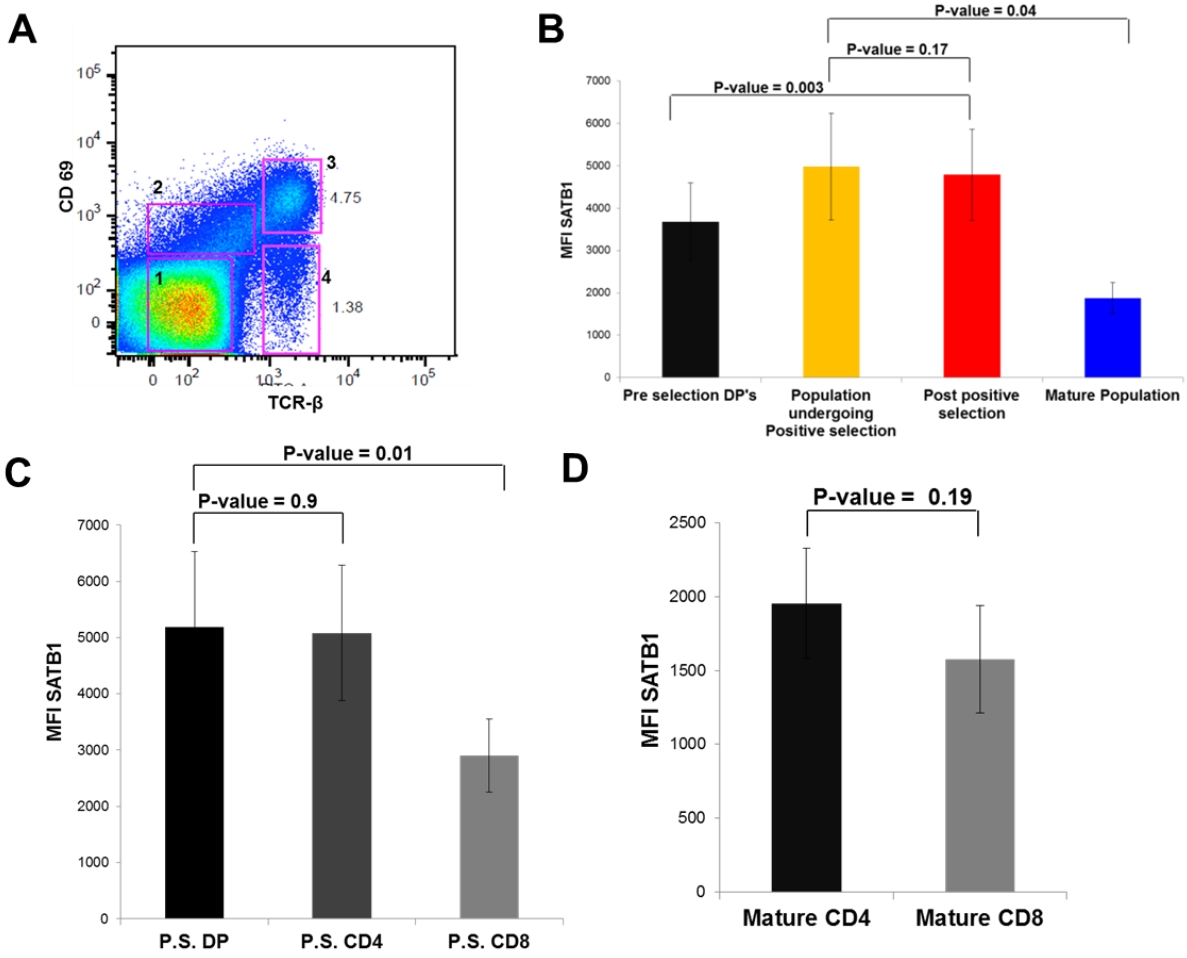

Figure 1.3.4: SATB1 expression is developmentally regulated during thymocyte development. (A**)**. Mouse thymocytes were stained with CD69 and TCR-β to distinguish them into developmental stages based on positive selection. Population 1 represents early thymocytes that have intermediate levels of TCR-β expression and negative for CD69 are thymocytes that have not undergone positive selection. Population 2 represents thymocytes that are undergoing positive selection and have upregulated CD69 expression. Population 3 are thymocyte population that are TCR-β^{Hi} and upregulated CD69 expression, these cells are thymocytes that have undergone positive selection. Population 4 thymocytes are mature thymocytes that have high TCR-β expression and downregulated CD69 expression. (B) Bar graph represents SATB1 expression in each population represented in panel (A) and the errors bars represent standard deviation calculated from n=5. (C) Bar graph represents SATB1 expression in DP, CD4 and CD8 subpopulations of the thymocytes that have undergone positive selection (Population 3). (D) Bar graph denotes SATB1 expression in CD4 and CD8 thymocytes of mature thymocyte population (population 4). P-values were calculated using students T-test.

1.3.3 Relevance of bimodal distribution in CD4 SP thymocytes

Since SATB1 is regulated in TCR-dependent manner, we therefore wished to understand the developmental relevance of bimodal distribution of SATB1 observed in $CD4^+$ SP gated thymocytes (Fig. 1.3.4A, B, C). We further compared the expression of SATB1 in CD4 mouse thymocytes with that of peripheral $CD4^+$ T cells isolated from spleen and lymph node and we observed that peripheral $CD4^+$ T cells do not exhibit dual population as seen in thymocytes and the expression pattern in peripheral cells coincides with the $SATB1^{Lo}$ population of $CD4^+$ thymocytes (Fig. 1.3.4D). Next, we wished to understand if the $SATB1^{Hi}$ and $SATB1^{Lo}$ populations of $CD4^+$ thymocytes are progressive stages during the development of $CD4^+$ thymocytes. Towards this, $CD4^+$ gated mouse thymocytes were analyzed for CD69 expression and we observed that $SATB1^{Hi}$ $CD4^+$ thymocytes were positive for CD69 expression whereas $SATB1^{Lo}$ $CD4^+$ thymocytes exhibited downregulated expression of CD69 (Fig. 1.3.5 A,B,C). When mouse thymocytes were analyzed for SATB1 expression separately in $CD69^{+ve}$ and $^{-ve}$ subsets, we observed about 3-fold higher expression of SATB1 levels in $CD69^{+ve}$ thymocytes compared to $CD69^{-ve}$ $CD4^+$ thymocytes (Fig. 1.3.5 D,E,F).

It has been shown that majority of $CD4^+$ SP thymocytes are functionally immature and heterogeneous population. A hematopoietic differentiation antigen - HSA (CD24), which is observed on all immature and DP thymocytes but not on peripheral T cells, interestingly a considerable fraction of CD4 SP thymocytes were shown to have expression HSA and are immature (Scollay et al., 1984). Further it was shown that mature CD4 SP thymocytes can be distinguished from immature CD4 thymocytes by the expression of non-classical class I antigen, Qa-2 which appears on cells that are HSA^{-ve} (Vernachio et al., 1989). Immature HSA^{Hi} $Qa\text{-}2^{Hi}$ CD4 thymocytes are incompetent for TCR activation contrasting to mature HSA^{Lo} $Qa\text{-}2^{+ve}$ thymocytes (Nikolic-Zugic and Bevan, 1990). Since maturation of SP thymocytes is associated with downregulation of HSA (Crispe and Bevan, 1987) and upregulation of Qa-2 surface expression (Ramsdell et al., 1991) we wished to determine the expression of these surface markers on $SATB1^{Hi}$ and $SATB1^{Lo}$ CD4 thymocyte populations. We observed that the expression of HSA in $SATB1^{Hi}$ CD4 thymocytes was similar to the profile seen in DP thymocytes. Whereas in $SATB1^{Lo}$ CD4 thymocytes the surface expression of HSA is downregulated indicating the developmental progression of CD4 thymocytes from $SATB1^{Hi}$ to $SATB1^{Lo}$ compartment (Fig. 1.3.7C, 1.3.7D). We also observed that $SATB1^{Hi}$ population is negative for the expression of Qa-2 (Fig. 1.3.7A, 1.3.7B). Of $SATB1^{Lo}$ CD4

thymocytes a significant fraction (> 80%) of thymocytes exhibited Qa-2 surface expression. These results suggested that SATB1 is downregulated in mature $CD4^+$ thymocytes and the expression of SATB1 is comparable to levels observed in peripheral $CD4^+$ T cells in spleen and lymph nodes (Fig. 1.3.6B). Other studies have indicated that CD5 expression is regulated on thymocytes by TCR signals and avidity of TCR-antigen interactions (Azzam et al., 1998). Intermediate levels of CD5 are observed in DP thymocytes and high levels of CD5 are observed in $CD4^+$ and $CD8^+$ thymocytes proportional to TCR signaling. We observed that CD5 expression in $SATB1^{Hi}$ $CD4^+$ thymocytes population is high compared to mature $SATB1^{Lo}$ $CD4^+$ thymocytes (Fig. 1.3.7E, F). In all these results suggest that SATB1 which is regulated in a TCR dependent manner (Fig. 1.3.5) is downregulated in mature $CD4^+$ SP thymocytes.

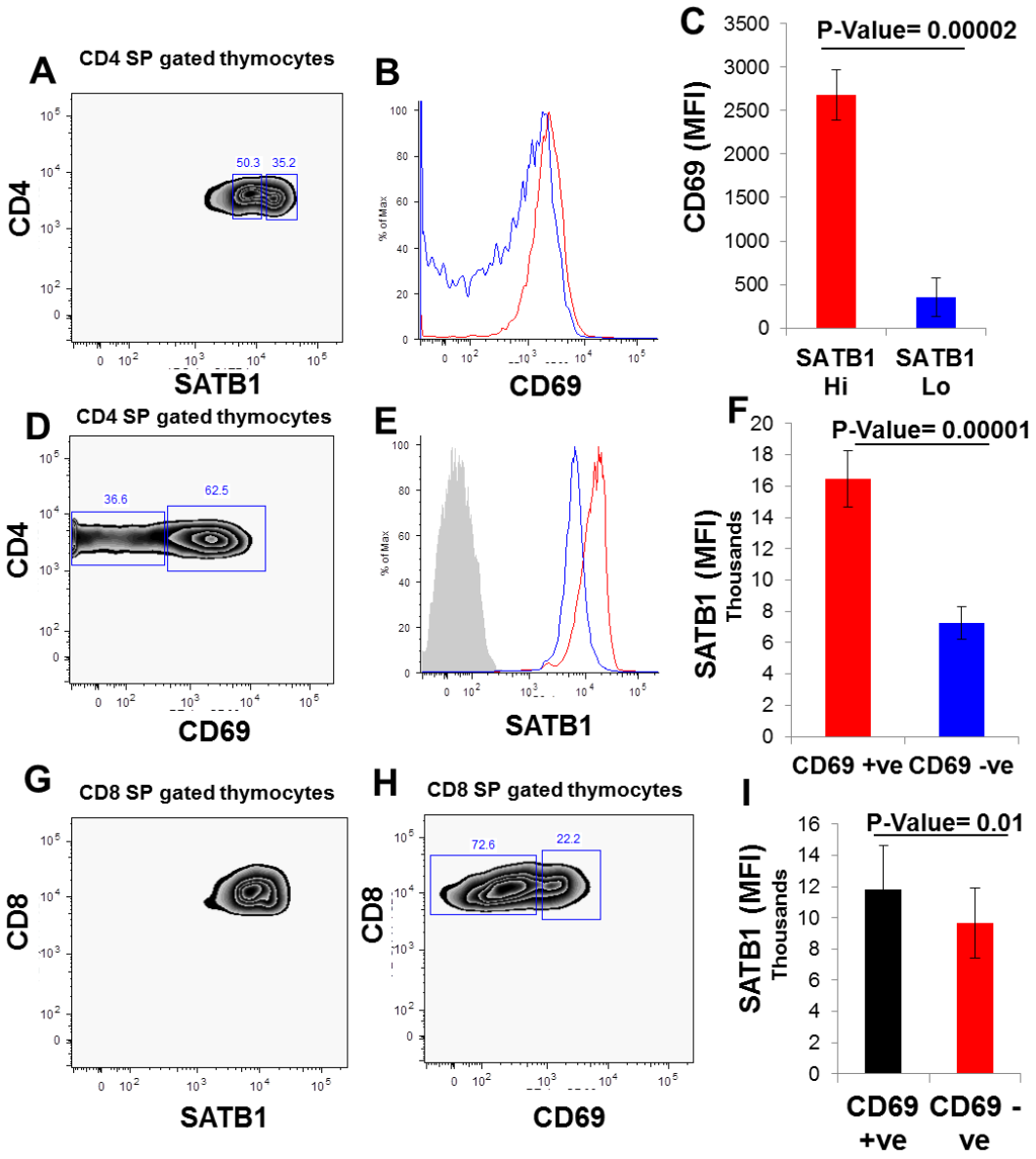

Figure 1.3.5: Expression of SATB1 in single positive thymocytes is regulated in TCR-dependent manner. (A) CD4 gated mouse thymocytes were analyzed for SATB1 expression and gates represent $SATB1^{Hi}$ (right) and $SATB1^{Lo}$ (left) populations of $CD4^{+}$ thymocytes. (B) Histogram represents CD69 expression in $SATB1^{Hi}$ (red) and $SATB1^{Lo}$ (blue) thymocytes. (C) Mean fluorescence Intensities (MFI) of CD69 expression in $SATB1^{Hi}$ and $SATB1^{Lo}$ populations are represented as bar graphs. (D) FACS plot representing CD69 expression in $CD4^{+}$ gated thymocytes. The right gate represents $CD69^{+ve}$ CD4 thymocytes and the left represents CD69-ve CD4 thymocytes. (E) Histogram represents SATB1 expression in CD69 positive (red) and CD69 negative (blue) subsets of CD4 thymocytes shown in figure (D). (F) Bar graph represents SATB1 expression (MFI) in $CD69^{+ve}$ and $CD69^{-ve}$ CD4 thymocytes. (G) CD8 gated thymocytes were analyzed for SATB1 expression profile. (H) FACS plot representing $CD69^{+ve}$ (right) and $^{-ve}$ (left) subsets in CD8 gated thymocytes thymocytes. (I) SATB1 expression (MFI values) plotted as bar graphs in '$CD69^{+ve}$' and '$CD69^{-ve}$' CD8 thymocytes. Data is generated from five individual mice thymii. Error bars denote standard deviation and P-value is calculated by student T-test.

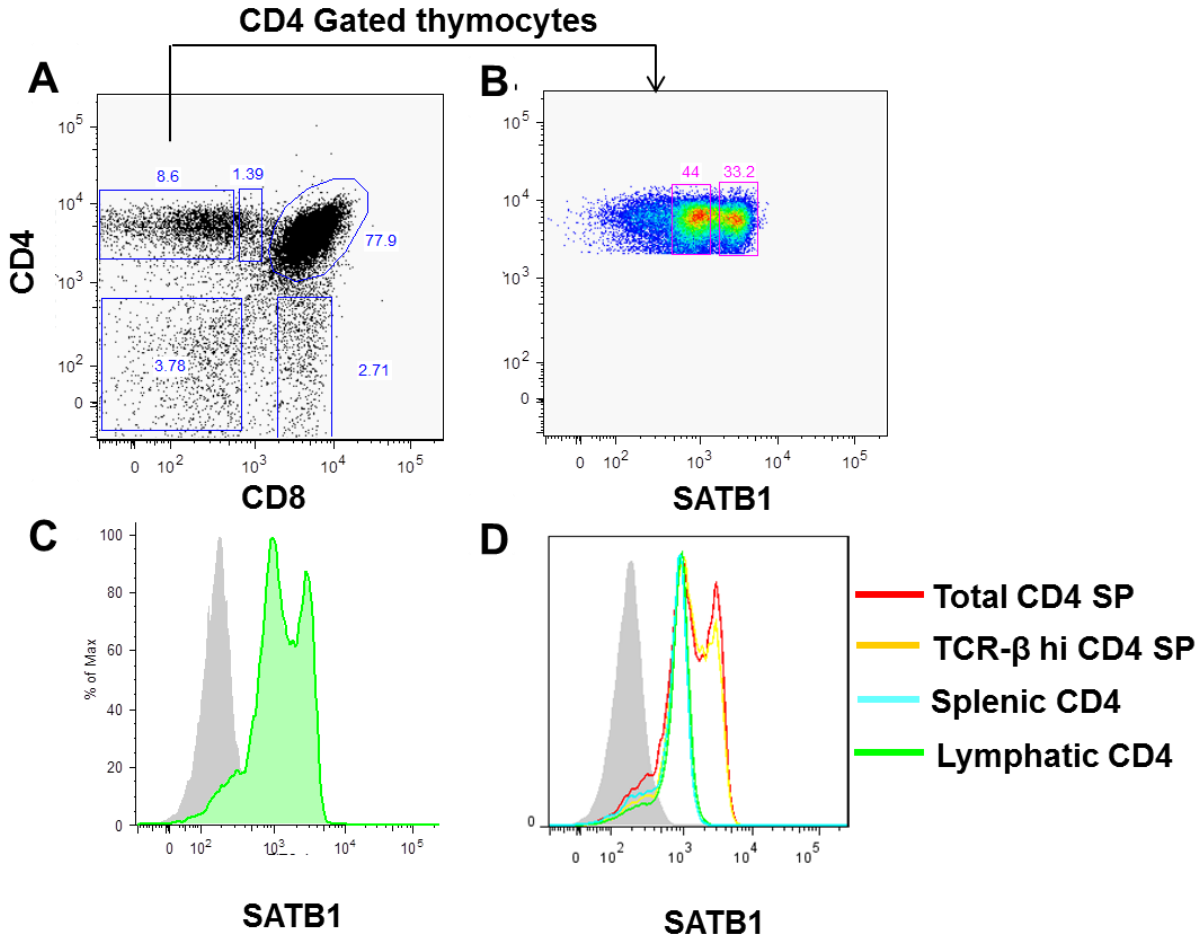

Figure 1.3.6: CD4 thymocytes exhibit bimodal distribution of SATB1 expression. (A) Representative flow cytometry scatter plot showing the gates applied on CD4 CD8 stained thymocytes that distinguishes them into DN, DP, CD4+ $CD8^{Lo}$ Intermediate, $CD4^{+}$ and $CD8^{+}$ populations. (B) $CD4^{+}$ gated thymocytes were analyzed for SATB1 expression and we observed bimodal distribution of SATB1 in $CD4^{+}$ positive thymocytes. (C) Histogram representing SATB1 expression in $CD4^{+}$ gated thymocytes. (D) Comparison of SATB1 expression in $CD4^{+}$ thymocytes with that of peripheral $CD4^{+}$ T cells. Peripheral lymphocytes were isolated from spleen and lymph node and stained with CD4, CD8 and SATB1. The plot represents overlay of SATB1 expression in $CD4^{+}$ thymocytes and peripheral $CD4^{+}$ T cells. The data is representative of three experimental replicates.

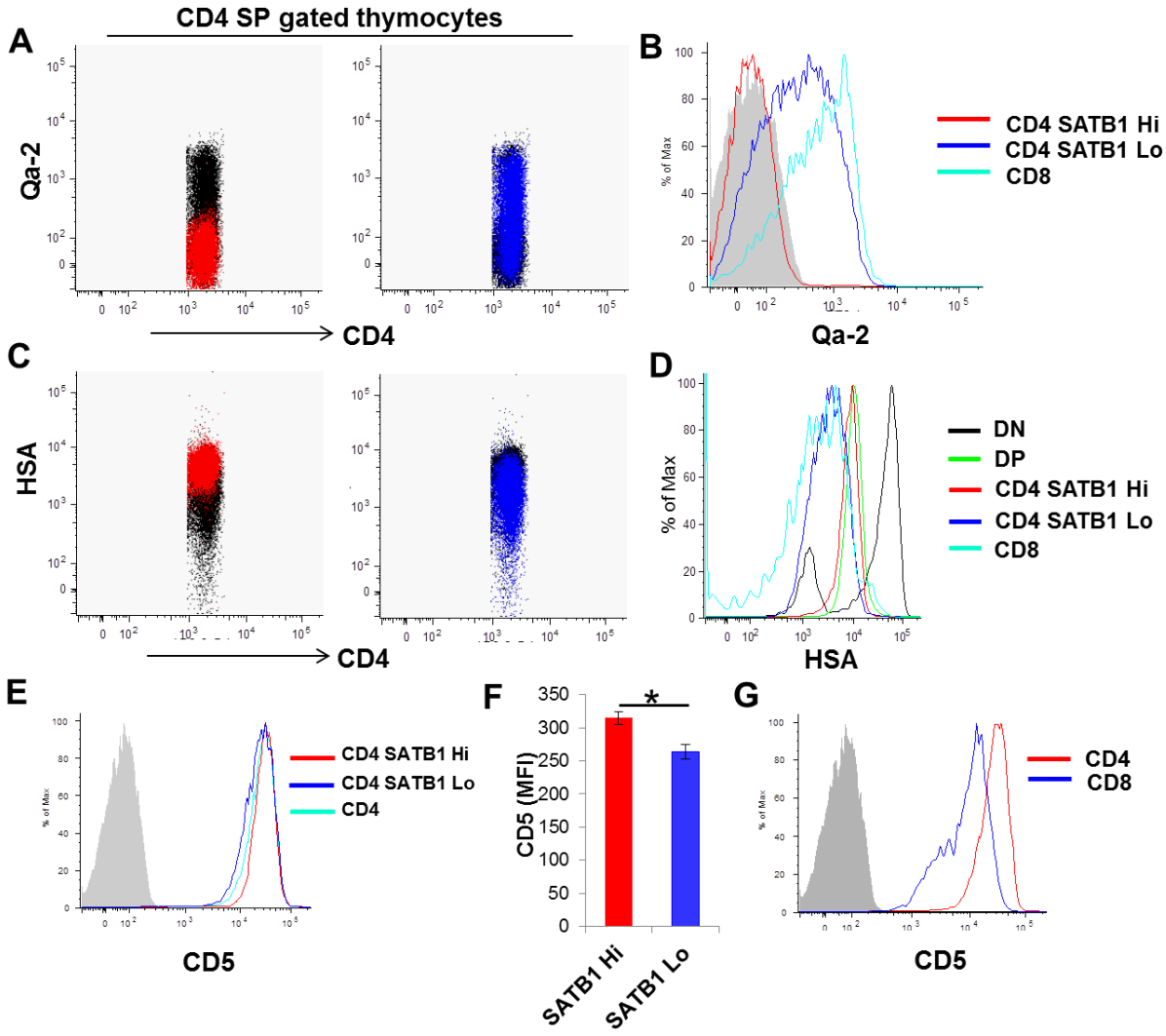

Figure 1.3.7: SATB1^{Hi} and SATB1^{Lo} population of CD4 thymocytes are distinct developmental stages of CD4 thymocytes development. (A) Gated CD4 thymocyte population was analyzed for Qa-2 expression and SATB1Hi (red) and SATB1^{Lo} (blue) CD4 thymocytes were overlaid on the plots. (B) Histogram represents the expression of Qa-2 in SATB1^{Hi} and SATB1^{Lo} CD4 thymocytes and CD8 thymocytes. (C) Expression profile for HSA/CD24 expression in CD4 thymocytes where SATB1^{Hi} (red) and SATB1^{Lo} (blue) populations are overlaid on the plot. (D) Histogram depicting the expression of HSA in all subpopulations of thymocytes including SATB1^{Hi} and SATB1^{Lo} CD4 thymocytes. (E) Histogram representing the expression of CD5 in SATB1^{Hi} and SATB1^{Lo} population of thymocytes. Expression of CD5 in total CD4 thymocytes is also shown (cyan). (F) Bar graph representing the average expression of CD5 (MFI values) in SATB1^{Hi} and SATB1^{Lo} populations of CD4 SP thymocytes. (G) Representation of CD5 expression in CD4 and CD8 thymocytes. Data is generated from five individual mice thymii. Error bars denote standard deviation and P-value is calculated by Students T-test.

1.3.4 Downregulation of SATB1 augments CD8 thymocyte development

CD8 SP thymocytes, unlike CD4 SPs have only a single population based on SATB1expression (Fig. 1.3.5G). We later analyzed expression of SATB1 in CD69 positive and negative fractions of CD8 gated thymocytes (Fig. 1.3.5H), and observed a significant downregulation of SATB1 expression in $CD69^{-ve}$ subset of CD8 thymocytes. Recent studies from Galande laboratory has shown that SATB1 interacts with β-catenin and together regulate expression of cytokine genes (Notani et al., 2010). Wnt/β-catenin signaling was shown to be important for $CD8^+$ T cell differentiation and memory formation. Enforced expression of ICAT (Inhibitor of β-catenin and Tcf) which disrupts interaction between β-catenin and TCF1 impairs the survival of $CD8^+$ T cells upon in vitro activation (Hossain et al., 2008). It was also shown that Wnt/β-catenin signaling can favor $CD8^+$ T cell memory formation by suppressing their maturation into terminally differentiated effector cells (Jeannet et al., 2010). Enforced expression of β-catenin (β-Cat Tg) mice have increased number of memory like $CD8^+$ SP thymocytes (Mulroy et al., 2003) (Fig. 1.3.8A) and also augments IL-7Rα chain expression in thymocytes undergoing positive selection (Yu et al., 2007). CD8 thymocytes in β-Cat Tg mice have been shown to have a memory like phenotype based on the expression of PLZF (Sharma et al., 2012). Since SATB1 null mice showed spatial-temporal dysregulation of genes such as IL-7Rα in DP thymocytes (Alvarez et al., 2000) and SATB1 was shown to interact with β-catenin in mouse thymocytes, we were interested in looking at the expression of SATB1 in β-Cat Tg thymocytes. Interestingly, expression of SATB1 in control and β-Cat Tg mice showed no significant difference upon comparison of whole thymocytes (Fig. 1.3.8 B). In contrast, CD8 SP fraction of β-Cat Tg thymocytes showed a significant downregulation of SATB1 expression (Fig. 1.3.8C) suggesting that low levels of SATB1 augments $CD8^+$ T cell development and memory like phenotype. This data corroborates with previous reports showing that IL-7Rα is upregulated in DP thymocytes of SATB1 null mice (Alvarez et al., 2000) and overexpression of β-catenin positively regulates IL7R-α expression (Yu et al., 2007). Our results suggest that SATB1 expression is dependent on TCR signaling and cessation of signal downregulates SATB1 expression in CD8 thymocytes. In β-cat Tg CD8 thymocytes, constitutive β-catenin expression increases the ability of cells to undergo positive selection by upregulating IL-7Rα expression. We propose that low levels of SATB1 expression in β-cat Tg CD8 thymocytes might be due to the rescue of Class I restricted DP thymocytes undergoing negative selection.

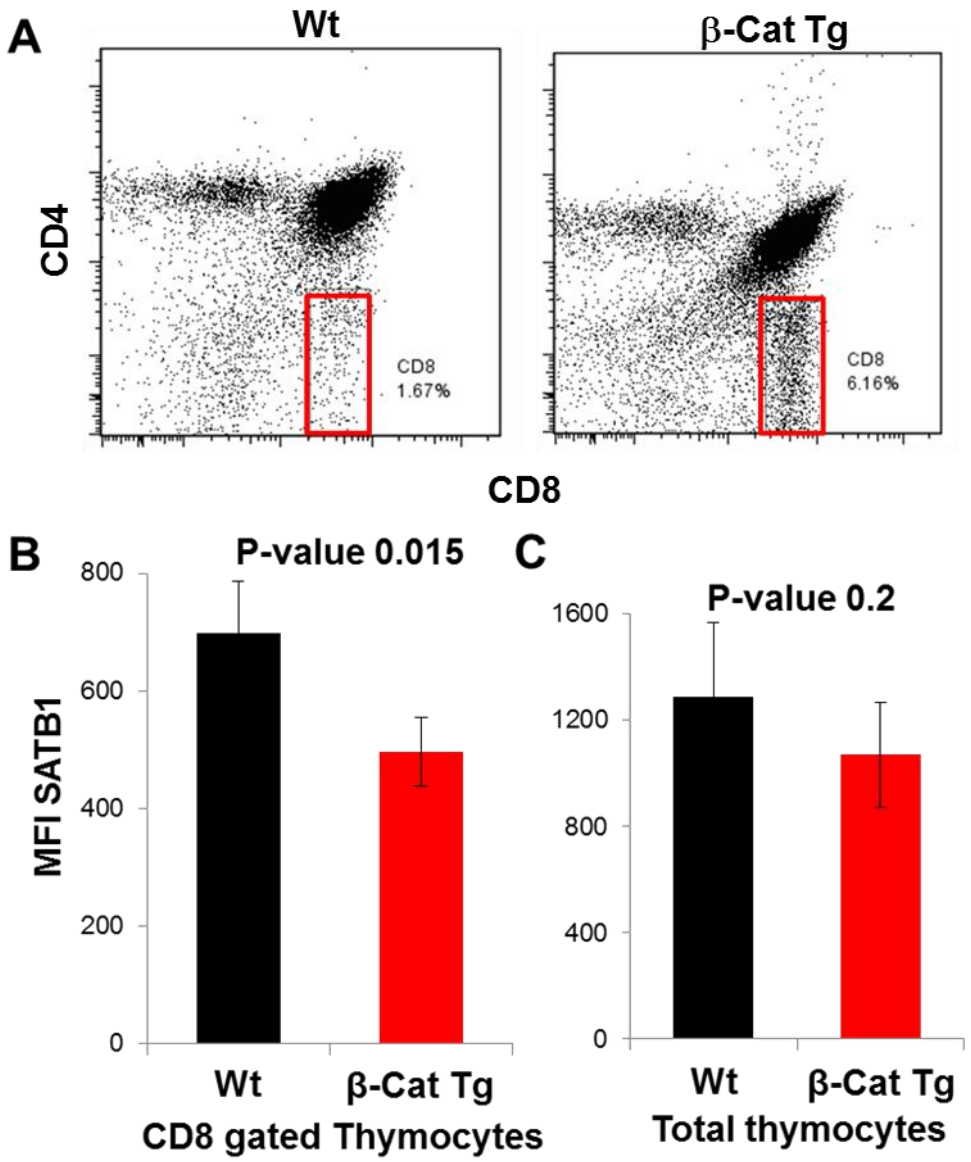

Figure 1.3.8: Downregulation of SATB1 augments CD8$^+$ SP thymocyte development. (A) Dot plot representing thymocyte profile in Wt and β-cat Tg mice. The gated population represents CD8 population in both the populations. (B) Bar graph representing SATB1 expression (MFI values) in Wt and β-cat Tg mice in CD8 populations. (C) Bar graph representing SATB1 expression (MFI) in total mouse thymocytes of Wt and β-cat Tg mice. Error bars represent standard deviation calculated from five independent mice and P-values are calculated using Students T-test.

1.3.5 SATB1 is repressed in FOXP3$^+$ natural regulatory T cells

Mouse thymocytes that express TCR with high affinity for self-peptide develop into CD4$^+$ CD25$^+$ regulatory T cells (nTregs) (Apostolou et al., 2002; Jordan et al., 2001). Regulatory T cells are distinguished from the positively selected thymocytes by virtue of elevated expression of the transcription factor FOXP3. Along with CD4$^+$ SP thymocytes a small percentage of DP thymocytes were shown to express Treg-specific transcription factor FoxP3 and act as precursors for CD4$^+$ Foxp3$^+$ Treg subset (Fontenot et al., 2003; Liston et al., 2008; Tai et al., 2005). Since we had noted that SATB1 expression is regulated in TCR-dependent manner, we speculated that SATB1 levels must be very high in nTregs since they are associated with strong TCR signals during their development (Carter et al., 2005). Paradoxically, we observed that SATB1 expression is downregulated in nTregs compared to effector CD4$^+$ T cells (Fig. 1.3.9). Similar results were reported by another group (Beyer et al., 2011) Furthermore we also observed that TCR-β^{Hi} thymocytes have two sub populations based on SATB1 expression that are either positive or negative for SATB1 expression (Fig. 1.3.9A). Interestingly, we observed that each subpopulation of DP, CD4$^+$ CD8^{Lo}, CD4$^+$ SP thymocytes that is negative for SATB1 expression exhibited Foxp3 expression (Fig. 1.3.9B) except for CD8 thymocytes. Whereas SATB1 positive fraction of TCR-β^{Hi} thymocytes do not express Foxp3 (Fig. 1.3.9B), indicating that expression of SATB1 and Foxp3 are mutually exclusive. It was suggested that SATB1 repression by Foxp3 and Foxp3 regulated miRNA is required for proper suppressive function of Tregs (Beyer et al., 2011). Since only a small fraction of SATB1 negative TCRβ^{Hi} thymocytes exhibited Foxp3 expression, our data opens up a new question of how SATB1 is downregulated in remaining larger fraction of Foxp3 negative subset of SATB1 negative population and CD8 thymocytes that do not express Foxp3 (Fig. 1.3.9B). Besides TCR signaling CD28 costimulatory signals also play an essential role during the differentiation of nTregs (Salomon et al., 2000; Tai et al., 2008) and clonal deletion of autoreactive thymocytes (Pobezinsky et al., 2012). In vitro studies in CD4$^+$ T cells from our own studies suggested that SATB1 expression is downregulated in response CD28 signals (Fig. 2.3.2). Our data suggests that SATB1 expression in nTregs is regulated in response to CD28 costimulatory signals along with Foxp3 and Foxp3 mediated miRNAs.

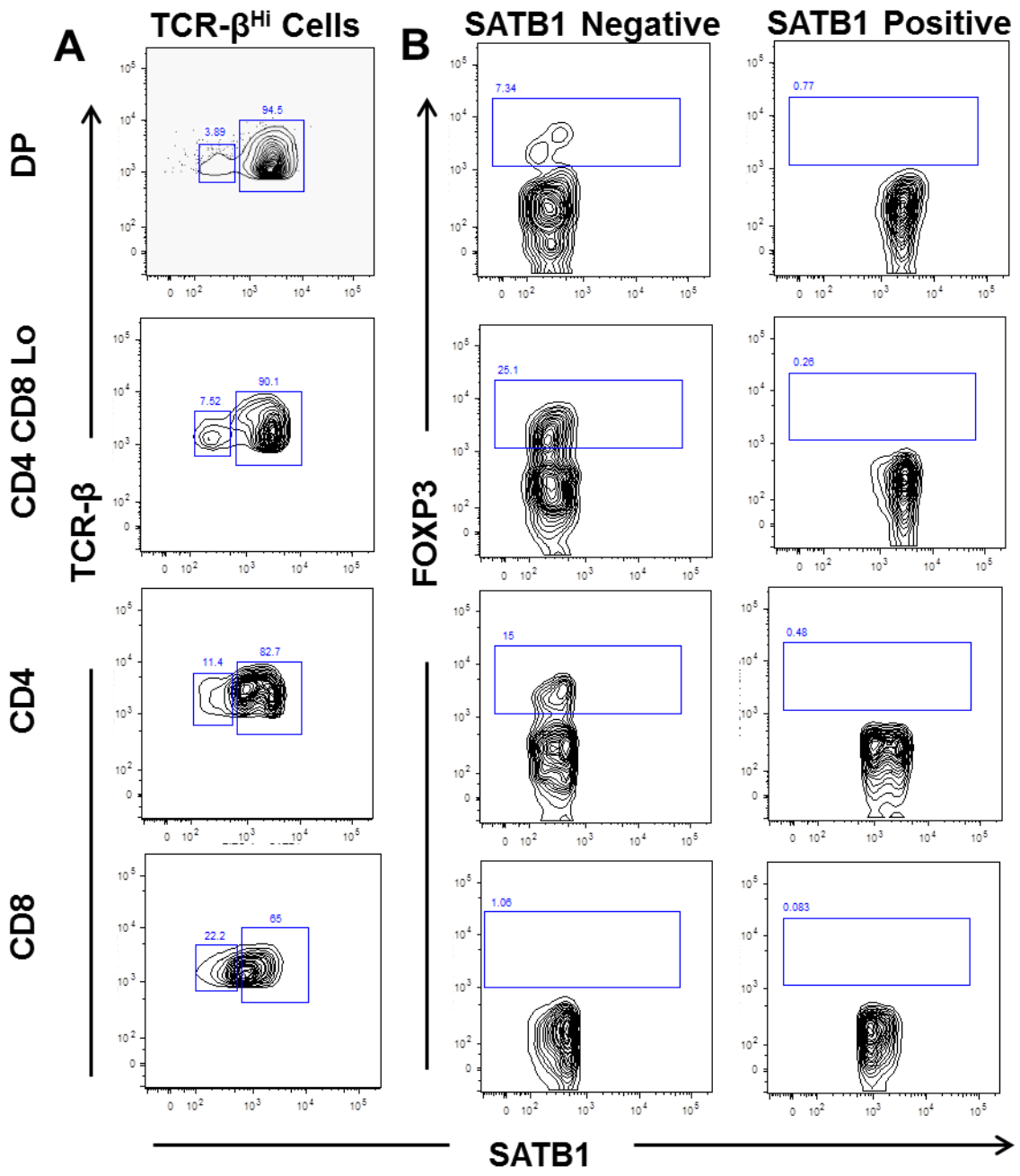

Figure 1.3.9: SATB1 is downregulated in Foxp3^{+} Regulatory CD4^{+} T cells. (A) Mouse thymocytes were stained with CD4, CD8, TCR-β, SATB1 and Foxp3 antibodies. Thymocytes were initially analyzed for TCR-β and SATB1 expression in each subpopulation of thymocytes as mentioned. The gated population on right represents SATB1 positive population and the population on the left represents TCR-β population that is negative for SATB1 expression. (B) FACS plots represent Foxp3 expression in both SATB1 positive and negative fractions of each population.

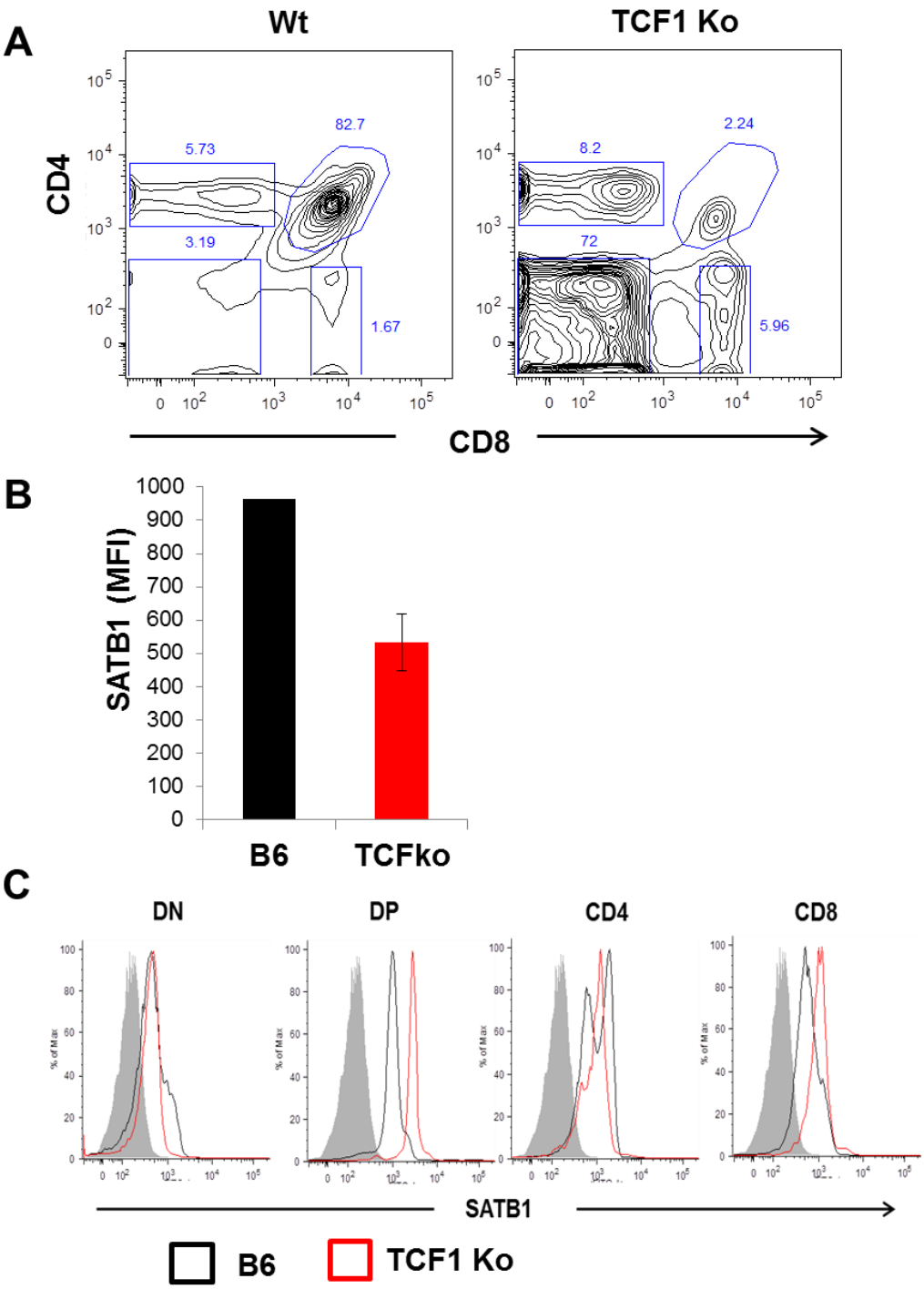

Figure 1.3.10: TCF1 regulates SATB1 expression in developing thymocytes. (A) FACS profile representing CD4-CD8 thymocyte profile in Wild type and TCF1 KO mice. (B) Bar graph representing SATB1 expression in Wt (B6 mice) and TCF1 KO mice. Error bars represent standard deviation calculated from 5 mice. (C) Histogram representing expression of SATB1 in each subset of mouse thymocytes.

1.3.6 Transcriptional regulation of SATB1 expression

Thymocyte development from early precursors to mature cells and further differentiation into effector cells depends on array of transcription factors that are expressed temporally at distinct stages of development. TCF1 and GATA3 transcription factors are indispensable for thymocyte development and overexpression studies of either TCF1 or GATA-3 in thymic precursors have been shown to initiate T lineage program (Rothenberg, 2011; Weber et al., 2011). We investigated if SATB1 expression is regulated by these core transcription factors utilizing TCF1 and GATA3 KO mice during thymocyte development. In TCF1 KO mice most of the thymocytes were blocked at the DN stage and have increased population of CD8 SP thymocytes which correspond to immature single positive (ISP) CD8 stage (Verbeek et al., 1995) (Fig. 1.3.10A). We observed that SATB1 is downregulated in TCF-1 null thymocytes compared to Wt B6 mouse thymocytes (Fig. 10B). Since most of the TCF1 KO thymocytes were blocked at DN stage, we further compared SATB1 expression in individual subsets of thymocytes between TCF1 KO and control mice (Fig 1.3.10C). We observed that SATB1 is downregulated in DN fraction of TCF1 KO thymocytes and the CD4 thymocytes do not have the characteristic bimodal distribution as observed in the control B6 mice (Fig 1.3.10C). Comparatively higher expression of SATB1 in CD8 fraction of TCF1 KO thymocytes could be ascertained Immature CD8 SP (ISP) thymocytes. The data suggests that SATB1 is regulated by TCF1 in thymocytes and is a downstream target of TCF1.

Recent studies have shown that TCF1 transcriptionally regulates Gata-3 expression (Weber et al., 2011) and we wished to test if Gata-3 has a direct effect on SATB1 expression. Gata-3 has been shown to regulate key factors involved in the thymocyte development and differentiation (Ho et al., 2009). Gata-3 activates or represses key genes by facilitating histone modifications such as H3K4me1, H3K4me2 and H3K27me3 on the enhancer regions of the target genes (Wei et al., 2011). Gata-3 is known to play a critical role during differentiation of DN to $CD3^{Lo}$, $CD3^{Hi}$ to CD4 and CD4 to Th2 phenotypes (Wei et al., 2011). Using the data from Wei et al., 2011, using GEO database (GSE20898) we monitored the expression of SATB1 in control and Gata3 null mice thymocytes at $CD3^{Hi}$ stage of thymocytes (Fig. 1.3.11A) and observed that SATB1 expression is downregulated in Gata-3 null mice in DP $CD3^{Hi}$ thymocytes. We further monitored if Gata-3 directly regulates SATB1 expression. *In silico* analysis revealed presence of the consensus WGATAA motifs on *Satb1* promoter. We therefore performed ChIP assay to monitor the occupancy of Gata-3 and

observed that it binds to *Satb1* promoter in mouse thymocytes (Fig. 1.3.11B). We further analyzed the ChIP-seq data sets for Gata-3 available in the public database (GEO20898) and observed similar results as seen in our ChIP analysis (Fig. 1.3.11C). Using peak identification software 'MACS' (Rothenberg, 2012; Zhang et al., 2008) to monitor the occupancy of Gata-3, we observed that *Satb1* promoter is bound by GATA3 in DN, DP and CD4 stages of thymocyte development (Fig. 1.3.11C). CD8 Gata-3 KO thymocytes sample was used as negative control, wherein we did not observe any binding of the protein on *Satb1* promoter (Fig. 1.3.11C).

Collectively these data suggest that TCF-1 and Gata-3 regulate SATB1 expression during thymocyte development. As TCF-1 regulates Gata-3 expression, it might be that down regulation of SATB1 in TCF-1 KO thymocytes may be because of downregulation of Gata-3 expression levels. Further investigation is required to confirm if TCF-1 directly regulates SATB1 expression. Current understanding suggests that TCF-1 transactivates Gata-3 expression, which in turn regulates SATB1 expression and all these processes are closely associated with TCR signaling.

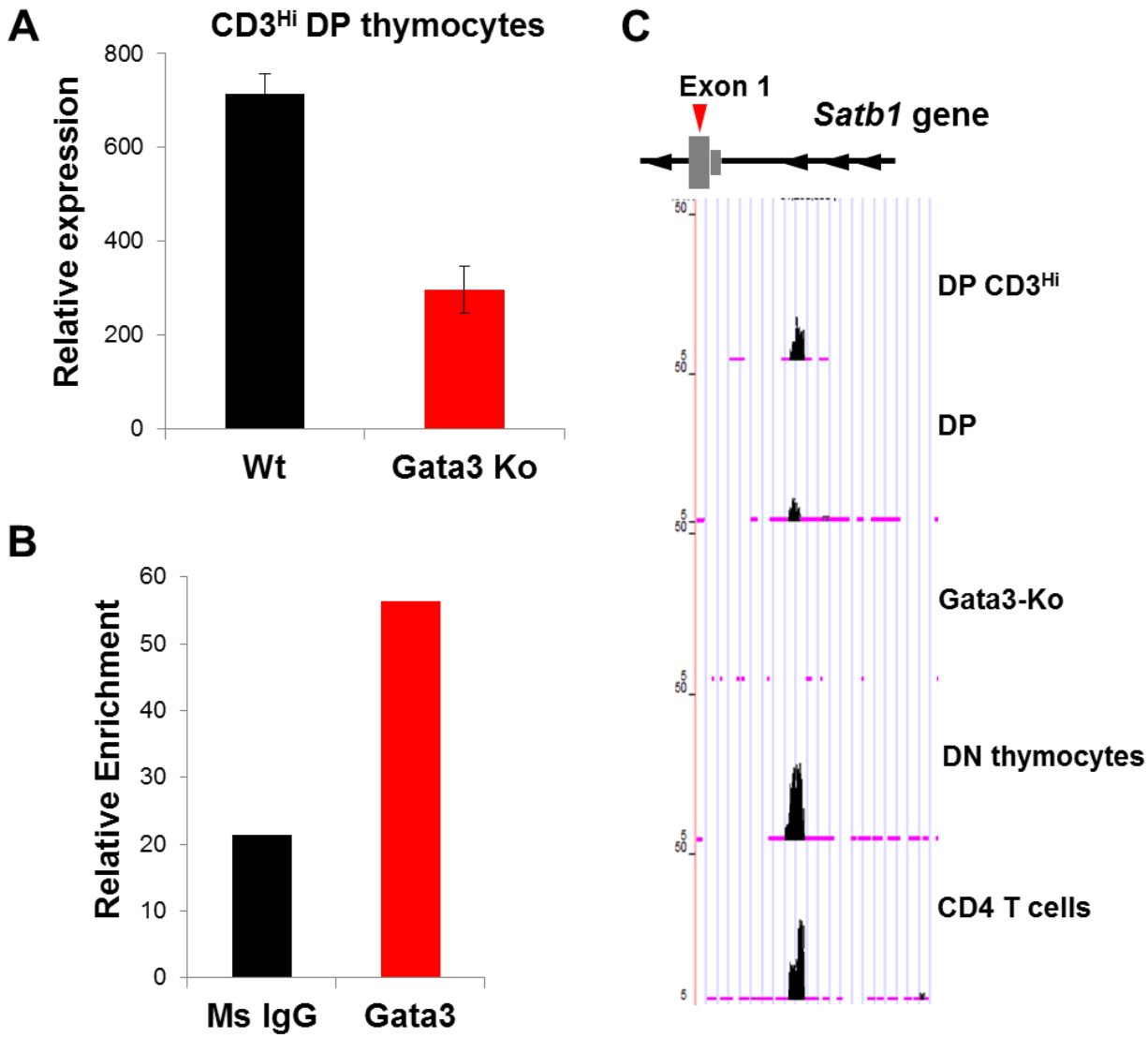

Figure 1.3.11: GATA3 regulates SATB1 expression in mouse thymocytes. (A) Relative expression of SATB1 in Wt and Gata-3 KO mouse thymocytes. The data was extracted from analysis of RNA-seq as reported in Wei et al., 2011. (B) Occupancy of Gata-3 on *Satb1* promoter was monitored by ChIP analysis. Chromatin was isolated from whole thymocyte populations and ChIP analysis was performed as described in 'Materials and Methods'. Relative occupancy was calculated by performing quantitative real-time PCR using and normalizing the C_t values with input chromatin. (C) Genome browser image showing the patterns of Gata3 on *Satb1* promoter in different subsets of thymocytes.

1.4 Discussion

The development of T lymphocytes from early thymic progenitors (ETPs) involves an ordered loss of alternative cell fates which in turn is determined by stage specific transcription factors (Rothenberg, 2012). SATB1 is a T lineage-enriched protein involved in chromatin organization and transcriptional regulation (Cai et al., 2003; Pavan Kumar et al., 2006). SATB1 null mice have block in the thymocyte development at DP stage and dysregulated expression of IL-2Rα and IL-7Rα on DP thymocytes (Alvarez et al., 2000). In this study we have focused on the understanding the role of SATB1 during the development of thymocytes and how levels of SATB1 are regulated during the process.

We have observed that SATB1 is differentially expressed in subsets of mouse thymocytes. The expression is very low in early DN (DN1-DN3) subsets and induced in DN4 subset. The early DN subsets in the thymus have a potential to develop into alternate fates like NK cells, dendritic cells, macrophages and γδ T cells (Rothenberg, 2012) and only those thymocytes that progress beyond DN3 stage are committed to the T lineage (Yui et al., 2010). DN3 thymocytes undergo TCR-β locus rearrangement and initiate the formation of pre-TCR complex which establishes the development of αβ T cells (Hoffman et al., 1996). This suggests that SATB1 is induced in thymocytes that are committed for T lineage development. We further gauged the expression of SATB1 during distinct developmental stages of thymocytes based on TCR-β and CD69 expression and observed that SATB1 is induced in cells that have undergone positive selection and later repressed in mature thymocytes. This data suggested us that SATB1 expression is dependent on TCR signaling. In vitro activation of CD4 T cells with anti TCR antibodies has upregulated the expression of SATB1 in a time dependent fashion and SATB1 binds to the transcriptionally active euchromatic regions and devoid from DAPI dense heterochromatic blobs (Chapter II, Fig. 1). We further observed that SATB1 levels are downregulated in Jurkat cells upon chemical inhibition of TCR signaling (Chapter II, Fig.2). This data suggests conclusively that SATB1 expression during thymocyte development is dependent on TCR signaling.

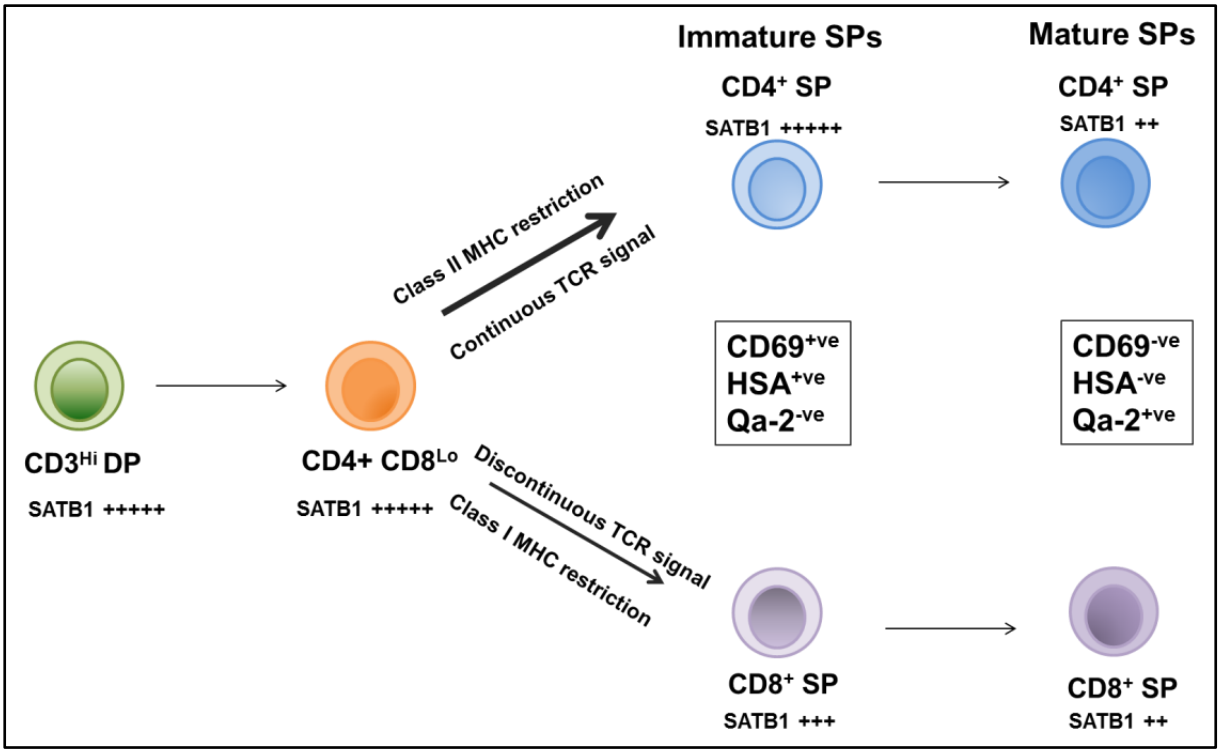

Figure 1.4.1: Role of SATB1 during CD4/CD8 lineage choice determination. SATB1 is required for the differentiation of DP thymocytes into either $CD4^+$ and $CD8^+$ SP thymocytes. The differentiation of DP to SP thymocytes is best explained by kinetic signaling model (Singer et al., 2008). Irrespective of TCR specificity and MHC restriction DP thymocytes undergoing positive selection terminate *Cd8* gene expression to convert into $CD4^+$ $CD8^{Lo}$ intermediate thymocytes. Persistent TCR signaling in the intermediate population blocks IL-7 mediated signaling and induces differentiation into $CD4^+$ thymocytes. Disruption of TCR signal in the intermediate thymocytes allows IL-7 mediated signaling and induces differentiation into $CD8^+$ thymocytes. In this study we show that during $CD4^+$ SP differentiation process as there is continuous TCR signaling, SATB1 expression levels are maintained at constant levels. Whereas in MHC class I restricted thymocytes where there is cessation of TCR signals SATB1 levels are downregulated. Newly differentiated SP thymocytes are immature and as TCR signaling wanes, SATB1 expression is downregulated and initiates the surface expression of Qa-2 which marks the mature thymocytes. The mature thymocytes subsequently migrate to the peripheral lymphoid organs spleen and lymph nodes.

Further analysis of SATB1 expression in SP thymocytes revealed that CD4 thymocytes have a characteristic bimodal distribution and SATB1 expression which we have mentioned them $SATB1^{Hi}$ and $SATB1^{Lo}$ populations. CD8 SP thymocytes have expression levels of SATB1 similar to that of $SATB1^{Lo}$ population. It is important to reiterate that in Satb1 KO mice single positive thymocytes fale to develop (Alvarez et al., 2000) and TCR signaling is indispensable for thymocyte development and maturation. One of the best accepted models to understand the development of CD4 and CD8 SP thymocytes is the kinetic signal model which proposes that MHC class II restricted CD4 thymocytes have persistent TCR signaling whereas CD8 thymocytes have early cessation in TCR signals (Singer et al., 2008). Based on the kinetic signaling model as CD4 thymocytes have a continuous and prolonged TCR signaling we observed higher expression of SATB1 ($SATB1^{Hi}$ population) and CD8 thymocytes exhibited lower expression of SATB1. These results were further confirmed by comparing and correlating with CD69 expression which is a well-established TCR activation marker, and observed that $CD69^{+ve}$ thymocytes have higher expression of SATB1 ($SATB1^{Hi}$) and $CD69^{-ve}$ thymocyte population have lower expression of SATB1 ($SATB1^{Lo}$). Similarly in CD8 thymocytes $CD69^{+ve}$ thymocytes have higher expression of SATB1 compared to $CD69^{-ve}$ subset of CD8 thymocytes. It is noteworthy to mention that since cytosolic tail of CD4 is known to associate with higher levels of LCK (which initiates TCR signal transduction) compared to CD8 coreceptor (Shaw et al., 1989; Wiest et al., 1993), thus CD4 TCR signaling is stronger as compared to CD8 TCR signaling. On a similar note we observed that expression of SATB1 in $CD69^{+ve}$ CD4 thymocytes is higher compared to that of $CD69^{+ve}$ CD8 thymocytes, these findings reinforce the fact that TCR signal transduction reciprocates the levels of SATB1 expression.

Earlier studies have shown that there are two subpopulations in CD4 SP thymocytes based on the expression of Qa-2 and HSA/CD24 (Bhandoola et al., 2000; Ramsdell et al., 1991). Qa-2 and HSA/CD24 markers distinguish mature form the immature populations and Qa-2. $SATB1^{Hi}$ thymocytes are negative for Qa-2 expression and have higher expression of HSA/CD24 whereas $SATB1^{Lo}$ population is positive for Qa-2 expression and have low levels of HSA/CD24 suggesting that $SATB1^{Hi}$ thymocytes are immature CD4 thymocytes and $SATB1^{Lo}$ population are majorly mature CD4 thymocytes. Mouse thymocytes upon treatment with DNA damaging agents such as Actinomycin D (ActD) which intercalates into DNA during replication or double-stranded breaks (DSB) causes cell death of mature thymocytes ($Qa\text{-}2^{+ve}$) but not in immature thymocytes ($Qa\text{-}2^{-ve}$) and this was attributed to ATM-

dependent death pathway (Bhandoola et al., 2000). Recent studies in MCF10-A, a non-malignant cell line obtained from two different sources has shown that SATB1 overexpression in the line which has high expression of ATM have not transformed into tumorogenic cells but those which have less expression of ATM have capability to form solid tumors (Ordinario et al., 2012). Taken together, these observations suggest that SATB1 is essential for maintaining a certain chromatin conformation which makes it accessible for the binding of ATM which is also expressed in high levels in immature thymocytes (Chen and Lee, 1996) and thus repairing the breaks which are might be induced by DNA damaging agents like ActD or breaks formed by physiological events such as the rearrangement of TCR-α locus (Petrie et al., 1993). SATB1 protein is discovered based on its propensity to bind the AT-rich sequences on the IgH locus (Dickinson et al., 1992) and it is also known that SATB1 binds to the major break point region on *Bcl2* gene which is responsible for translocations in lymphomas and mediated by V(D)J recombination process (Ramakrishnan et al., 2000). In a non-malignant cell line overexpression of SATB1 has led to two contrasting outcomes i.e. the cells become transformed or remain non-malignant based on the levels of ATM expression. In cells that have low levels of ATM upon overexpression of SATB1 transforms the cells into a malignant cell line. Whereas in cells that have low levels of ATM overexpression of SATB1 does not have any affect (Ordinario et al., 2012). In light of these findings it might be possible that in immature thymocytes that have undergone *Tcr* locus rearrangements have DSBs and SATB1 that is upregulated in TCR-dependent manner might recruit ATM and help in repairing of DSBs and this hypothesis requires further experimental evidence.

Since we have shown that SATB1 levels are regulated by TCR signaling, it is pragmatic that nTregs should have even higher expression compared to other conventional CD4 thymocytes, as nTregs originate from high affinity TCR engagement of thymocytes with self-peptide MHC (Itoh et al., 1999). Paradoxically we observed that SATB1 expression is downregulated in nTregs compared to conventional CD4 thymocytes and similar observations were reported by Beyer et al. (Beyer et al., 2011). It was shown that Foxp3 along with Foxp3 regulated miRNA miR-155 downregulates SATB1 expression and thus promotes Treg differentiation. Foxp3 was shown to directly bind to SATB1 promoter and downregulates its expression, further miR-155 binds to SATB1 3' UTR of mRNA and leads to degradation of the transcripts. It was also shown that overexpression of SATB1 in Tregs inhibited the suppressive function of the cells (Beyer et al., 2011). All the subsets of TCR-β^{Hi} thymocytes

(DP, CD4CD8^{Lo}, CD4 and CD8) have two populations of which one population is negative for SATB1 expression (this does not include SATB1^{Lo} population). SATB1 negative population might probably be the population which undergoes negative selection and also includes anergic T cells. All subsets of SATB1 negative thymocytes have a certain percentage of Foxp3^{+} thymocytes. CD28 costimulation has been shown to be necessary for the conventional CD4^{+} thymocytes to undergo negative selection (Punt et al., 1994) and induce of Foxp3 expression (Tai et al., 2005). In vitro activation of CD4 T cells with only plate bound anti-CD28 ab has not induced SATB1 expression (Chapter II, Fig. 1) suggesting that stronger CD28 costimulatory signaling during thymocyte development inhibits SATB1 expression and may lead to alternate fates. Recent studies have shown that Treg cell development is a two-step process where initially TCR signaling is succeeded by epigenetic modifications of chromatin and at later stage induction of Foxp3 occurs, which are both mutually exclusive but necessary for Treg lineage development (Ohkura et al., 2012; Samstein et al., 2012a). As nTreg cell development is dependent on strong CD28 costimulatory signals along with TCR signals its might be that SATB1 is downregulated in the initial step and further complete repression of SATB1 occurs in presence of Foxp3 and Foxp3 regulated miRNAs. Since factors such as Helios are shown to be important for the development of Tregs it might be possible that these factors suppresses the expression of SATB1 during the development of T reg phenotype (Grzanka et al., 2013).

The genes required for the T cell development and function are regulated by transcription factors such as RBPJ, TCF1 and Gata-3 (Rothenberg et al., 2010). Deletion of any of these key factors in the thymic progenitors has blocked the thymocyte development. Thymocyte development in *TCF1* and *Gata-3* null mice is blocked at early stages of thymocyte development and either of TCF1 or Gata-3 null mice have downregulated expression of SATB1. Since TCF1 transcactivates Gata-3 expression and Gata-3 from our studies has shown to directly regulate SATB1 expression. These studies suggest that Gata-3 which itself is known to be upregulated in response to TCR signaling (Hernandez-Hoyos et al., 2003) also regulates SATB1 expression. Gata-3 is known to regulate the expression of Thpok, a transcription factor upregulated specifically in CD4+ CD8^{Lo} intermediate stage thymocytes. During the development of class-II restricted thymocytes the requirement of Gata-3 precedes that for Thpok, constitutive expression of Thpok in Gata-3 null mice does not rescue the development CD4^{+} lineage (Wang et al., 2008). Gata-3 binding on *Thpok* locus has been identified and the region was shown to be essential for Thpok expression (Wang et al., 2008).

As both Thpok and SATB1 null mice have a similar phenotype where there is no development of $CD4^+$ SP thymocytes and since both are under control of Gata-3 expression, the question arising is whether there is a functional relation between SATB1 and Thpok. ChIP-on-Chip studies in mouse CD4 results from our studies have shown that SATB1 binds to *Thpok* promoter, and the results suggest that SATB1 regulates Thpok expression.

Taken together, this study shows that expression of SATB1 is induced during T cell development and a downstream effector of TCR signal transduction is required for the development and lineage commitment in thymocytes. We propose that SATB1 mediated chromatin organization is necessary for thymocyte survival. Further investigation would be required to address if SATB1 has a role in processing of DSB repair during *Tcra* recombination process. Although SATB1 is highly expressed in thymus the levels of expression are elevated in multiple types of cancers (Han et al., 2008; Mir et al., 2012). It would be interesting to dissect what causes upregulation of SATB1 in malignant cells which lack a surface TCR. The findings might shed light on relationship in the signaling pathways between T cells and cancerous cells. It would also be interesting to further understand the lineage-specific genes that are regulated by SATB1 in individual subsets of thymocytes.

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Chapter II

Regulation of SATB1 During T helper Cell Differentiation

2.1 Introduction

Mature single positive $CD4^+$ and $CD8^+$ thymocytes migrate to the periphery. Peripheral lymphoid organs otherwise known as secondary lymphoid organs - Spleen and lymph node, are the tissues where naïve T cells are harbored. In cases of infection these T cells differentiate into multiple types of effector T cells which are responsible for pathogen clearance and long-term immunity (Zhu et al., 2010). T cell response during an acute infection consists of 3 steps – (i) clonal expansion, (ii) contraction and (iii) memory T cell formation. T cells differentiate into effector T cells during a pathogenic encounter and facilitate the clearance of the pathogen. Infections with intracellular pathogens such as viruses and bacteria promote the differentiation of $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs). CTLs secrete cytotoxins, Perforin and Granzymes which responsible for the clearance of the pathogen (Berke, 1995). $CD4^+$ T cells differentiate into distinct classes of effector T cells commonly known as T helper (Th) lineages. T helper cells perform wide range of functions such as - (i) they help the maturation of B cells and enhance the production of antibody (ii) they enhance the effector function of $CD8^+$ T cells (iii) they control the amplitude of immune responses so as to prevent any detrimental effects on the host organs. $CD4^+$ T cells are important in mediating immunological memory and loss of these $CD4^+$ T cells as seen in clinical conditions such as human immuno deficiency virus (HIV) infections leads to opportunistic infections.

2.2.1 Functions of T helper cells

Upon engagement of T cell receptor with appropriate peptide-MHC complex, $CD4^+$ T cells differentiate into various T helper lineages. Initial identification of T helper lineages was reported by Mosmann and Coffman (Mosmann et al., 1986) designated as Th1 and Th2 cells. Mouse $CD4^+$ Th clones were mainly distinguished based on the expression pattern of the effector cytokines IFN-γ and IL-4 respectively. Th1 cells are required for cell mediated immune responses and provide immunity against intracellular protozoan parasites such as *Leshmania* species and *Toxoplasma* species. Mycobacterial infection induces the development of Th1 cells and are required for the protection against the infection in mouse and human (Khader and Cooper, 2008). Th2 cells are responsible for immunity against extracellular pathogens such as helminth parasites (Murphy and Reiner, 2002). Th2 cells

isolated from human blood when activated in vitro, produce TNF-α (Tumor necrosis factor – α) a pro-inflammatory cytokine along with Th2 cytokines. TNF-α^+ Th2 cells are also observed in human breast cancer biopsies and are believed to contribute to inflammation and tumor progression (Pulendran and Artis, 2012). Bacterial Lipopolysaccharides (LPS) can also promotes the development of Th2 cells in a Toll like receptor (TLR) dependent mechanism but they produce only IL-13, IL-5 and IL-10 but not IL-4 (Pulendran et al., 2001). Recent studies have identified the occurrence of various other T helper cells - Th17, iTregs, Follicular T helper (Tfh) cells and Th9 cells. Th17 cells are critical for protection against extracellular bacteria and fungi (Weaver et al., 2006) by recruiting neutrophils and induction of antimicrobial peptides (O'Connor et al., 2010). Effector cytokines IL-17A and IL-17F serve as chemo attractants and induce inflammatory responses (O'Connor et al., 2010). IL-22, another effector cytokine secreted by Th17 cells is known to protect from bacterial infection by increasing the proliferation rate of epithelial cells and providing transepithelial resistance to injury (Aujla et al., 2008). Higher expression of IL-17 cytokine by Th17 cells is associated with disease conditions such as Crohn's disease, ulcerative colitis and multiple sclerosis (O'Connor et al., 2010). Induced T regs (iTregs) arise from the peripheral $CD4^+$ T cells (Chen et al., 2003) and function in concert with the thymic regulatory T cells (nTregs) in suppression of different types of inflammatory responses, auto immunity and tumor immunity (Josefowicz et al., 2012). Loss of function mutation in Foxp3 results in immunodeficiency, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), where the individual shows autoimmune symptoms and elevated production of Th2 cytokines (Wildin et al., 2001). It was shown that extrathymic differentiation of Tregs has emerged in placental mammals to facilitate fetal tolerance (Samstein et al., 2012b). Follicular T helper cells (Tfh) are helpful in the formation of the germinal centers in the lymph node and they aid B cells to produce antibody (King and Mohrs, 2009). Tfh cells express various surface molecules like ICOS, PD-1, CD40 ligand, and BTLA that are required for the activation, survival and differentiation of B cells and $CD4^+$ T cells (Rasheed et al., 2006; Vinuesa et al., 2005). Insufficient Tfh generation leads to impaired B cell responses and excessive generation of this cell type contributes to development of autoimmune diseases (Ma et al., 2012). Differentiation to these various T helper lineages along with the TCR signaling requires the cognate cytokines which are secreted by the innate immune cells at the site of infection.

T helper Subset	Key Transcription factors	Effector cytokines	Effector functions	References
Th1	T-bet	IFN-γ	• Immunity against intracellular pathogens	Glimcher and Murphy, 2000.
Th2	Gata-3	IL-4, IL-5, IL-13	• Protection against heliminth parasites • Provide help to B cells	Zhang et al., 1997
Th17	RORγt	IL-17 A, IL17-F	• Inflammation • Immunity against fungal pathogens	Park et al., 2005; Harrington et al., 2005
Tfh	Bcl-6	IL-21	• Provide help to B-cells to elicit antibody response	King et al., 2008
iTreg	Foxp3	IL-10, IL-35	• Immuno suppression • Gut microbiome homeostasis	Chen et al., 2003

Table 2.1.1: Table summarizing the key transcription factors involved during differentiation, effector cytokine produced by the helper cells and their functions.

2.1.2 Determinants of T helper cell differentiation

A. Cytokine environment during T helper cell differentiation.

Cytokine environment plays a critical role during the differentiation of T helper cells. In vitro studies of $CD4^+$ T cells have been important in showing the role of distinct cytokine signals in achieving different helper fates. IL-12 cytokine is important for the differentiation of naïve $CD4^+$ T cells to Th1 phenotype (Hsieh et al., 1992). However, subsequent studies revealed that Interferon-γ (IFN-γ) is also sufficient for the differentiation of $CD4^+$ T cells to Th1 phenotype (Lighvani et al., 2001). Differentiation of $CD4^+$ T cells to Th2 phenotype requires IL-4 signals. In vitro differentiation of $CD4^+$ T cells to Th17 phenotype was most efficient in the presence of IL-6 and TGF-β (Veldhoen et al., 2003; Bettelli et al., 2006; Magan et al., 2006). Other studies have shown that IL-21 could also drive the cells to Th17 phenotype in

IL-6 knockout by cooperating with TGF-β (Korn et al., 2007; Nurieva et al., 2007). TGF-β signaling is also found to be important in the generation of induced Treg (iTreg) (Liu et al., 2008) by inducing Foxp3, a master transcription factor required for Treg functions. Combination of multiple cytokines such as IL-6, IL-21, IL-27 and IL-12 can induce the differentiation of naïve $CD4^+$ T cells to follicular T helper cells (Tfh) (Ma et al., 2012). Although it seems that there is reciprocity of various cytokines required for T helper cell differentiation, the final effector cells are quite distinct from each other by virtue of not only the effector cytokines they secrete but also have a gross distinction in their transcriptional programs.

B. Transcriptional regulators of T helper differentiation

Cell identity is primarily determined by constituent macromolecular components of the cell such as proteins, RNA and lipids. This in turn is the readout of the transcriptional state of that particular cell type (Egli et al., 2008). Transcriptional program that determines the fate of a particular cell type is regulated by specific transcriptional regulators, non-coding RNAs, chromatin remodelers and DNA methylation. Relevance of transcription factors in cell fate determination is exemplified by the discovery of four transcription factors – Oct4, Sox2, Klf-4 and c-Myc requirement in conversion of a terminally differentiated fibroblast cells into induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Many studies have demonstrated the role(s) of a specific transcription factor in lineage commitment or differentiation. Early studies on Pax5 have shown that ablation of Pax5 in B cell progenitors activates the genes which are required for differentiation to T lineage and other myeloid cells (Nutt et al., 1999). Factors such as Pax5 whose action is sufficient to induce the transcriptional program of a particular lineage are known as ‘Master regulators’. Similarly in T helper cells a few transcription factors are shown to be required and indispensable for the differentiation to a particular lineage (Kanno et al., 2012). The roles of master transcription factors contributing to each T helper lineage are discussed below.

2.1.3 STATs in T helper differentiation

Signal transducers and activators of transcription (STATs) play an important role as the name suggests in bridging the external cellular signals with transcriptional activation of genes (Levy and Darnell, 2002). Extracellular signals activate membrane receptor bound protein tyrosine kinases which phosphorylate STAT proteins. Activated STAT proteins translocate to the nucleus and regulate transcription. A group of cytokines which includes IFN-γ, Type-I interferons, colony stimulating factors bind to receptors known as type I and type II cytokine receptors. These cytokine receptors are associated with various tyrosine kinases commonly known as JAKs (Janus Kinases) (O'Shea and Plenge, 2012). Early studies in Th1 and Th2 cells have shown the importance of STAT molecules in their differentiation. IL-12 signaling activates JAK2, which in turn activates STAT4 and results in Th1 phenotype. Similarly JAK1, JAK3 and STAT6 are activated upon IL-4 signaling and are deterministic in Th2 differentiation (Darnell et al., 1994). STATs are also shown to play a role in the recently discovered T helper lineages. STAT3 is shown to be critical for differentiation to Th17 phenotype and it directly regulates the expression of the effector cytokines (Milner et al., 2008). IL-2 signaling mediated STAT5a and STAT5b are negative regulators of Th17 cells and they achieve this by directly competing with the binding of STAT3 molecules with RORγt on Th17 target genes (Laurence et al., 2007). IL-6 and IL-21 signals together via STAT3 and promote the differentiation to Tfh lineage by directly upregulating Bcl-6. STAT4 and IL-12 signaling were also found to be sufficient for the differentiation to Tfh cells. Recent analyses of genome-wide occupancy of STAT molecules using ChIP-sequencing have revealed that these regulators have thousands of genomic targets and regulate expression of their targets by controlling their epigenetic landscape (Durant et al., 2010; Elo et al., 2010). Recent data shows that STAT proteins are involved in the activation of lineage-specific enhancers and inhibit enhancers associated with alternatives fates. Overexpression of master regulators in STAT deficient cells could not establish the active enhancer landscape of that particular lineage (Vahedi et al., 2012). Thus, all of the above findings establish that STAT proteins are very crucial in relaying the external cues to reprogram the chromatin and cause lineage commitment to a particular effector phenotype in $CD4^+$ T cells.

2.1.4 Master regulators of T helper lineage differentiation

Cell extrinsic signals received by naïve CD4 T cells are converted to cell intrinsic changes by TCR signals and STAT molecules. Multitude of transcription factors are involved in the differentiation of T helper cells. Master transcription factors are those that directly regulate the expression of effector cytokines, by whose coordinated action on target cells T helper lineages are determined. STAT4 induced transcription factor T-bet is important for downregulation of Gata-3 expression and thereby facilitates Th1 lineage commitment (Glimcher and Murphy, 2000). T-bet acts in concert with Runx3 in regulating IFN-γ production, an effector cytokine of Th1 cells (Djuretic et al., 2007). T-bet inhibits the function of Gata-3 and Rorγt and thus inhibits the differentiation to Th2 and Th17 phenotypes respectively. Overexpression of T-bet in Th2 cells inhibits the production of IL-4 and induces IFN-γ expression (Szabo et al., 2000). Phosphorylated T-bet binds to Gata-3 and inhibits transcriptional activity of Gata-3 (Hwang et al., 2005). Runx3 which is involved during the development of CD8 thymocytes by inhibiting *cd4* gene locus is also shown to have an important role in Th1 differentiation. Runx3 deficiency in Th1 cells produce reduced levels of IFN-γ in Th1 cells (Naoe et al., 2007). STAT3 activation along with RORγt is shown to have a critical for Th17 effector lineage (Harris et al., 2007). Mice lacking RORγt are shown to have attenuated development of experimental autoimmune encephalomyelitis (EAE), an autoimmune disorder promoted by Th17 cells (Ivanov et al., 2006). Forced expression of RORγt in CD4 T cells leads to upregulation of IL-17, a phenotypic cytokine of Th17 cells. In absence of pro-inflammatory signals TGF-β signals differentiation of naïve CD4 T cells into induced regulatory T cells (iTregs) (Davidson et al., 2007). IL-2 signaling induced STAT5 is shown to be necessary for the differentiation to iTreg cells (Yao et al., 2007).

Gata-3 is a master transcription factor which is known to have an important role in the development of $CD4^+$ thymocytes and is also shown to have a critical role in T helper differentiation (Zheng and Flavell, 1997). Gata-3 is differentially expressed in Th1 and Th2 cells (Zhang et al., 1997). Enforced expression of Gata-3 induced Th2 responses which are independent of IL4/STAT6 signaling pathway (Ouyang et al., 1998). Deletion of Gata-3 in CD4 T cells differentiating to Th2 phenotype leads to drastic reduction in their capacity to produce IL-4. Further, deletion of Gata-3 in cells that have differentiated to Th2 phenotype exhibited a modest effect on IL-4 cytokine production (Zhu et al., 2004). Enforced expression of Gata-3 in Th1 cells leads to upregulation of the Th2 cytokines (Ouyang et al., 1998) and

also upregulates endogenous Gata3 expression (Ouyang et al., 2000). The chromatin landscape of Th2 cytokine locus is very dynamic and has been characterized extensively over past years. The Th2 cytoline locus undergoes histone H3 lysine 4 trimethylation (H3K4me3) and H3K14 acetylation which in turn renders the locus more accessible for the transcription factors and generates DNaseI hypersensitive sites (HS) (Ansel et al., 2006). Gata-3 has been shown to bind several regulatory elements including conserved non-coding sequence (CNS) and HSVa site on promoter of *Il-5* and intronic regions of *Il-4* gene (Yamashita et al., 2002). In addition to increasing the accessibility of the chromatin of the Th2 cytokine locus, Gata-3 is also known to regulate the expression of individual cytokine genes by directly binding to the promoter regions of *IL-5* and *IL-13* genes (Zhu et al., 2004). Gata-3 not only regulates the expression of Th2 cytokine locus but also induces the expression of other transcription factors such as Dec2 which in turn activates the expression of IL-4 (Yang et al., 2009).

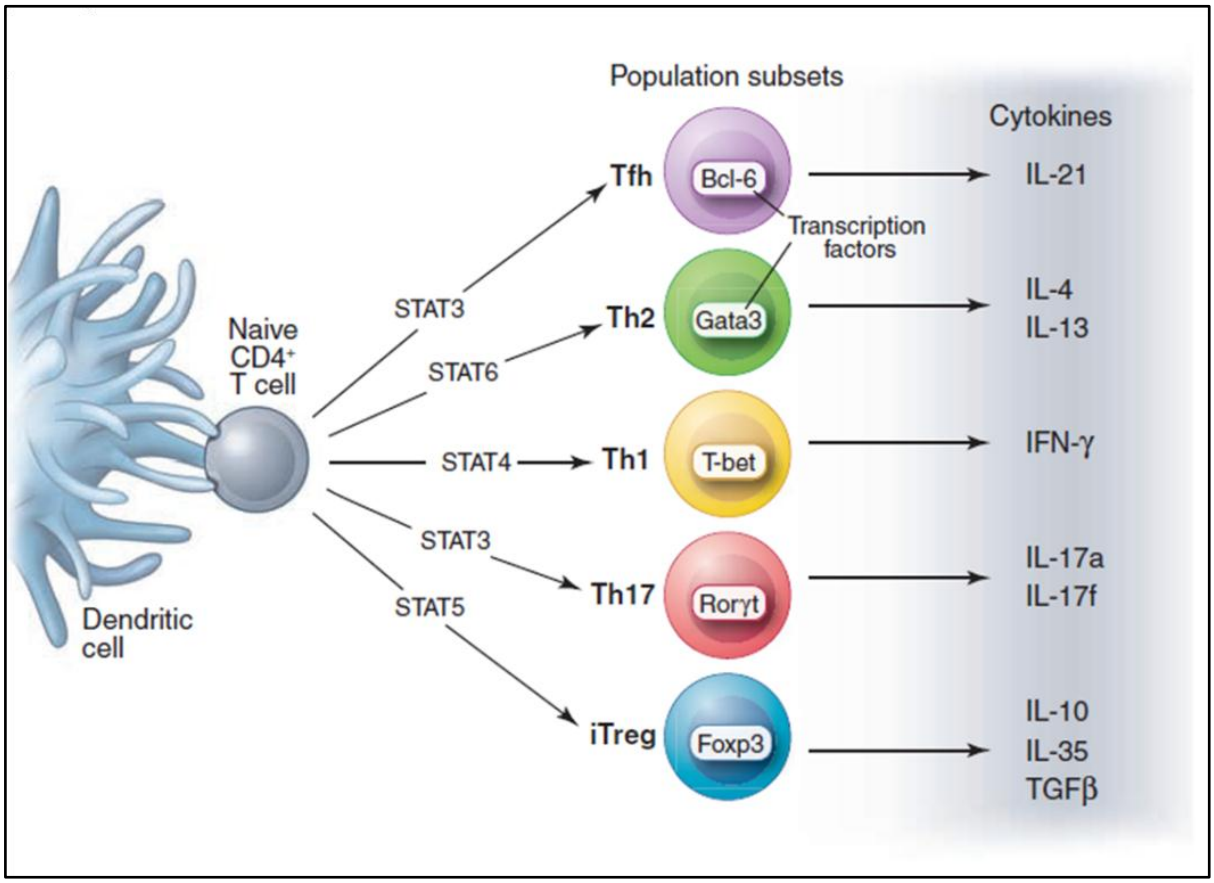

Figure 2.1.1: T helper cell differentiation. Upon antigenic stimulation of naïve $CD4^+$ T cells along with cytokine signals activate various STAT molecules. STAT molecules play an important role in upregulation of the key transcription factor genes particular to each phenotype. Lineage-specific transcription factors positively regulate the expression of effector cytokines that are characteristic of a particular T helper lineage. (Adapted from O'shea and Paul, 2010).

2.1.5 Other mediators of T helper cell differentiation

Notch is a cell surface receptor that is involved in broad range of cell differentiation processes (Bray, 2006). Ligation of notch receptor facilitates the cleavage of intracellular domain of notch receptor by γ-secretase. The cleaved product, Notch intracellular domain (NICD), translocates to the nucleus and cooperates with DNA-binding proteins CSL (CBF-1, Su(H) and LAG-1) and Mam (Master mind like proteins) to convert a repressor complex into an activator complex (Schweisguth, 2004). CD4 T cells express four different Notch molecules which perform their downstream functions via RBPJ (Kato et al., 1996). Exposure to bacteria such as *Acinobacter* species and *Lactobacillus Lactis* induces DLL4 (Notch ligand) and promotes dendritic cell mediated Th1 differentiation of $CD4^+$ T cells (Shimizu et al., 2000). How Notch signaling regulates differentiation to Th1 lineage is not well understood. In T cell hybridomas it was shown that Notch directly binds to *T-bet* promoter and upregulates its expression. Further, Notch is also known to interact with NF-κB complexes on *IFN-γ* promoter and thereby promotes Th1 differentiation (Minter et al., 2005). In contrast, in primary T cells Notch does not bind to *T-bet* promoter (Fang et al., 2007). Overexpression of intracellular Notch in CD4 T cells promoted Th2 lineage (Amsen et al., 2004; Tu et al., 2005). Th2 lineage commitment was defective in cells lacking Notch receptors (Amsen et al., 2004). Notch signaling in CD4 T cells was shown to transcactivate *Gata3* and *IL-4* genes required for Th2 differentiation. Notch signaling has been shown to induce Gata-3 expression in STAT6 deficient cells and promote Th2 differentiation (Amsen et al., 2007; Fang et al., 2007). Furthermore, Notch and Gata-3 synergize and upregulate IL-4 expression. Thus, Notch signaling plays an important role in the differentiation of $CD4^+$ T cells to various T helper phenotypes by regulating the expression of key transcription factors and cytokine genes.

mTOR signaling pathway plays central role in regulation of metabolism, energy balance, protein synthesis, proliferation and survival (Guertin and Sabatini, 2007). mTORC forms two distinct signaling complexes mTORC1 and mTORC2. CD4 T cells lacking mTORC fail to differentiate into effector T cells; instead they become FOXP3 expressing Tregs (Delgoffe et al., 2009). Inability of mTORC deficient T cells to become effector T cells is due to reduced activation of transcription factors STAT4, STAT6, and STAT3. Rheb – small GTPase is a component and crucial regulator of mTORC1 signaling. Selective ablation of Rheb differentiation to Th1 and Th17 is abrogated but there is no effect on Th2 polarization

(Delgoffe et al., 2011). Deletion of mTORC2 signaling components inhibited the development of Th2 cells but not Th1 or Th17 cells (Delgoffe et al., 2011; Lee et al., 2010).

Wnt signaling is one of the widely studied signaling mechanism in the context of development, cell fate specification and cell division (Clevers, 2006). In canonical Wnt signaling, the binding of Wnt ligands to the cognate frizzled receptors initiates the signaling cascade wherein glycogen synthase kinase-3β (GSK-3β) is inhibited. Under Wnt off conditions active GSK-3β phosphorylates β-catenin, phospho-β-catenin is then targeted to the proteosomal degradation pathway. When the signaling cascade is active un-phosphorylated β-catenin translocates to the nucleus and associates with T cell factor (TCF)/ lymphoid enhancer factor (LEF) to induce transcription (Logan and Nusse, 2004). Although the role of β-catenin and TCF1 in context of T cell development in thymus is well understood (Mulroy et al., 2003; Verbeek et al., 1995; Weerkamp et al., 2006), the role of Wnt signaling in differentiation of $CD4^+$ T cells is less understood. Recent reports from Galande laboratory and Jyoti Sen's group have shown that Wnt signaling pathway is important for the differentiation of CD4 T cells to Th2 phenotype (Notani et al., 2010; Yu et al., 2009). Wnt/β-catenin signaling along with SATB1 induces Gata-3 expression and propels $CD4^+$ T cells towards Th2 phenotype.

2.1.5 Plasticity of T helper differentiation

As the differentiation of $CD4^+$ T cells to various effector lineages is well established, the arising question that remains fairly unresolved is the plasticity between different T helper cells. With increasing number of T helper subsets being discovered there is an observed promiscuity between different T helper subsets. Initially discovered Th1 and Th2 subsets are quite distinct from each other with respect to the inherent transcriptional program and the effector cytokines they produce. Interestingly, Th2 cells that are generated in response to lymphocytic choriomeningitis virus (LCMV) when transferred into mouse that is infected with LCMV produce IFN-γ (Lohning et al., 2008). Th17 and iTregs require the same cytokine TGF-β for their differentiation process (Lee et al., 2009). Tfh which perform the same function as that of the Th1/Th2 that is helping B cells in generating antibody responses, express low levels Gata-3 and T-bet along with Bcl-6 which is a key transcription factor of the Tfh lineage (Chtanova et al., 2004; Ma et al., 2012). T helper lineages not only share the

inducing cytokines and transcription factors between the lineages they also have common effector cytokines. IL-10 was initially thought to be a Th2 cytokine, is also known to be expressed by Th17, Th1 and iTregs (Lee et al., 2009; Zhou et al., 2009). IL-9, a Th17 cytokine is also shown to be expressed in Th9 cells, a subset that is derived from Th2 cells upon TGF-β stimulation (Veldhoen et al., 2008). The Th17 cells are capable of expressing IL-17 in vivo along with IFN-γ which is a Th1 cytokine, further studies have shown that Th17 cells can shutdown IL-17 production and continue to remain as IFN-γ producers (Wilson et al., 2007). The plasticity of T helper subtypes is also supported by epigenetic changes including H3 lysine 4 (H3K4) and H3 lysine 27 (H3K27) in Th1, Th2, Th17, and iTregs where they observe a lineage-specific pattern for cytokine specific genes but the transcription factors exhibit a broad spectrum of epigenetic states, and observe the expression of T-bet and IFN-γ in Treg cells (Wei et al., 2009). All these studies imply that there is an area of flexibility and redundancy in the functions of these T helper lineages. It has been suggested that although each subset of T helper cells have a distinct set of transcription factors their differentiation into a particular subset is determined by the ratio of transcription factors which in turn is determined by intrinsic and extrinsic factors (O'Shea and Paul, 2010).

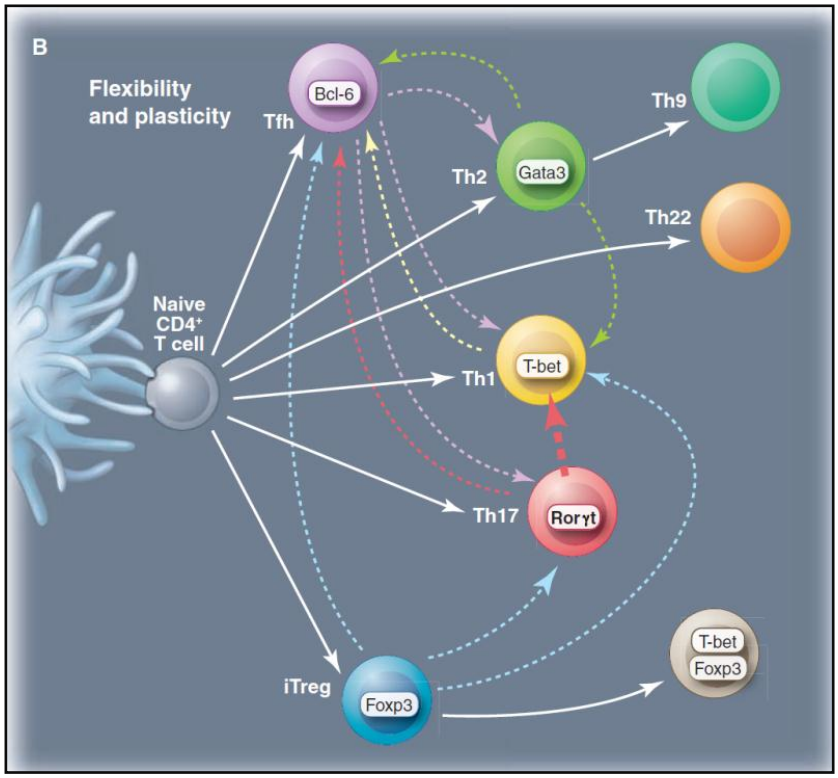

Figure 2.1.2: A model depicting the plasticity between different T helper lineages. Studies show that that each T helper lineage has flexibility in cytokine production and a particular T helper lineage is shown to express more than one master regulator. (Reproduced from O'Shea and Paul, 2010).

2.1.6 SATB1 in T helper cell differentiation

Genome-wide approach involving ChIP-seq and gene expression analyses in Th17 and T regs have shown that phenotype of a particular cell type is not solely dependent on a master regulator but is assisted by additional regulatory factors and epigenetic marks (Ciofani et al., 2012; Ohkura et al., 2012). Gene expression analysis using Th1 and Th2 cells revealed differential expression of SATB1, TCF7 and Bcl6 along with the well-studied key regulators such as Gata3 and T-bet (Lund et al., 2005). Studies using a mouse Th2 cell line - D10.G4.1, revealed that SATB1 was rapidly induced and forms a transcriptionally active chromatin structure at the Th2 cytokine locus (Cai et al., 2006). Knockdown of SATB1 revealed that it controls the expression of multiple genes involved in Th2 differentiation including the effector cytokine IL-4 (Ahlfors et al., 2010; Notani et al., 2010). IL-5 cytokine which is predominantly expressed by Th2 cells during asthma is shown to be repressed by direct binding of SATB1 to the *IL-5* promoter (Ahlfors et al., 2010). The NH_2-terminal PDZ-like domain of SATB1 interacts with several other proteins in signal-dependent manner and regulates gene expression (Notani et al., 2011). Since the PDZ-like domain of SATB1 lacks DNA-binding activity, overexpression of the PDZ-like domain would sequester all interacting partners and would serve as a dominant negative effector. This strategy has shown to affect the expression of various genes which are downstream of various signaling pathways such as Wnt, TGF-β, MAPK and many other pathways (Notani et al., 2010). In thymocytes, SATB1 regulates the expression of both SATB1 target genes and Wnt target genes via interaction with β-catenin and its recruitment (Notani et al., 2010). During Th2 differentiation, TCF1 and its cofactor β-catenin bind to the proximal promoter of *Gata3* and regulate its expression. TCF-1 was also shown to inhibit IFN-γ expression and negatively regulate Th1 differentiation (Yu et al., 2009). Similar studies from Galande laboratory have shown that SATB1 and β-catenin complex together bind to non-TCF binding sites and regulate expression of Gata-3, the key factor for Th2 differentiation (Notani et al., 2010). Although these studies demonstrated the role of SATB1 during Th2 differentiation, there is virtually no information pertaining to how SATB1 expression levels are specifically regulated in Th2 cells. Moreover, it is also not known whether SATB1 is differentially expressed in various effector lineages of $CD4^+$ T helper cells. Hence, to gain further insights into the function of SATB1 in T helper cell differentiation, it was important to elucidate the signaling pathways that regulate SATB1 expression. Additionally, we were interested in examining whether SATB1 is essential for the expression of effector cytokines.

2.2 Materials and Methods

2.2.1 Antibodies and reagents

Anti-SATB1 antibody was generated in the lab and described in Kumar et al., 2006 (Pavan Kumar et al., 2006). Anti-IL4, anti-IFN-γ PE conjugated antibodies were obtained from BD biosciences (CA, USA). Gata-3 (sc 268) and Stat6 (sc 981) antibodies were obtained from Santra Cruz Biotechnology (CA, USA). Chemical inhibitors of PI3K pathway, MEK and ERK pathway are obtained from Sigma and Calbiochem (USA).

2.2.2 Cell culture

$CD4^+$ T cells were isolated from spleen and lymph nodes of young mice (2 month old) using magnetic bead selection (BD) or MACS cell separation (Miltenyi). Mouse $CD4^+$ T cells were stimulated using anti-CD3 and anti-CD28 coated plates in RPMI media with 10% fetal bovine serum (FBS). $CD4^+$ T cells were polarized to various T helper types using appropriate cytokine stimulus, Th1 (10 ng/ml IL-12), Th2 (20 ng/ml IL-4), Th17 (20 ng/ml IL-6, TGF-β 10 ng/ml), iTregs (TGF-β and IL-2) along with TCR stimulation using anti-CD3 and anti-CD28 antibodies. Jurkat T cells were cultured in RPMI-1640 with 10% FBS and the cells were cultured in the presence of inhibitors LY294002, PD98509 and PD0325901.

2.2.3 RNA isolation and RT-PCR

CD4+ T cells polarized under Th1 and Th2 and were harvested after 2 days of culture. Total RNA is isolated using RNeasy isolation Kit (Qaigen). Five hundred ng of RNA was used for first strand cDNA synthesis using Dynamo cDNA synthesis kit (Finnzymes). Quantitative real-time PCRs are performed using Sybr-green mix (Applied biosystems). Changes in threshold cycle were calculated using the formula $\Delta Ct = (Ct_{\text{Target gene}} - Ct_{\text{Actin}})$. The fold difference in gene expression is calculated using the formula, Fold change = $2^{\textbf{(treatment –loading control)}}/2^{\textbf{(Control –loading control)}}$.

2.2.4 Immunoblotting

Cell lysates were prepared using RIPA lysis buffer (Tris pH 7.4, NP40 1%, Na Deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM). The protein lysates were quantitated using Bio-Rad DC protein assay (Bio-Rad). Equal amounts of protein lysates were resolved on 10% SDS-PAGE gels and transferred onto PVDF membrane (Millipore). Blots were incubated with anti-SATB1 antibody and anti Actin (Sigma) or Tubulin (Sigma), followed by incubation with horseradish peroxidase (HRP)-conjugated anti mouse IgG or HRP-conjugated anti rabbit IgG. The signals were visualized using ECL reaction (Thermo) on LAS 4000 (Fuji Film).

2.2.5 Nucleofection

Freshly isolated mouse $CD4^+$ T cells were transfected using Nucleofector II (Lonza). Approximately 2-3 million cells were transfected with siSATB1 (20 nM) or control scrambled siRNA. The transfected cells were polarized under Th1 and Th2 conditions for 48 h and harvested at end of the assay and were utilized for FACS staining.

2.2.6 Immunostaining and FACS analysis

Mouse $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 (eBioscience, San Diego, CA, USA), and harvested at different time points. The cells were fixed using 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and permeabilized using 0.1% Triton-X-100 (Sigma) followed by staining with anti-SATB1 antibody. Secondary antibody used was conjugate with Alexafluor 594. DNA counter staining was performed using DAPI. Cells were visualized under confocal microscope (Carl Zeiss). Image analysis was performed using Zen 2011 software (Carl Zeiss).

siSATB1 and siSCR transfected $CD4^+$ T cells polarized under either Th1 or Th2 conditions were harvested at the end of 48h time point. Six h prior to harvest, the cells were activated with PMA (50 ng/ml) and Ionomycin (1 μM) and after 3 h of activation the cells were treated with Brefeldin-A (3 μg/ml) (BD Biosciences, San Jose, CA, USA). After 48 h cells were fixed and stained with anti-IL-4 (BD Biosciences, San Jose, CA, USA) and anti- IFN-γ (BD Biosciences, San Jose, CA, USA). The cells were analyzed using FACS Canto II flow

cytometer (Becton Dickinson, NJ, USA). The data was analyzed using FlowJo (Treestar, Ashland, OR, USA).

2.2.7 Electrophoretic Mobility Shift Assay (EMSA)

The regions of *Satb1* promoter that either possess or do not possess consensus STAT6 binding sites were used as probes for the assay. The probes were amplified using PCR in the presence of α-P^{32} ATP (BRIT, India) and the probes were purified by gel excision followed by phenol-chloroform extraction. Protein lysates were prepared as described above. EMSA binding reactions were performed in a 10 μl volume containing 5XC buffer (10 mM HEPES pH 7.9, 1 mM DTT, 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.5 μg poly dI/dC). In supershift assays the reaction mixtures were incubated with anti-STAT6 antibody (Santa Cruz biotechnology, Dallas, TX, USA) or normal Rabbit IgG in control reactions. The complexes were incubated at room temperature for 30 min and resolved on a 6% native polyacrylamide gel. Dried gels were subjected to autoradiography.

2.2.8 ChIP analysis

ChIP was performed as described previously (Jayani et al., 2010). Briefly, purified mouse $CD4^+$ T cells were crosslinked by addition of formaldehyde to 1% final concentration in media and incubation at room temperature for 10 min, neutralized with 125 mM glycine, and then subjected to sonication using Bioruptor (Diagenode, Belgium) for 15 cycles with 30 sec on and 30 sec off to fragment the chromatin to obtain 200–500 bp fragments. Sonicated chromatin was precleared with a cocktail containing 50% protein A/G beads slurry (Thermo scientific, IL, USA.), Salmon sperm DNA, and BSA. Precleared chromatin was incubated with specific antibodies overnight at 4^0C and respective IgG types were used as isotype controls. Protein A/G bead cocktail was then added to pulldown the antibody-bound chromatin and was subjected to elution using sodium biocarbonate buffer containing SDS and DTT (Sigma, USA.). Eluted chromatin was de-crosslinked and protein was removed by treating with proteinase K. Purified immunoprecipitated chromatin was subjected to PCR amplification using specific primers. Input chromatin was used as a control.

2.3 Results

2.3.1 TCR signaling positively regulates SATB1 in peripheral CD4$^+$ T cells

Peripheral CD4$^+$ T cells differentiate into various T helper lineages in response to TCR signals, cytokine milieu and other ligands in the microenvironment. We wished to determine the expression levels of SATB1 in response to TCR signals in vitro. Mouse CD4$^+$ T cells were activated in presence of plate-bound anti-CD3 or anti-CD28 or together with anti-CD3 plus anti-CD28. Immunoblot analysis has shown that SATB1 expression is induced within 2 h of anti-CD3 or anti-CD3/CD28 and levels are upregulated until 24 h of activation (Fig. 2.3.1 A; lanes 1-3 & 7-9). In contrast, activation with only anti-CD28 antibody did not induce the expression of SATB1 in CD4$^+$ T cells (Fig. 2.3.1 A; lanes 4-6). It is interesting to note the kinetics of SATB1 expression was steep in cells activated by TCR (anti-CD3) alone, in comparison to cells activated with TCR and co-stimulatory signals (anti-CD3 and anti-CD28). Further CD4$^+$ T cells cultured under control conditions have no induction of SATB1 expression (Fig. 2.3.1 B; lanes 4-8). The bar graph (Fig. 2.3.1 C) summarizes the expression pattern of SATB1 in CD4 T cells upon TCR signaling. We observed that SATB1 is specifically upregulated by TCR signaling (CD3) but not CD28 signaling. Immunostaining of SATB1 in CD4$^+$ T cells activated in presence of anti-CD3 or anti-CD3 plus anti-CD28 depicted a characteristic cage-like structure as seen in thymocytes (Fig. 2.3.2 A&B). Whereas cells activated in the presence of only anti-CD28 showed poor induction of SATB1 expression (Fig. 2.3.2 A). In activated CD4$^+$ T cells there is compaction of chromatin and few pockets of densely DAPI stained heterochromatic regions in the nucleus. SATB1 is localized exclusively in lightly stained euchromatic regions and virtually absent in the heterochromatic regions. In naïve CD4 T cells SATB1 expression is almost undetectable and prominent heterochromatic blobs as seen in activated nuclei are also not observed indicating that the gross chromatin organization could be very different (Fig 2.3.2 B). These results suggest that SATB1 expression in peripheral CD4$^+$ T cells is regulated by TCR signaling.

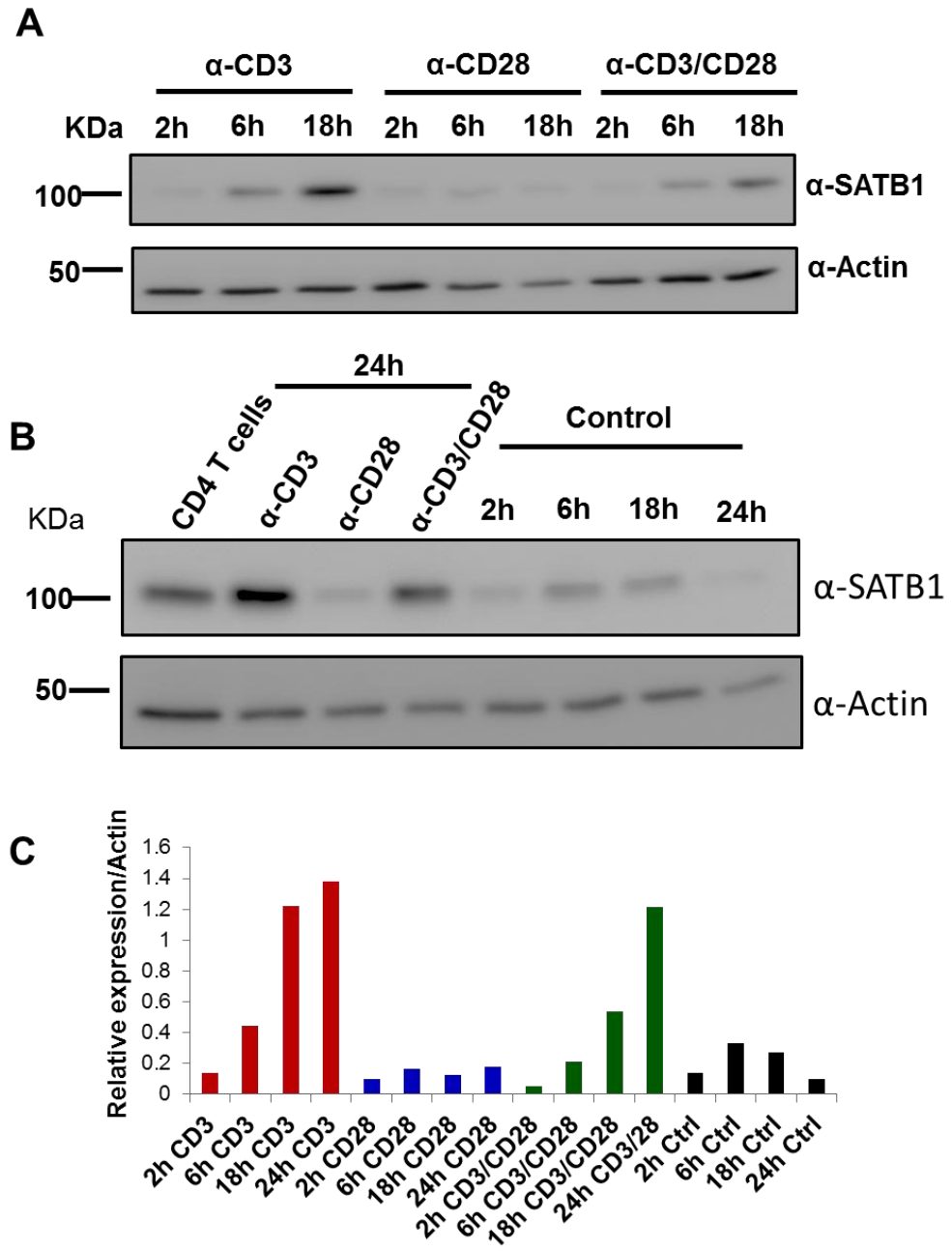

Figure 2.3.1: SATB1 levels are upregulated in CD4 T cells response to TCR signaling. (A, B) Immunoblot analysis (representative figure) in Naïve CD4 T cells isolated from spleen and lymph node are activated either with plate-bound anti-CD3 or anti-CD28 or with anti-CD3 plus anti-CD28 together for 2h, 6h, 18h and 24h as described in 'Materials and Methods'. Cells were harvested and whole cell lysates were prepared using RIPA buffer. Thirty μg of protein was loaded per lane and anti β-Actin was used as loading control. (C) Bar graph represents the densitometric analysis of above data using ImageJ software (NIH).

2.3.2 Chemical Inhibition of TCR signaling suppresses SATB1 expression

To confirm if SATB1 is downstream effector of TCR signaling we wanted to determine if SATB1 levels are affected when TCR signaling is perturbed. Jurkat T cells are known to have a tonic TCR signal and inhibition of TCR signaling by chemical inhibitors affects the expression of downstream targets such as recombinant activation genes 1 and 2 (Roose et al., 2003). Inhibition of TCR signaling by chemical inhibitors PD98509 which is an ERK inhibitor and PD0325901 MEK inhibitor and also an ERK inhibitor downregulated SATB1 expression in Jurkat cells (Fig. 2.3.3 A&B). SATB1 expression was unaltered at 3 hour time point in presence of any of the inhibitors in comparison with the vehicle control, whereas SATB1 was downregulated within 6 hours post treatment with ERK and MEK inhibitors (Fig. 2.3.3 A&B; compare lanes 3, 7 & 4, 8). PI3K inhibitor LY294002 has no effect on SATB1 expression in Jurkat cells since they are deficient in phosphatases PTEN (Phosphatase and tensin homolog) and SHIP (SHP-1 and SHP-2, inositol 5-phosphatase) that cause dephosphorylation of PIP3 served as internal control (Fig 2.3.3 B; lane 2 & 6). These results together suggested that SATB1 expression is induced in peripheral $CD4^+$ T cells in response to TCR signaling and inhibition of TCR signaling suppresses SATB1 expression.

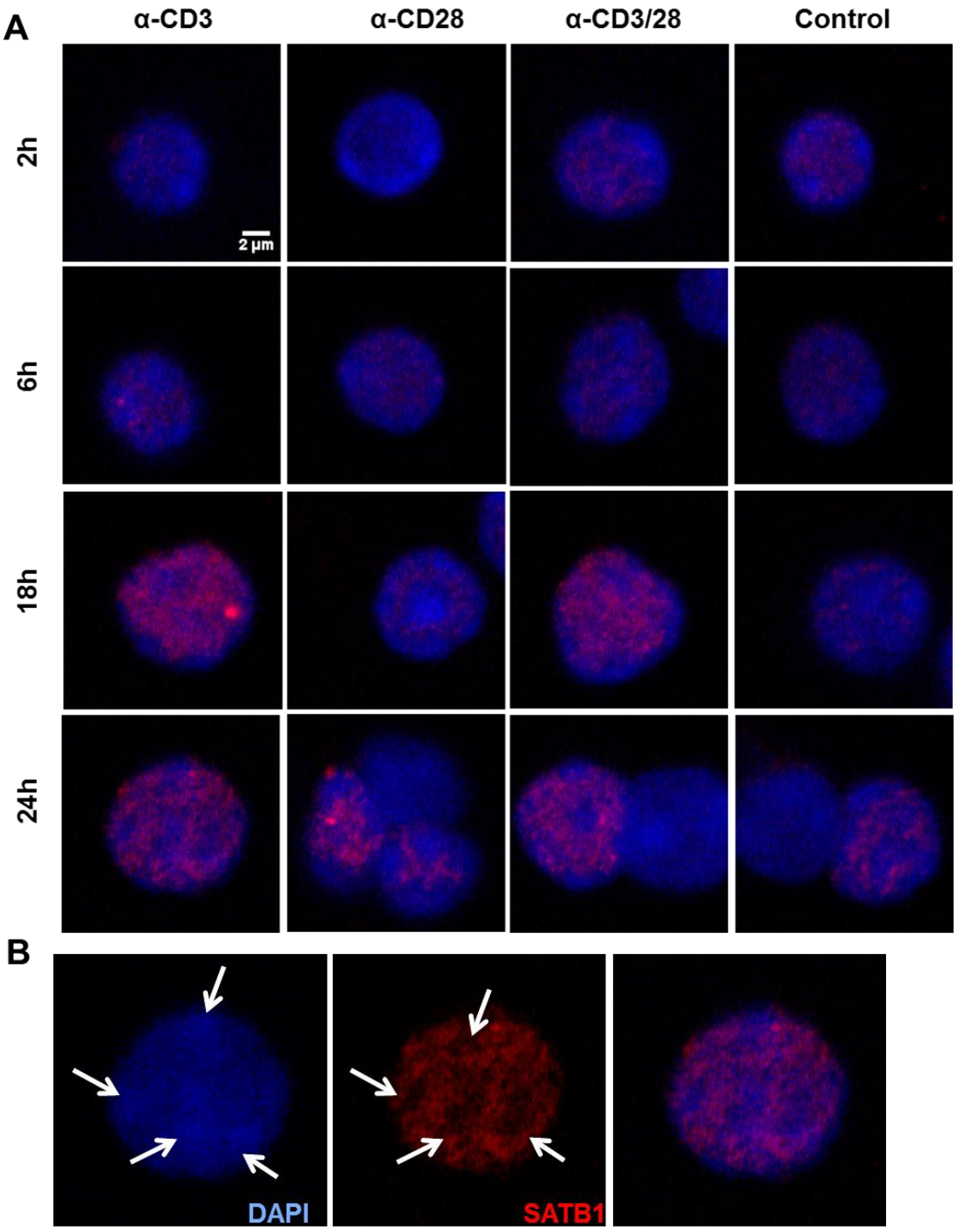

Figure 2.3.2: TCR induced SATB1 forms a typical nuclear network in activated CD4 T cells. (A) Mouse CD4 T cells were activated in presence of plate-bound anti-CD3 or anti-CD28 or anti-CD3 plus anti-CD28 or control (culture media) conditions, and cultured for 2h, 6h, 18h and 24h as described in 'Materials and Methods'. The cells were stained with DAPI (blue) and SATB1 (red) and immunofluorescence images were observed by Zeiss confocal microscope. (B) Representative image of CD4 T cells cultured for 24 h under TCR conditions, the image represents staining pattern in individual DAPI (blue), SATB1 (red) channels along with merged image (extreme right). Arrows indicate positions of heterochromatic blobs within the nucleus.

2.3.3 Differential expression of SATB1 in distinct T helper populations

$CD4^+$ T cells can be polarized to various T helper cells in vitro following activation with anti-CD3 plus anti-CD28 and providing the corresponding cytokine cues. We wanted to analyze the expression of SATB1 and later its role in T helper cell differentiation. In this experiment we have polarized $CD4^+$ T cells to Th2, Th9, iTregs and Th17 cells. Quantitative RT-PCR analysis in cells differentiated to various phenotypes revealed differential expression of SATB1. SATB1 was upregulated in Th2, Th9 and Th17 cells, however SATB1 was dramatically downregulated in iTregs (Fig. 2.3.4A). The downregulation of SATB1 expression in iTregs is similar to that observed in the natural Tregs (Beyer et al., 2011). Expression of SATB1 is highly upregulated in Th17 in comparison to other helper phenotypes (Fig 2.3.5A, lane 5). Th9 cells which are closely related to Th2 cells (Veldhoen et al., 2008) have higher expression of SATB1 compared to Th2 cells. We then focused on deciphering the role of SATB1 in Th2 differentiation. $CD4^+$ T cells were polarized to Th2 phenotype and monitored the expression of SATB1 in time-dependent manner. Gata-3 1-b and interferon-γ (Fig. 2.3.5B) were used as controls to confirm proper polarization to Th2 phenotype. Gata-3 is transcribed from two alternative promoters and resultant mRNA transcripts can be distinguished based on promoter specific exon 1-a or exon 1-b (Asnagli et al., 2002). Gata-3 1-b was upregulated in response to TCR signaling by 24 h whereas Gata3 1-a was minimally transcribed (Yu et al., 2009). We observed that Gata3 1-b was induced by 48 h and further upregulated by 5 day time point in Th2 polarized cells. Interferon-γ expression, which correlates with Th1 differentiation, is downregulated in Th2 cells and there is no significant change in its expression during the course of Th2 differentiation (Fig. 2.3.4B). These results indicated that Gata-3 1-b and SATB1 have a similar expression pattern and are required during Th2 differentiation. We then performed immunoblot analysis to monitor the expression of SATB1 in $CD4^+$ T cells differentiated to Th0, Th1 and Th2 cell types. Th0 cells receive only activating signals without any polarizing cytokines. We observed that SATB1 was induced in cells polarized in Th2 conditions but downregulated in cells skewed towards Th1 phenotype (Fig. 2.3.4D, lane 4 vs. lane 3) in comparison to Th0 cells (Fig. 2.3.4D, lane 2). Ku70 blot was used as loading control to show that equal amount of protein was loaded in all lanes (Fig 2.3.4D). These data demonstrated that SATB1 is differentially expressed in various T helper cells in response to TCR stimulation and various cytokine cues.

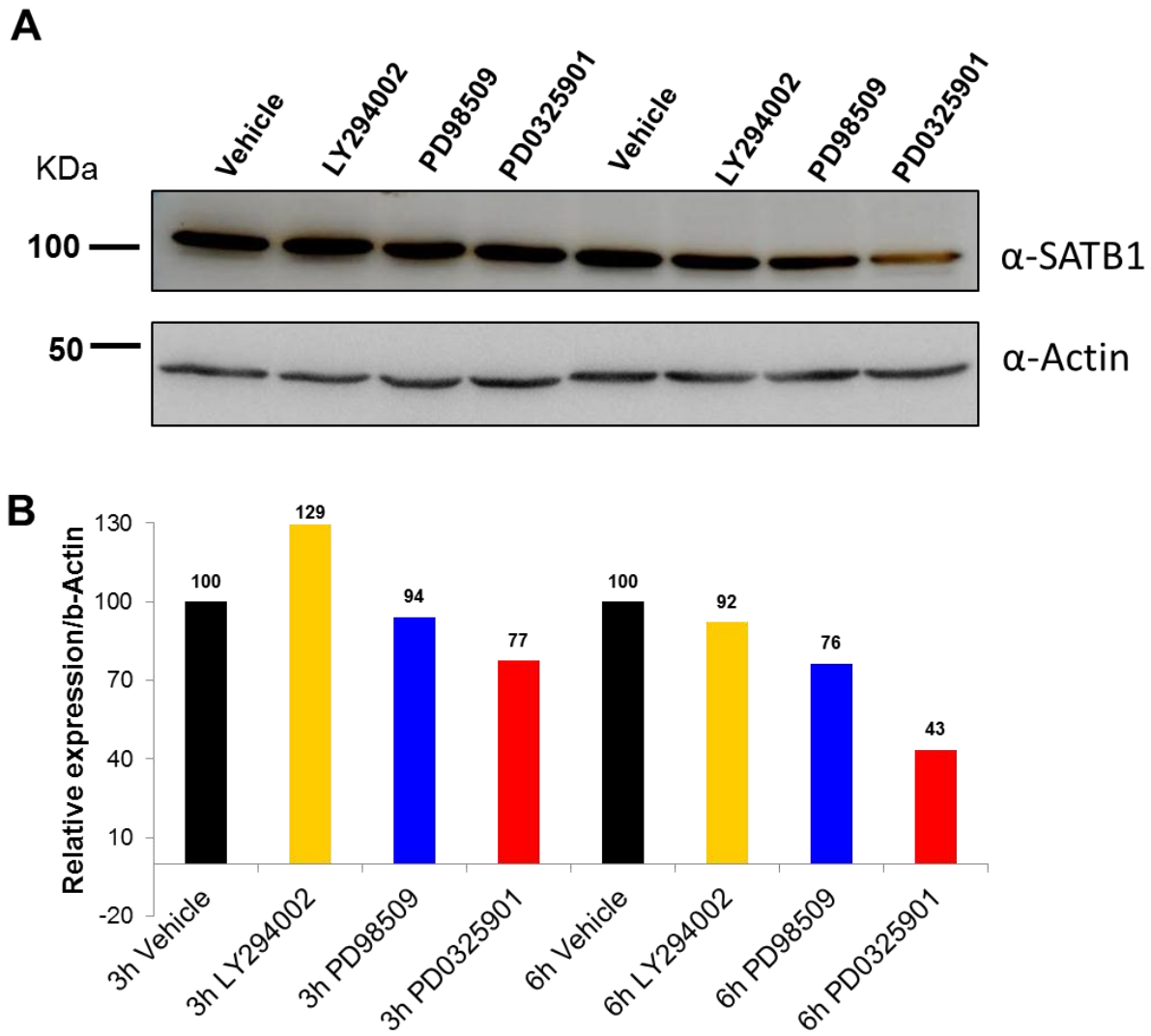

Figure 2.3.3: Inhibition of TCR signaling downregulates SATB1 expression in T cells. (A) Jurkat cells were treated with various chemical inhibitors that affect the downstream targets of TCR signaling pathway. Jurkat cells were cultured with PI3K inhibitor (LY294002), ERK1/2 inhibitor (PD98509), MEK/ERK inhibitor (PD0325901) and DMSO which served as vehicle control. The cells were harvested after 3 h or 6 h and whole cell lysates were prepared using RIPA buffer. Representative image of immunoblot using anti-SATB1 is depicted on top. Actin blot served as loading control. (B) Bar graph below represents densitometric analysis performed using ImageJ software. Expression values of SATB1 were normalized with β-Actin expression.

2.3.3 SATB1 is essential for Th2 differentiation

Since SATB1 is preferentially upregulated in Th2 cells compared to Th1, we wished to examine whether SATB1 is necessary for Th2 cell fate determination. Earlier studies in D10.G4.1 (Th2) T cells demonstrated that SATB1 binds across the Th2 cytokine locus and regulates the coordinated expression of IL-4, IL-5 and IL-13 (Cai et al., 2006). It was also shown that knockdown of SATB1 in D10.G4.1 cells led to downregulation of IL-4 expression (Cai et al., 2006). To extend these studies in primary cells we transfected $CD4^+$ T cells with scrambled and siSATB1 RNA duplexes using nucleofection and then polarized the cells to Th2 phenotype. After confirming effective knockdown of SATB1 we monitored the transcript levels of IL4 in scr and siSATB1 transfected cells. The expression of IL-4 in scr or siSATB1 transfected Th0 polarized control cells did not exhibit any relative change. In contrast, knockdown of SATB1 in Th2 cells significantly downregulated IL-4 expression in comparison with scr transfected Th2 cells (Fig. 2.3.6 A). To further confirm whether expression of IL-4 cytokine in Th2 cells is regulated by SATB1 we quantified the expression of IL-4 by intracellular cytokine staining. Towards this $CD4^+$ T cells were transfected with scrambled siRNA and siSATB1 and then were polarized under Th2 conditions for 2 days. The cells were activated with PMA and Ionomycin 6 h prior to harvesting time and later treated with Brefeldin-A which prevents the secretion of recently synthesized proteins and blocks them at Golgi bodies. Harvested cells were stained with fluorescently-tagged IL-4 antibody and analyzed via flow cytometry. We observed that upon knockdown of SATB1 there is complete loss of IL-4 positive cells. However, scr transfected cells exhibit IL-4 expressing cells (Fig. 2.3.6). These results suggested that SATB1 expression is necessary for the differentiation of $CD4^+$ T cells to Th2 phenotype by regulating the expression of effector cytokine IL-4.

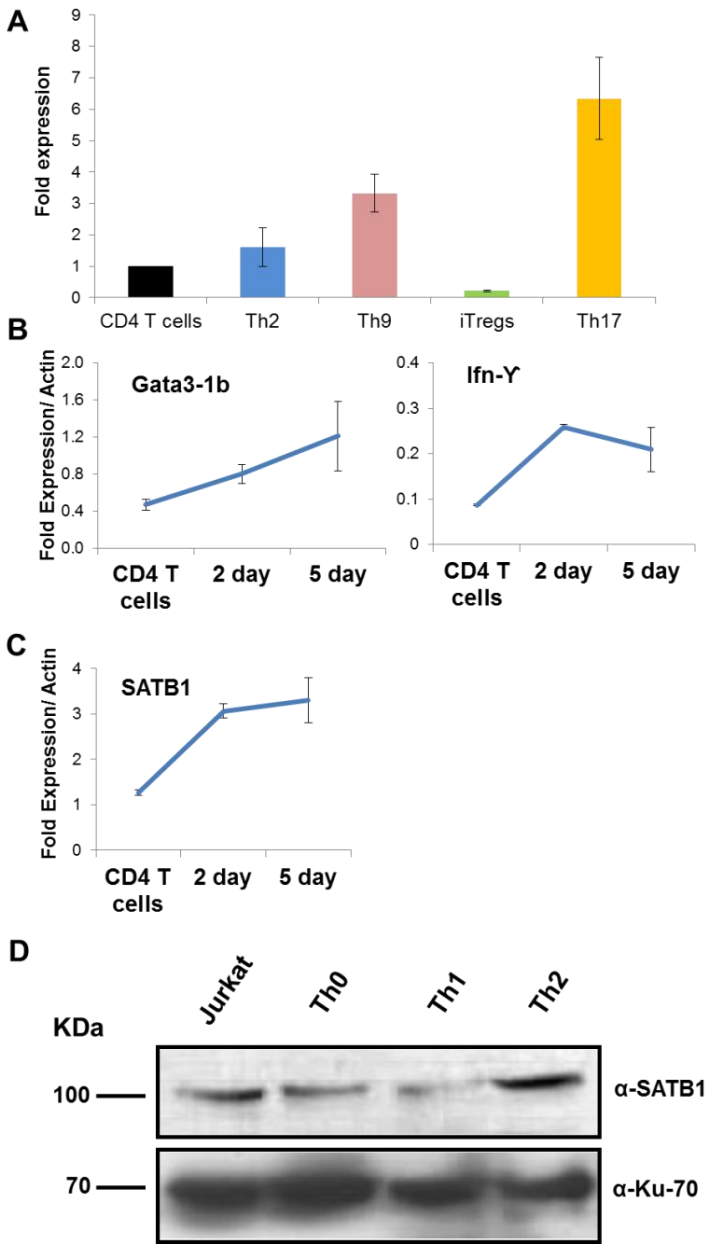

Figure 2.3.4: SATB1 is differentially expressed in different T helper populations. (A) Mouse $CD4^+$ T cells were polarized to different T helper populations as described in 'Materials and Methods'. Cells were polarized in respective culture conditions for 5 days. Quantitative RT-PCR analysis was performed using RNA isolated from cultured CD4 T cells. The bar graph represents fold gene expression in different T helper cells, and expression is normalized with untreated control $CD4^+$ T cells, which was set to 1. Error bar indicates standard deviation calculated from triplicates. (B & C) Mouse $CD4^+$ T cells were polarized in Th2 conditions and the expression of target genes was analyzed on day 0, 2 and 5. Each line graph represents the relative expression of the gene at respective days. Gata-3 1-b transcript serves as positive control and Ifn-γ served as negative control for Th2 polarization. Error bars indicate standard deviation calculated from triplicates. (D) Mouse $CD4^+$ T cells were polarized to Th0, Th1 and Th2 conditions for 3 days and cells were harvested. Protein lysates were resolved on SDS-PAGE gels. Immunoblot was performed using anti-Satb1 antibody. Ku70 was used as loading control.

2.3.4 SATB1 negatively regulates IFN-γ expression

Signals that induce Th2 differentiation in $CD4^+$ T cells inhibit the expression of interferon-γ (IFN-γ) (Murphy and Reiner, 2002). Since we observed that SATB1 is specifically upregulated in Th2 cells as compared to Th1, we wanted to address if SATB1 has any effect on IFN-γ expression in Th2 cells. Towards this we have performed ChIP analysis in Th1 and Th2 cells polarized for 3 days, and assessed for the occupancy of SATB1 on *Ifn-*γ promoter. We performed quantitative PCR of ChIP products and observed that SATB1 is highly enriched on *Ifn-*γ promoter in Th2 cells as compared to Th1 and Th0 cells (compare lanes 2, 4 with lane 6) (Fig. 2.3.5A). Rabbit IgG represents negative control for the ChIP experiment and did not reveal any enrichment. To further validate the effect of SATB1 on the expression of IFN-γ in Th2 cells, we knocked down SATB1 expression in $CD4^+$ T cells and polarized them to Th2 phenotype. We observed that ablation of SATB1 in Th2 cells has increased the percentage of IFN-γ positive cells in comparison to scr transfected $CD4^+$ T cells (Fig. 2.3.5B). Whereas in $CD4^+$ T cells transfected with either siSATB1 or scr and polarized under neutral conditions (Th0) i.e. with only TCR stimulation and no polarizing cytokines, the IFN-γ positive cells were unchanged. These results suggested that SATB1 is not only required for coordinated expression of the *IL-4* effector cytokine locus in Th2 cells, but is also necessary for inhibition of IFN-γ gene expression.

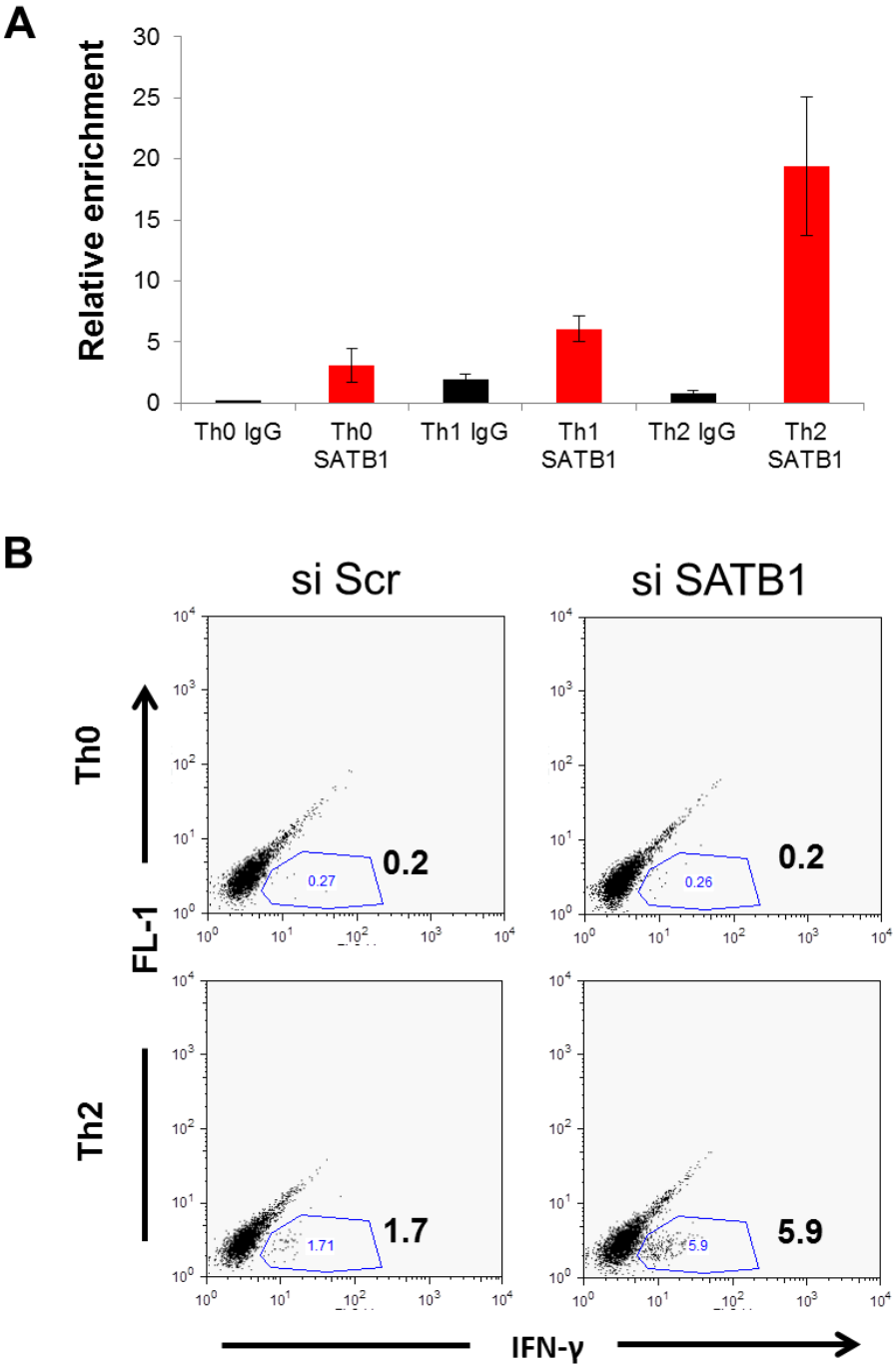

Figure 2.3.5: SATB1 represses Interferon-γ expression in Th2 cells. (A) Mouse $CD4^+$ T cells were polarized under Th1 and Th2 conditions as described in 'Materials and Methods'. Th0 cells served as control. Post polarization for 3 days cells were harvested and occupancy of SATB1 on *Ifn-γ* promoter was determined by ChIP analysis. Bar graph represents relative enrichment by SATB1 antibody i.e. SATB1 occupancy on the *Ifn-γ* promoter locus. IPs performed using IgG serve as negative control. Error bar depicts the standard deviation calculated from triplicates. (B) $CD4^+$ T cells were transfected with Scr or siSATB1 and cells were polarized in Th0 or Th2 conditions. AFter 48 h cells were harvested and stained for nterferon-γ and analyzed by flow cytometry. Gated populations in the FACS plots represent the percentage of IFN-γ positive cells.

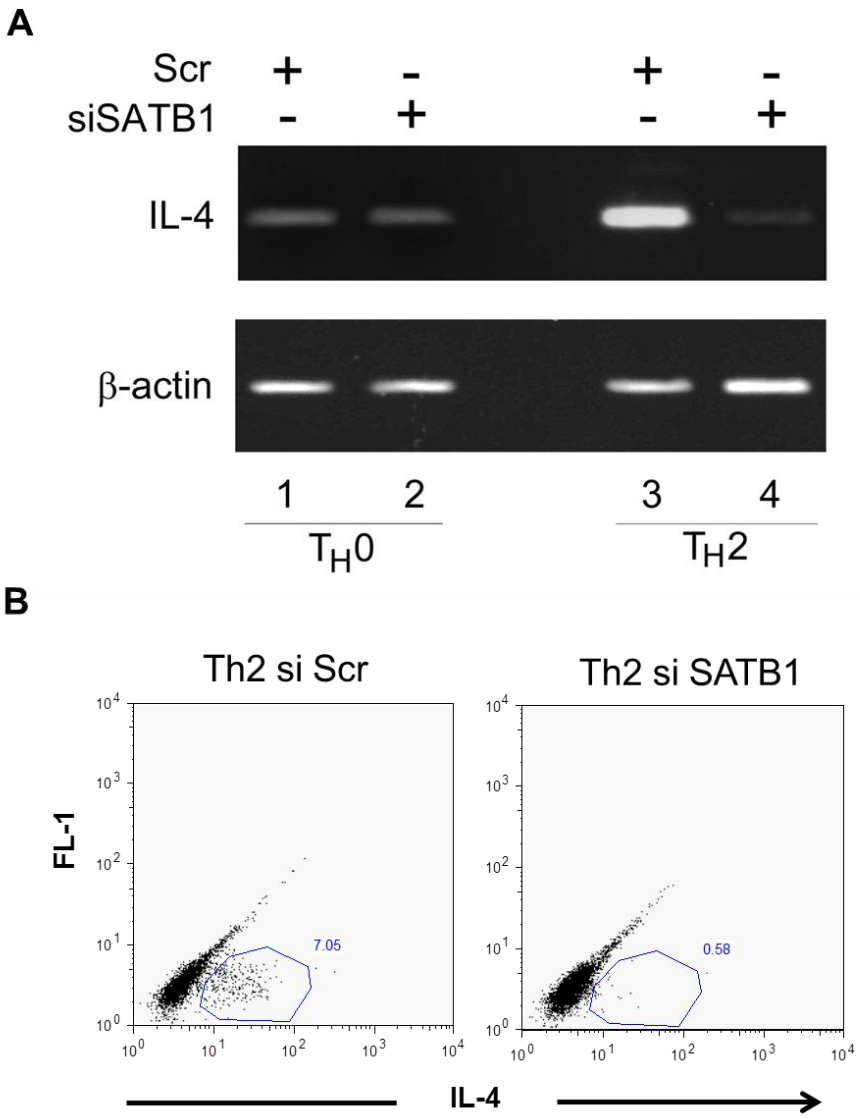

Figure 2.3.6: SATB1 regulates IL-4 expression in CD4$^+$ T helper cells. (A) CD4$^+$ T cells were transfected with Scrambled (Scr) siRNA or siSATB1 and then polarized to Th2 phenotype as described in 'Materials and Methods'. The cells were harvested 48 h post transfection and used for monitoring the expression of IL-4. IL-4 expression was quantified by RT-PCRs. Actin transcript was used as loading control. (B) Scr and siSATB1 transfected cells were analyzed for intracellular IL-4 cytokine expression as described in 'Materials and Methods'. Gated populations represent the cells expressing cells positive for IL-4 cytokine expression.

2.3.5 STAT6 signaling regulates SATB1 expression in Th2 cells

Since SATB1 levels are specifically upregulated in Th2 and we observed that it is necessary for Th2 differentiation, we wished to understand the regulation of SATB1. Studies from our laboratory have shown that IL-4 dependent regulation of SATB1 is mediated by STAT6 (Ahlfors et al., 2010). It was also shown that RNAi induced silencing of STAT6 in mouse $CD4^+$ T cells and later polarization under Th2 conditions downregulated SATB1 expression. We therefore wished to test if STAT6 directly binds to SATB1 and regulates its gene expression. Towards this we performed gel mobility shift assays using radioactively labeled SATB1 promoter probes of which two probes 644-741 and 644-643 harbor consensus STAT6 binding sites. As control, probe 741-748 which does not harbor the consensus STAT6 binding site (Fig. 2.3.7 A) was used. If STAT6 protein complex from the lysate binds to the consensus STAT6 binding site on SATB1 promoter we should observe a greater shift in the complex when incubated with STAT6 antibody. We observed that probe 644-643 and 644-741 have retarded mobility of the complex and observed a greater shift when the whole complex is incubated with anti-STAT6 antibody compared to control R-IgG. We observed no further retardation of the complex with probe 741-748 in comparison to R-IgG or with anti-STAT6 antibody, which does not have a consensus STAT6 binding site. We then performed ChIP analysis to verify the in vivo binding of STAT6 on SATB1 promoter. As observed in gel mobility shift studies we found significant enrichment of STAT6 on SATB1 promoter using quantitative PCR analysis (Fig. 2.3.7 B). A recent study has elucidated the role of STAT proteins in dictating T helper differentiation by modulating the epigenetic changes and regulating gene expression (Wei et al., 2010). We further analyzed the data pertaining to the expression pattern of SATB1 from Wei et al., and observed that STAT6 deletion had profound effect on SATB1 expression compared to STAT4, although deletion of either STAT proteins led to downregulation of SATB1 (Fig. 2.3.7C). These results indicated that SATB1 expression during Th2 differentiation is positively regulated by STAT6 signaling.

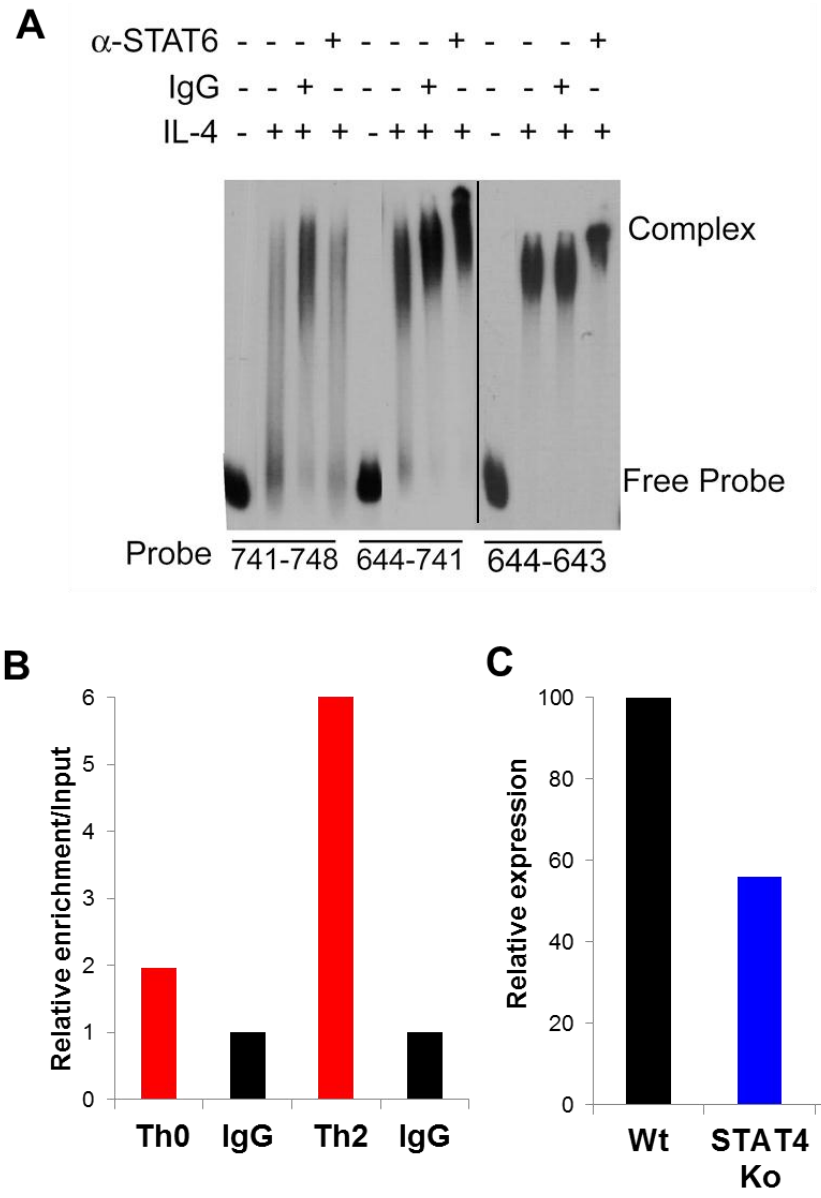

Figure 2.3.7: STAT6 signaling regulates SATB1 expression in Th2 polarized cells. (A) Increasing amounts of $CD4^+$ T cell lysate were incubated with three different radioactively labeled DNA probes. Probe 741-748 of SATB1 promoter has no STAT6 binding site and served as negative control. Probe 644-741 and 644-643 are two different probes with the STAT6 consensus binding sites. Probe along with lysates were incubated for 1 h at room temperature and later with STAT6 antibody or rabbit IgG. The complexes are resolved on native polyacrylamide gel and analyzed by autoradiography as described in 'Materials and Methods'. (B) ChIP analysis was performed in $CD4^+$ T cells polarized to Th0 and Th2 phenotype. IPs were performed using either STAT6 antibody or rabbit IgG. The bar graph represents the relative occupancy of STAT6 on SATB1 promoter. (C) Bar graph represents the relative expression of SATB1 in $CD4^+$ T cells in Wt, STAT4 and STAT6 knockout mice. The data is analyzed using the ChIP seq data from Durant et al., 2010.

2.3.6 TCF1 and Gata3 transcriptionally regulate SATB1 expression

We were further interested in identifying the transcriptional regulators of SATB1 during Th2 polarization. Along with Gata-3 which is master regulator of Th2 differentiation various other factors such as TCF1 and Notch transcription factors have been shown to be necessary and regulate the Th2 differentiation (Zhu et al., 2010). $CD4^+$ T cells from TCF KO mice when polarized under Th2 conditions have downregulated levels of Gata-3 and have inhibition of IL-4 cytokine expression. Further it was shown that TCF1 positively regulates Gata-3 expression and promotes Th2 differentiation (Yu et al., 2009). We then wished to determine if TCF1 and Gata-3 have any role in regulating SATB1 expression during Th2 differentiation. Towards this we polarized $CD4^+$ T cells from TCF1 KO mice under Th2 conditions for 3 days and then determined SATB1 expression in those cells. We observed that there is about five fold reduction of SATB1 transcripts in TCF1 KO mice compared to control Th2 cells (Fig. 2.3.8 A). As observed earlier (Yu et al., 2009), we found that Gata-3 1b transcription is downregulated in TCF1 KO cells polarized under Th2 conditions. Our own results from mouse thymocytes have shown that Gata-3 binds to *Satb1* promoter and regulates its expression. We have performed ChIP analysis using Gata-3 in Th2 cells polarized for 5 days under Th2 conditions and observed that Gata-3 binds to *Satb1* promoter (Fig. 2.3.8 B) . We scanned across the *Satb1* promoter locus for binding sites and identified only a single Gata-3 binding site as evident from the quantitative PCRs (Fig. 2.3.8 C). These results argued that TCF1 regulates Gata-3 expression, which in turn activates SATB1 expression by directly binding to the SATB1 promoter.

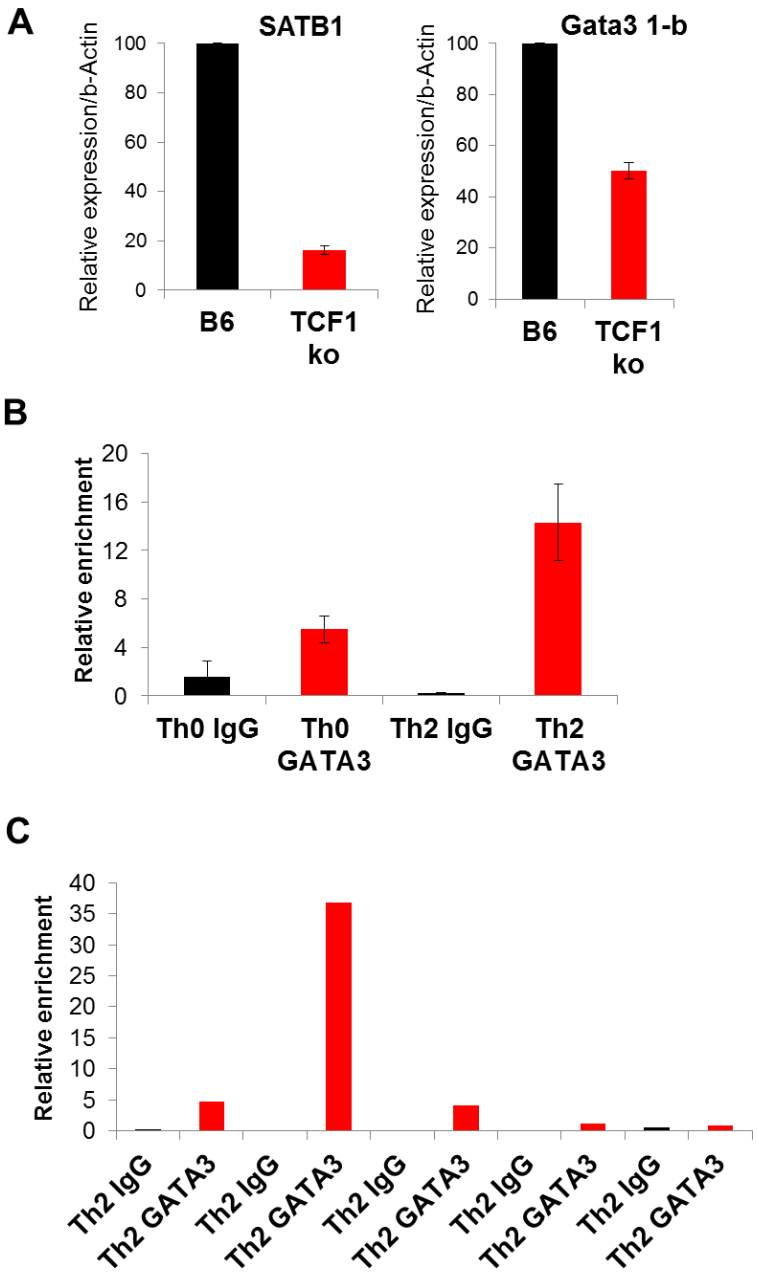

Figure 2.3.8: Transcriptional regulators of SATB1 expression in Th2 cells. (A) Mouse $CD4^+$ T cells and Wt and TCF1 KO mice were polarized to Th2 phenotype. Expression analysis is performed in Th2 cells after 48 h of polarization of cells for SATB1 and Gata-3 1-b. The bar graph represents relative expression in Wt and TCF1 KO mice, and error bars represent standard deviation calculated from triplicates. (B) ChIP analysis is performed in mouse $CD4^+$ T cells polarized to Th0 and Th2 cells using GATA3 antibody. Bar graph represents the relative enrichment of GATA-3 on SATB1 promoter, and error bars represent standard deviation calculated from triplicates. (C) Bar graph represents the occupancy of GATA-3 across the 3 kb *Satb1* promoter locus observed after ChIP analysis.

2.4 Discussion

$CD4^+$ T cells that develop in the thymus migrate to the periphery where they differentiate into multiple lineages of effector cells, which help in clearance of the antigen (Zhu et al., 2010). The differentiation of $CD4^+$ T cells to T helper cells is orchestrated by master transcription factors specific to each lineage, along with certain common signaling processes such as TCR and costimulatory signals and transcription factors such as STATs, NFATs (Hermann-Kleiter and Baier, 2010) and NF-κB. NFAT was shown to be important during the T helper differentiation by transcriptional regulation of lineage-specific transcription factors such as T-bet (Th1), Gata-3 (Th2), RORγt (Th17) and Foxp3 (iTregs) (Hermann-Kleiter and Baier, 2010). In this study we explored the role of chromatin organizer SATB1 during T helper cell differentiation using an in vitro culture system wherein $CD4^+$ T cells are differentiated into distinct helper T lineages.

In chapter I we reported that SATB1 levels are regulated in TCR-dependent fashion in developing thymocytes. Next, we wished to determine if SATB1 expression is upregulated upon TCR signaling using peripheral $CD4^+$ T cells. Since an effective TCR signal transduction pathway involves signaling via the TCR and coreceptors such as CD28 (Acuto and Michel, 2003) we activated $CD4^+$ T cells in time-dependent manner using only anti-CD3 or anti-CD28 or both of them together. We observed that SATB1 expression is induced specifically with anti-TCR but not with costimulatory signals. Further activation with both anti-CD3 and anti-CD28 instead resulted in downregulation of SATB1 expression, showing that anti-CD3 signals activate SATB1 expression and anti-CD28 inhibits SATB1 expression. We also observed by immunostaining that SATB1 in expressed only in TCR activated cells and localizes in the euchromatic regions and almost absent in the DAPI intense heterochromatic regions. Similar findings were observed in mouse thymocytes wherein Kohwi-Shigematsu's group reported that SATB1 forms a 'cage-like network' circumscribing heterochromatin and binds to specific loci such as *Myc* in the euchromatic regions (Cai et al., 2003). Thus, these results suggested that SATB1 functions as a gene regulator by creating distinct nuclear compartments. Further, to conclusively prove that SATB1 expression is directly regulated by TCR signaling, we chemically inhibited TCR signaling in Jurkat T cells and observed that SATB1 is downregulated upon inhibition of the ERK and MAP kinase pathways. Jurtkat T cells and thymocytes are known to have basal TCR signaling and RAG genes are shown to be target genes of this signaling pathway. Perturbing TCR signaling with

chemical inhibitors has shown to upregulate the expression of RAGs (Roose et al., 2003). We observed that SATB1 levels are downregulated in Jurkat cells when cultured with inhibitors of MEK and ERK which are downstream mediators of TCR signal transduction pathway. Since Jurkat cells are mutant for phosphatases such as 3'-phosphatase and tensin homolog deleted on chromosome 10 (PTEN) which causes deposphorylation of phosphatidyl inositol (3,4,5)-tri phosphate (PIP3) have very high levels of inositol phospholipids (Astoul et al., 2001). Thus, inhibition of phosphoinositide 3' kinase (PI3K) which has antagonistic function of PTEN in Jurkat cells has no effect. Similarly we observed no effect on SATB1 expression upon treatment of Jurkat cells with PI3K inhibitor LY294002 and thus acted as an internal control. Collectively the findings in thymocytes and those we observed in peripheral cells strongly suggest that SATB1 expression levels in T lineage are regulated by TCR signaling which in turn is indispensable for T cell development and differentiation.

Previous studies from Galande laboratory and others have shown the role of SATB1 in Th2 cell differentiation (Ahlfors et al., 2010; Notani et al., 2010) and its regulation of Th2 cytokine locus (Cai et al., 2006). We further investigated the expression levels in other T helper lineages and observed that SATB1 is also upregulated in other T helper subsets such as Th9 and Th17. A recent report using Th17 polarized cells demonstrated that knockdown of SATB1 resulted in downregulation of IL-17 showing the importance of SATB1 during Th17 differentiation (Ciofani et al., 2012). As observed in nTregs peripheral induced Tregs (iTregs) also have downregulated expression of SATB1. These results suggested that SATB1 might be important during the differentiation of $CD4^+$ T cells to various helper phenotypes.

We further wished to establish if SATB1 is necessary for the differentiation of $CD4^+$ T cells into T helper lineages. Towards this we abrogated the expression of SATB1 using RNAi mediated silencing and further differentiated these cells under Th2 conditions. Interestingly, knockdown of SATB1 led to reduction in the percentage of IL-4 positive cells, IL-4 being the key effector cytokine of Th2 lineage cells and increased the percentage of IFN-γ expressing cells, IFN-γ being the effector cytokine of Th1 cells. During the development of Th2 cells *IFN-*γ locus is known to be bound by STAT6 and Gata-3 which further recruit chromatin remodelers such as the Polycomb proteins which initiate methylation of histones at H3K27 and cause stable silencing of *IFN-*γ locus (Chang and Aune, 2007). In this study we showed that SATB1 directly binds to *Ifn-*γ promoter in Th2 cells and inhibits its expression. Our

study revealed that SATB1 is essential for the differentiation of $CD4^+$ T cells to Th2 phenotype via regulation of effector cytokine expression.

Transcription factors such as T-bet and Gata-3 were considered as master transcription factors that are necessary and sufficient to induce either Th1 or Th2 lineage respectively (Lee et al., 2001; Mullen et al., 2001). However, large body of evidence in recent years suggests that concerted effect of minimum pool of factors, and not any single factor, is necessary for the commitment of a particular lineage (Ciofani et al., 2012; Ohkura et al., 2012; Vahedi et al., 2012). STAT proteins act as adaptor molecules during T helper cell differentiation by sensing the extracellular cytokine signals and induce the expression of factors required for the differentiation process (O'Shea and Plenge, 2012). Upon cytokine signaling specific STAT molecules are activated. STAT4 and STAT6 in particular are well characterized and are known to transactivate the expression of transcription factors and effector cytokine genes in Th1 and Th2 cells respectively (Thieu et al., 2008; Zhu and Paul, 2008). Interestingly, RNAi mediated knockdown of STAT6 downregulated SATB1 expression in human $CD4^+$ T cells (Ahlfors et al., 2010). Here, we have further demonstrated the molecular mechanism for this observation and show that STAT6 directly regulates SATB1 expression by directly binding to its promoter region. Next, we wished to address if the key transcription factors involved during Th2 differentiation are also required for Th2 differentiation. It was shown that TCF1 KO $CD4^+$ T cells have defective Th2 differentiation (Yu et al., 2009) and this is due to downregulated expression of Gata-3. Similar results were also observed during the early thymocyte development (Weber et al., 2011). In Cat-Tg mice which have the constitutive expression of β-catenin, $CD4^+$ T cells produce higher levels of IL-4 in Th2 cells. This is because β-catenin complexes with TCF1 on *Gata-3* promoter and upregulates its expression. Galande laboratory has shown that β-catenin complexes with SATB1 in Th2 cells and regulates *Gata-3* promoter (Notani et al., 2010). As the consensus binding sites of TCF1 and SATB1 differ it might be that both are important in recruiting β-catenin to the *Gata-3* promoter. However, what remains to be understood is how do the β-catenin:TCF1 and β-catenin:SATB1 complexes differ in terms of their target specificities and what are the other members of each of the complexes. In silico analysis of SATB1 promoter revealed consensus binding site for Gata-3. As observed in mouse thymocytes we also found that Gata-3 binds to *Satb1* promoter and regulates its expression in Th2 cells. Therefore, we speculate that Gata-3 might be involved in upregulation of SATB1 expression in Th2 cells during the initiation phase. Whereas during the maintenance phase of Th2 cells SATB1 binds to *Gata-3* promoter

and has a feedback loop, which we speculate might be necessary for the stable expression of Gata-3 expression in Th2 cells.

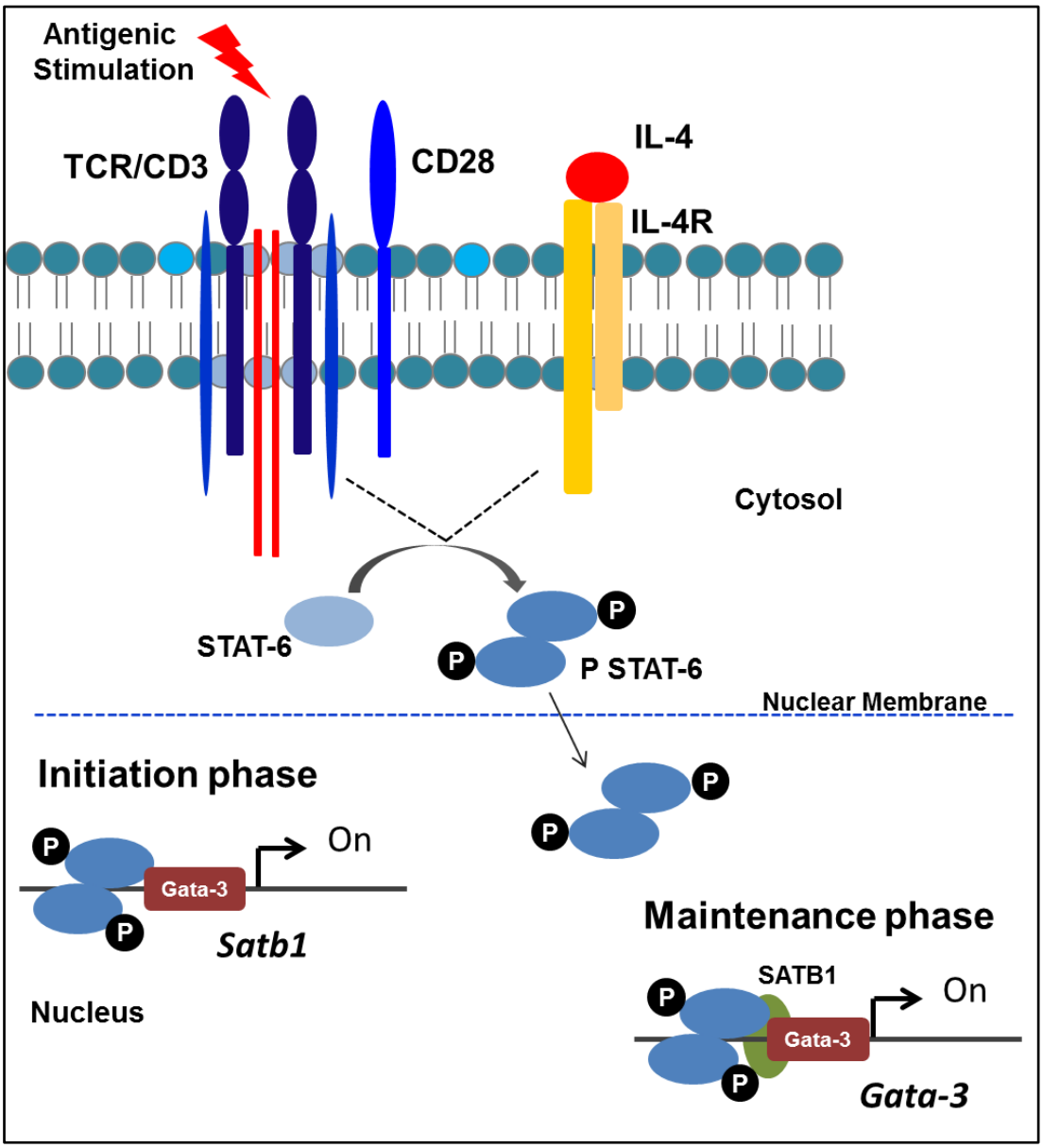

Figure 2.4.1: Model depicting the role of SATB1 during Th2 differentiation. Upon antigen stimulation and appropriate cytokine signaling naïve $CD4^+$ T cells activate STAT-6, which then translocates into the nucleus. Inside the nucleus, STAT-6 along with Gata-3 transactivates SATB1 expression which is required for the expression of IL-4 and suppression of IFN-γ and thus initiates Th2 differentiation. Once the cells are committed to Th2 lineage SATB1 binds to *Gata-3* promoter along with Gata-3 itself and is required for the maintenance of Gata-3 expression.

We show that SATB1 is necessary not only during thymocyte development but is also essential for the differentiation of $CD4^+$ T cells to various T effector lineages. However, question remains that why SATB1 is differentially expressed in different T helper lineages if its expression levels are dependent on TCR signaling. Our data suggest that along with the TCR signals the cytokine signaling also plays a role in regulating the expression of SATB1 in Th2 cells. The strength of TCR signaling has also been shown to be important for the differentiation of $CD4^+$ T cells to Th1/Th2 phenotype (Boyton and Altmann, 2002; Constant and Bottomly, 1997; Iezzi et al., 1999). Transgenic mouse models that have a Cytochrome specific TCR, when polarized to either Th1 or Th2 at varying concentrations of antigen revealed that high doses of peptide induces Th1 response and low doses of antigenic peptide induces Th2 response (Constant et al., 1995). Low strength of TCR signaling has also shown to be important for the differentiation of $CD4^+$ T cells to Th17 lineage (Purvis et al., 2010). Further, the role of costimulatory signals was elucidated using CD28 KO and it was observed that IL-2 production by these cells was greatly reduced and inhibited the proliferation of Th1 cells whereas there was no effect on Th2 cell differentiation (Seder et al., 1994). In contrast, In vitro studies using CD28 KO mice with a transgenic TCR have shown that they have selective impairment of Th2 differentiation (Lenschow et al., 1996). During Th17 polarization of $CD4^+$ T cells, it was shown that CD28 signaling induced IL-2 and IFN-γ inhibits the differentiation process (Bouguermouh et al., 2010). In this study we show that SATB1 is upregulated in Th2 and Th17 cells which are associated with low strength of TCR signaling but downregulated in Th1 and iTregs that require higher strength of TCR signaling. Our studies show for the first time that SATB1 is regulated by TCR signaling in both thymocytes and peripheral T cells during T helper cell differentiation. Although what remains unanswered is the role of cytokine signaling in modulating TCR and costimulatory signals which determine the differentiation of $CD4^+$ T cells to distinct T helper subsets.

In conclusion, SATB1 has emerged as a key factor in regulating higher-order chromatin organization and gene expression by modulating epigenetic changes. SATB1 is also known to orchestrate the gene expression program by organizing higher-order chromatin ‘loopscape’ in a dynamic manner (Galande et al., 2007; Kumar et al., 2007). The post-translational modifications of SATB1 and its interaction partners together play an important role in regulating gene expression. The nature of modifications and their specific role(s) in different T cell subsets and during differentiation process remains to be elucidated. Understanding how

various signaling pathways that regulate the molecular switches would provide novel insights into the signal-dependent regulation of gene expression in various T cells. SATB1 is shown to be regulated in IL-4/STAT6 dependent manner in Th2 cells but how it is regulated in other T helper subsets needs to be further investigated. Newly discovered mechanism of regulation of SATB1 by miRNAs is an exciting new direction which needs to be explored further during differentiation process (Beyer et al., 2011; Burute et al., 2012; McInnes et al., 2012). Since SATB1 forms a cage-like structure demarcating active and inactive chromatin domains in thymocytes, it would be interesting to delineate how SATB1 organizes the chromatin loopscape. Understanding the regulation of SATB1 expression in various cell types would provide insights into the function of SATB1 in T helper subsets.

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- **Gottimukkala KP**, Sultana DA, Sen JM, Galande S. (2013) Induction of SATB1 expression via TCR signaling regulates T cell development and function.

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