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TRANSCRIPTIONAL AND EPIGENETIC REGULATION OF HUMAN CD4+ T HELPER LINEAGE SPECIFICATION

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ABSTRACT

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Transcriptional and Epigenetic Regulation of Human CD4+ T Helper Lineage Specification

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Activated T helper (Th) cells have ability to differentiate into functionally distinct Th1, Th2 and Th17 subsets through a series of overlapping networks that include signaling and transcriptional control and the epigenetic mechanisms to direct immune responses. However, inappropriate execution in the differentiation process and abnormal function of these Th cells can lead to the development of several immune mediated diseases. Therefore, the thesis aimed at identifying genes and gene regulatory mechanisms responsible for Th17 differentiation and to study epigenetic changes associated with early stage of Th1/Th2 cell differentiation. Genome wide transcriptional profiling during early stages of human Th17 cell differentiation demonstrated differential regulation of several novel and currently known genes associated with Th17 differentiation. Selected candidate genes were further validated at protein level and their specificity for Th17 as compared to other T helper subsets was analyzed. Moreover, combination of RNA interference-mediated downregulation of gene expression, genome-wide transcriptome profiling and chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq), combined with computational data integration lead to the identification of direct and indirect target genes of STAT3, which is a pivotal upstream transcription factor for Th17 cell polarization. Results indicated that STAT3 directly regulates the expression of several genes that are known to play a role in activation, differentiation, proliferation, and survival of Th17 cells. These results provide a basis for constructing a network regulating gene expression during early human Th17 differentiation. Th1 and Th2 lineage specific enhancers were identified from genome-wide maps of histone modifications generated from the cells differentiating towards Th1 and Th2 lineages at 72h. Further analysis of lineage-specific enhancers revealed known and novel transcription factors that potentially control lineage-specific gene expression. Finally, we found an overlap of a subset of enhancers with SNPs associated with autoimmune diseases through GWASs suggesting a potential role for enhancer elements in the disease development. In conclusion, the results obtained have extended our knowledge of Th differentiation and provided new mechanistic insights into dysregulation of Th cell differentiation in human immune mediated diseases.

Keywords: T helper cell differentiation, gene regulation, TF, epigenetic regulation, RNA interference, ChIPseq, STAT3, enhancer, GWAS, SNP, immune mediated disease

TIIVISTELMÄ

Subhash Kumar Tripathi

Ihmisen auttaja-T-solujen erilaistumisen transkriptionaalinen ja epigeneettinen säätely

Lääketieteellinen mikrobiologia ja immunologia, Turun yliopisto
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Auttaja-T-solut erilaistuvat toiminnallisesti erilaisiksi Th1-, Th2- ja Th17-soluiksi. Erilaistumista ohjataan useiden, osittain päällekkäisten, verkostojen kautta, joihin liittyy mm. soluviestintää, transkription säätelyä ja epigeneettisiä mekanismeja. Vääränlainen erilaistumisprosessi ja auttaja-T-solujen epänormaali toiminta voivat johtaa immuunivälitteisten tautien kehittymiseen. Tämän tutkimuksen tavoitteena oli tunnistaa Th17-solujen erilaistumiseen vaikuttavia geenejä ja niiden ilmenemistä sääteleviä mekanismeja. Lisäksi tutkittiin mitkä epigeneettiset muutokset liittyvät Th1- ja Th2-solujen alkuvaiheen erilaistumiseen. Th17-solujen erilaistumisen käynnistymisvaiheessa tehdyn transkriptomianalyysin avulla tunnistimme useita tunnettuja, mutta myös täysin uusia geenejä, joiden ilmeneminen vaikuttaa Th17 solujen erilaistumiseen. Osaa näistä tutkittiin edelleen proteiinitasolla sen selvittämiseksi, miten spesifisiä ne ovat Th17-soluille verrattuna muihin auttaja-T-soluihin. Osoitimme, että transkriptiofaktori STAT3 on keskeinen Th17-solujen erilaistumiselle. Ymmärtääksemme molekyylitason mekanismeja Th17 solujen erilaistumisen aikana, määritimme nyt ensimmäistä kertaa STAT3:n ensimmäiset kohdegeenit ihmisen T-soluissa, joissa on juuri käynnistetty Th17-solujen erilaistuminen. Tätä varten hyödynsimme siRNA-välitteistä geeninhiljennystä, transkriptomianalyysiä ja ChIP-sekvensointia. Yhdistämällä aineistot laskennallisesti onnistuimme tunnistamaan STAT3:n suorat ja epäsuorat kohdegeenit ja havaitsimme, että STAT3 säätelee suoraan useita geenejä, joiden tiedetään osallistuvan Th17-solujen aktivaatioon, erilaistumiseen, jakautumiseen ja ylläpitoon. Tuloksiamme voidaan jatkossa hyödyntää selvitettäessä niitä monimutkaisia verkostoja, jotka säätelevät geenienekspressiota Th17-solujen erilaistumisen aikana. Määritimme myös genominlaajuisesti histonimodifikaatioita Th1- ja Th2-solujen erilaistumisen aikana ja tunnistimme useita Th1- ja Th2-spesifisiä geenienekspressiota vahvistavia alueita, eli ns. enhancer-alueita. Näiden alueiden tarkempi analyysi osoitti, että niissä on mahdollisia sitoutumispaikkoja useille transkriptiofaktoreille joiden tiedetään osallistuvan Th1- ja Th2-solujen erilaistumiseen, mutta myös sellaisille transkriptiofaktoreille, joiden ei ole aiemmin tiedetty osallistuvan erilaistumisprosessiin. Lisäksi havaitsimme, että näillä enhancer-alueilla esiintyy sellaista nukleotidipolymorfiaa, joiden on GWAS-analyyseissä osoitettu assosioituvan autoimmuunitautien kanssa. Tämä puolestaan viittaa siihen, että genomin näillä alueilla olisi merkitystä autoimmuunitautien syntymiselle. Tämä väitöskirjatutkimus on lisännyt tietoa auttaja-T-solujen erilaistumisesta ja tuonut esiin sellaisia mekanismeja, jotka voivat johtaa auttaja-T-solujen vääränlaiseen erilaistumiseen ja sitä kautta immuunivälitteisiin sairauksiin.

Avainsanat: T-auttajasolujen erilaistuminen, geenien säätely, transkriptiotekijä, epigeneettinen säätely, RNA-interferenssi, ChIPseq, STAT3, enhancer, genominlaajuinen assosiaatiokartoitus, yhden nukleotidin monimuotoisuus, immuunivälitteinen sairaus.

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ABBREVIATIONS

AHR, aryl hydrocarbon receptor
APC, antigen-presenting cell
AMD, age-related macular degeneration
AREDS, age-related eye disease study
AS, ankylosis spondylitis
BATF, basic leucine zipper transcription factor, ATF-like
BCL6, B-cell CLL/lymphoma 6
β-ME, β-mercaptoethanol
bp, base pair
BRG1,brahma related gene 1
BSA, bovine serum albumin
BTLA, B and T lymphocyte associated
CCR, C-C chemokine receptor
CD, crohn's disease
CD, cluster of differentiation
cDNA, complementary DNA
ChIP, chromatin immunoprecipitation
ChIP-seq, chromatin immune-precipitation followed by high throughput DNA sequencing
CIA, collagen-induced arthritis
CLPs, common lymphoid precursors
CNS, conserved non-coding sequences
CRTH2, chemoattractant receptor homologous molecule expressed on Th2 cells
CRMs,cis-regulatory modules
Ct, cycle threshold
DAPA, DNA affinity precipitation assay
DGE, digital gene expression
DHS, DNase1 hypersensitive sites
DNA, deoxyribonucleic acid
DTT, ditiotreitol
EAE, experimental autoimmune encephalomyelitis
EDTA, ethylenediaminetetraacetic acid
ENCODE, encyclopedia of DNA elements
eQTL, expression quantitative trait loci
ES, embryonic stem
FAM, carboxy-fluorescein
Fc, constant region of an antibody
FC, fold change
FCS, fetal calf serum
FDR, false discovery rate
FOXP3, forkhead box P3
FLT1, Fms-related tyrosine kinase 1
FSC, forward scatter

GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GATA3, GATA binding protein 3
GC, germinal centre
GFI1, growth factor independent 1 transcription repressor
GO, gene ontology
GM-CSF, granulocyte-macrophage colony-stimulating factor
GWAS, genome wide association studies
H, histone
HATs, histone acetyltransferases
HDACs, histone deacetylases
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIES, hyper-immunoglobulin E syndrome, Job's syndrome
HSc, hematopoietic stem
IBD, inflammatory bowel disease
ICOS, inducible T-cell co-stimulator
ID3, inhibitor of DNA binding 3
IFN, interferon
Ig, immunoglobulin
IL, interleukin
ILR, IL receptor
iPS, induced pluripotent stem
iTreg, inducible regulatory T cell
LCRs, locus control regions
LPS, lipopolysaccharide
lincRNA, long intergenic non-coding RNAs
MAF, v-maf musculoaponeurotic fibrosarcoma oncogene homolog
MAMPs, microbe-associated molecular patterns
me, methylation
MHC, major histocompatibility complex
MIP3α, Macrophage inflammatory protein 3α
miRNA, microRNA
mRNA, messenger RNA
MS, multiple sclerosis
ncRNAs, non-coding RNAs
NFR, nucleosome free region
NHGRI, National Human Genome Research Institute
NLRs, Nod like receptors
NK cell, natural killer cell
nTreg, natural regulatory T cell
PAGE, polyacrylamide gel electrophoresis
PALLD, Palladin
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PcG, polycomb group
PD-1, programmed cell death 1
PE, phycoerythrin

PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
PHLDA1, pleckstrin homology-like domain, family A, member 1
PRRs, pattern recognition receptors
PRKCQ, Protein kinase C, theta
PS, psoriasis
PU.1, spleen focus forming virus (SFFV) proviral integration oncogene spi1
PVDF, polyvinylidene difluoride
qPCR, quantitative PCR
RA, rheumatoid arthritis
RNA, ribonucleic acid
RNAi, RNA interference
RORC, RAR-related orphan receptor C
RPMI, roswell park memorial institute medium
RT, room temperature
RT-PCR, reverse transcriptase polymerase chain reaction
RUNX, runt-related transcription factor
SAP, SH2 domain containing 1A
SDS, sodium dodecyl sulphate
Seq, massively parallel sequencing
siRNA, small interfering RNA
SLE, systemic lupus erythematosus
SNP, single nucleotide polymorphism
STAT, signal transducer and activator of transcription
TAMRA, tetramethylrhodamine
TBX21, T-box 21, synonym T-bet
Tc, cytotoxic T cell
TCR, T cell receptor
T1D, type 1 diabetes
TFs, transcription factors
Tfh, T follicular helper
TFBS, transcription factor binding site
TGF, tumor growth factor
Thp, T helper precursor
Th , T helper cells
TLRs, toll like receptors
TNFα, tumor necrosis factor alpha
TRANSFAC, transcription factor database
TrxG, trithorax group
TSS, transcription starting sites
UC, ulcerative colitis
VEGFR, vascular endothelial growth factor receptor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by roman numerals (I-III).

I Soile Tuomela,*, Verna Salo*, **Subhash K. Tripathi**,*, Zhi Chen, Kirsti Laurila, Bhawna Gupta, Tarmo Äijö, Lotta Oikari, Brigitta Stockinger, Harri Lähdesmäki, and Riitta Lahesmaa. Identification of early gene expression changes during human Th17 cell differentiation. Blood 04/2012; 119 (23):e151-60. (* Equal contribution/ shared first authorship)

II **Subhash K. Tripathi***, Zhi Chen*, Tarmo Äijo, Antti Larjo, Isis Ricaño-Ponce, Verna Salonen, Soile Tuomela, Cisca Wijmenga, Harri Lähdesmäki, Riitta Lahesmaa. STAT3 mediated transcription regulation during early human Th17 cell differentiation. (Original Manuscript). (* Equal contribution/ shared first authorship)

III R. David Hawkins*[#], Antti Larjo*, **Subhash K. Tripathi***, Ulrich Wagner, Ying Luu, Tapio Lönnberg, Sunil K. Raghav, Leonard K. Lee, Riikka Lund, Bing Ren, Harri Lähdesmäki [#], and Riitta Lahesmaa[#]. Global Chromatin State Analysis Reveals Lineage-Specific Enhancers During the Initiation of Human Th1 and Th2 Polarization. Immunity. 2013 Jun 27; 38(6):1271-84. (* Equal contribution/ shared first authorship, [#] Corresponding authors)

INTRODUCTION

The immune system has evolved to defend our body from different intra and extracellular pathogenic organisms, such as bacteria, virus and fungi, as well as to eradicate faulty host cells. The hierarchy of distinct cell types performs the specific function either independently or synergistically to mount targeted response. The first line of defense is provided by innate immune cells, including monocytes, macrophages, dendritic cells, natural killer (NK) cells, neutrophils, basophils, eosinophils, and mast cells. These cells recognize specialized structures on foreign pathogens and transformed host cells and mount quick responses to eliminate them through phagocytosis or complement system. Though, innate immunity is not specific and long lasting, it activates the cells of adaptive immune system to mount specific and long lasting response. Thus, adaptive immunity provides specialized and long lasting immune responses that systematically eradicate the pathogenic or intracellular antigen. Lymphocytes are an integral component of the adaptive immune system and play a key role in the regulation of immune response. Lymphocytes are classified into two classes, antibody secreting B lymphocytes and T lymphocytes. T lymphocytes are categorized into two groups; CD4+ T helper (Th) cells and CD8+ cytotoxic killer T (Tc) cells. Depending on the nature of antigen signal, local cytokine milieu, TCR activation, and co-stimulatory signals, CD4+ Th precursor (Thp) cells differentiate into functionally different effector cells including Th1, Th2, and Th17 cells and regulatory T (Treg) cells. Each cell subset is characterized by the expression of key transcription factors (TFs) and secretion of signature cytokines. Controlled regulation of lineage specification and commitment during differentiation and development of these effector Th and Treg cell lineages is required for proper functioning and protection against pathogenic infections. However, inappropriate execution of lineage specification and commitment program during the activation and differentiation of Th cells can result in the pathogenesis of inflammatory autoimmune and allergic diseases.

Molecular basis for Th cell lineage specification and commitment relies on transcriptional and epigenetic mechanisms that modulate gene expression patterns to determine the fate of specific cell-type while opposing the fate of alternative subsets (Rothenberg, 2007). Thus, different Th cell subsets can be distinguished from each other based on their unique transcriptional and epigenetic profiles. During T cell development, TFs play a significant role in coordinating developmental events by priming the transcription of lineage specific regulatory genes which restrict the multi-lineage potential and drive the development potential towards specific T cell lineage fate (Evans and Jenner, 2013; Kanno et al., 2012; Zhou et al., 2009). However, it is becoming clear that integrated networks of regulatory TFs are needed to understand the complete differentiation program. In the past, efforts have been made to

understand the complete picture of cellular specification during differentiation of the specific phenotype using system-wide approaches to construct and decode the gene regulatory networks of TFs co-expressed within the cell (Ciofani et al., 2012; Novershtern et al., 2011). On the other hand, epigenetic factors mediate cellular specificity and plasticity. For example, Th1 and Th2 specific cytokine loci are marked with specific epigenetic states: *Ifng* and *Il18r1* loci marked with H3K4me3 in Th1 cells and H3K27me3 in Th2 cells (Hatton et al., 2006; Schoenborn et al., 2007; Wei et al., 2009). Likewise in Th2 cells, *Il4* and *Il13* loci are marked with distinct epigenetic modifications (Ansel et al., 2003, 2006). In past few years, the advent of new high throughput microarray and sequencing technologies and functional genomics approaches have opened the door to decode the TF mediated transcriptional regulatory networks and profile epigenetic modification throughout the genome during cellular differentiation and development program including T cells.

The objective of this thesis is to study the transcriptional and epigenetic regulation of human Th cell differentiation - with a special focus on transcriptional regulatory mechanisms responsible for Th17 cell differentiation and epigenetic regulation of human Th1/Th2 cell differentiation. The aim is to capture the early changes in gene expression profiles during human Th17 cell differentiation using Illumina Beadarrays (*Publication I*), to identify the immediate targets of the TF STAT3 by using RNA interference (RNAi) and gene expression profiling and chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) (*Publication II*). In addition, epigenetic changes in histone modification during the early stage of human Th1 and Th2 cell differentiation through ChIP-seq were investigated (*Publication III*).

REVIEW OF THE LITERATURE

2.1 General overview of the immune system

The immune system represents an integrated network of distinct cells, tissues, and organs that function to defend the host by mounting an immune response against invading pathogens, such as bacteria, virus, parasites, and fungi as well as its role in elimination of cancer cells. An immune system works on the basis of two principles, firstly recognition of a microbial pathogen or foreign substances, and secondly by mounting a response to kill and eliminate the invading pathogen. Recognition is a vital feature of a healthy immune system which must discriminate between 'self' and 'nonself'. Normally the immune system lives in serenely with cells harboring distinctive "self" marker molecules, and when it encounters foreign cells or pathogens, which display 'nonself' marker molecules, it quickly launches an immune response to destroy and neutralize them. However, under abnormal circumstances, the immune system can commit an error in recognition, and mount an immune attack against the body's own cells or tissues, resulting in autoimmune disease such as rheumatoid arthritis (RA), multiple sclerosis (MS), and type 1 diabetes (T1D). In the other situation, the immune system responds to innocuous substances, resulting in an inflammatory condition called allergy. This kind of innocuous substance is called an allergen (Alberts et al., 2002; Janeway, 1989; Kindt et al., 2006). The immune system is composed of two parts: the innate immune system, which accounts for nonspecific part, and the adaptive immune system which accounts for the specific component.

Innate (nonspecific) immune system

Innate immune system exerts the first line of immune protection against pathogenic infections. Upon encounter with a pathogen, the innate immune system launches a quick non-specific response to destroy the pathogen. The innate immune system operates its defense mechanisms through four types of defensive barriers. The first, anatomic barrier against invading pathogen uses the skin or surface of the mucus membrane to kill them. The second is a physiologic barrier, which includes temperature, pH (gastric acid), and various soluble factors (lysozyme, interferons, and complement proteins). Third, the phagocytic barrier ingests extracellular particulate material, or sometimes whole microorganisms, which is further destroyed by a mechanism called phagocytosis (a form of endocytosis). The fourth barrier is the inflammatory barrier, which involves induction of the complex sequence of events upon tissue damage due to a wound or by an infectious pathogen known as inflammatory response. The specialized cells involved in innate protection against invading pathogens are basophils, mast cells, eosinophils, dendritic cells, neutrophils, blood monocytes, macrophages and NK cells. These innate immune cells

nonspecifically recognize foreign molecules associated with groups of microbes know as microbe-associated molecular patterns (MAMPs), which include bacterial lipopolysaccharides (LPS), unmethylated CpG motifs, fungal chitins, and other ligands. These cells crosslink these MAMPs through MAMP receptors, such as Toll like receptors (TLRs) and other pattern recognition receptors (PRRs), for example, Nod like receptors (NLRs) and RIG-like helicases (RLHs) expressed by these cells and initiate an innate immune response against microbial pathogens. Basophils, eosinophils and mast cells are activated by crosslinking with antibodies or complement proteins to destroy microbial pathogens through release of antimicrobial compounds upon degranulation. If a pathogen evades innate immune responses, innate immune cells such as macrophage and dendritic cells function as antigen presenting cells (APCs) that present the antigen to the cells of the adaptive immune system to attain the next level of immune protection. Thus, innate and adaptive immunity work cooperatively in many ways to mount a more effective immune response.

Adaptive (specific) immune system

The adaptive immune system provides specific immunity with specialized cells that selectively mount an immune response for a specific foreign pathogen. Unlike innate immunity, adaptive immunity takes several days to mount a specific immune response for the pathogen. The adaptive immune system displays four characteristic features while mounting an immune response for a specific antigen: First, antigen specificity, provided by antibodies and T cells which discriminate subtle differences among various antigens. Secondly, the adaptive immune system exhibits a remarkable diversity in its recognition molecules, which helps in recognizing an enormous number of uniquely different structures on foreign microorganisms and molecules (foreign antigens). Thirdly, and an important feature of adaptive immune system is immunologic memory for a specific antigen, i.e. a second encounter with the same antigen induces a quick and a high level of the immune response. Finally, the adaptive immune system has the capability of self/nonself recognition during antigen presentation, i.e. it mounts an immune reaction only for the foreign antigen.

Lymphocytes are the components of an adaptive immune response. They mediate two broad types of response—humoral (antibody) and cell-mediated immune responses. These responses are carried out by two major types of lymphocytes, B cells and T cells, respectively. These cells possess antigen receptors with high specificity for processed antigens to mediate immune response. Upon antigenic challenge, lymphocytes are activated, differentiated and clonally amplified into functionally mature cells to produce pathogen specific antibodies or cytokines, in a sequence of events that take several days. In humoral responses, B cells mature in the bone marrow and express membrane bound antibodies (a class of proteins called immunoglobulins) on their surface. Upon their first encounter with a foreign antigen,

such as viruses and microbial toxins, naive B cells undergo clonal expansion and differentiate into antibody secreting effector B cells or plasma cells. Secreted antibodies neutralize antigen by blocking their ability to bind to receptors on host cells, or destroy by ingesting them via phagocytosis. Humoral immunity is especially good at processing and eliminating extracellular microbes.

Cell-mediated immune responses are mediated mainly by T lymphocytes and their effector lineages. T cell precursors are produced in the bone marrow and travel to the thymus for full maturation. During maturation, T cells start to express an antigen specific T cell receptor (TCR) on the cell membrane. These TCRs can only recognize an antigen that is presented by major histocompatibility complex (MHC) molecules, a type of polymorphic glycoproteins expressed on the cell membrane of APCs such as dendritic cells or B-cells. APCs express two major types of MHC molecules; MHC class I (MHC I) and MHC Class II (MHC II) molecules. T cells can be classified into two subpopulations depending on the class of MHC molecule presenting the antigen and type of the CD (cluster of differentiation) proteins on the surface; First: T cells displaying CD4 only recognize antigens bound to MHC II molecules on APCs, are known as CD4+ Th cells, and second: T cells that express CD8 only recognize antigens combined with MHC I molecules on APCs, are called cytotoxic CD8+ T cells (Tc). Upon antigen presentation by their respective MHC molecules, naive CD4+ Th cells and Tc cells have the ability to proliferate and differentiate into functionally distinct memory and effector T cells which secrete various growth factors known as cytokines or are involved in cell-mediated killing, respectively. Cytokines play a key role in the activation of other cells that are involved in immune response. However, inappropriate activation and regulation of Th and Tc cells may result in inflammatory and autoimmune diseases (Alberts et al., 2002; Janeway et al., 2001; Kindt et al., 2006)

2.2 CD4+ T- helper lineages in the immune system

CD4+ T cells are an integral component of adaptive immune responses. Upon antigen stimulation signals received from APCs, naïve CD4+ cells differentiate into functionally distinct effector Th and Treg cells (Abbas et al., 1996; Bettelli et al., 2007; Coffman, 2006; Coffman and Mosmann, 1991; Mosmann et al., 1986). Although these defend the body from various infections, they are also involved in the pathogenesis of various inflammatory and autoimmune diseases (Bettelli et al., 2007; Nicholson and Kuchroo, 1996; Umetsu and DeKruyff, 1997; Blom et al., 2011; Chang et al., 2010; Perumal and Kaplan, 2011; Staudt et al., 2010; Veldhoen et al., 2008) The initial dogma of the involvement of CD4+ Th cells in the protection against pathogens was limited to the Th1 and Th2 cell subsets (Amsen et al., 2007; Coffman, 2006; Liew, 2002; Romagnani et al., 1997). However, during the past decade, studies have identified and characterized new subsets of CD4+ cells, which include Th17, Treg, follicular helper T cell (Tfh), Th22 and Th9 (Bettelli et al., 2006, 2007; Crotty, 2011; Eyerich et al., 2009;

Fontenot et al., 2003; Fujita et al., 2009; Gavin and Rudensky, 2003; Kaplan, 2013; Korn et al., 2009; Perumal and Kaplan, 2011; Soroosh and Doherty, 2009; Stassen et al., 2012; Trifari and Spits, 2010; Vinuesa and Cyster, 2011) (*Figure 1*). These functionally distinct Th cell subsets are characterized by the expression of lineage specific transcriptional regulators, cell surface chemokine receptors and secretion of key cytokines (*Table 1*). These subset specific transcriptional regulators and cytokines induce several upstream signaling pathways and downstream transcriptional and epigenetic regulatory mechanisms to initiate and amplify Th cell differentiation and oppose the alternative fates (Ansel et al., 2003; Chen et al., 2003; Evans and Jenner, 2013; Gavin et al., 2007; Goswami et al., 2012; Hirahara et al., 2011; Jenner et al., 2009; Kanhere et al., 2012; Liu et al., 2013; Lund et al., 2003, 2004, 2007, 2005; Placek et al., 2009; Wang et al., 2008; Zhu, 2010). Th1 cells express STAT4 and TBX21 (T-bet) as their key TFs, that promote the secretion of proinflammatory cytokines, such as IFNγ, IL18, Tumor necrosis factor beta (TNFβ), and proliferation factor (growth factor) IL2 (Mosmann et al., 1986)(Gökmen et al., 2013; Good et al., 2009; Liberman et al., 2003; Placek et al., 2009). Th2 cells express the TFs STAT6 and GATA3, which induce the transcription of *Il4, Il5, Il13* and *Il25* (Bettelli et al., 2006; Elo et al., 2010; Jenner et al., 2009; Liberman et al., 2003; Shulman et al., 2013; Wei et al., 2009; Yagi et al., 2011; Zhu, 2010). Th17 cells express TFs, such as STAT3, BATF, RORA and RORC, and secrete IL17A, IL17F, IL21, IL22, and IL9 cytokines (Annunziato et al., 2013; Durant et al., 2010; Ivanov et al., 2006; Schraml et al., 2009). Tfh cells express BCL6 and STAT3, which increase the transcription of *Il21* and *Cxcr5* and secretion of TGF-β (Baumjohann et al., 2011; Johnston et al., 2009; Kroenke et al., 2012; Liu et al., 2013; Ma et al., 2012; Nurieva et al., 2008). Th9 and Th22 cells are named after cytokines they secrete, i.e. IL9 and IL22, respectively, although they as of yet are relatively poorly characterized (Blom et al., 2011; Chang et al., 2010; Perumal and Kaplan, 2011; Staudt et al., 2010; Veldhoen et al., 2008). Overall, these Th subsets serve specific immune functions in response to the wide range of pathogens.

Cytokines secreted by Th1 cells contribute to adaptive immunity against intracellular pathogens, for example, *Mycobacterium tuberculosis, Listeria monocytogenes*, and *Leishmania major*, through cell mediated killing or inducing macrophages to digest them (Adorini, 1999; Curtis et al., 2010; Harris et al., 2009; Hovav et al., 2003; Jankovic et al., 2007; Quiroga et al., 2004). Th2 cells mediate humoral responses and are involved in the destruction of extracellular pathogens such as helminths. Th2 secreted cytokines, such as IL4 and IL13, help in enhancing B cell proliferation and function. IL5 binds to its receptor on eosinophils and basophils and control their growth, differentiation and function, which in turn produce inflammatory mediators, such as histamine and leukotrienes and promote proliferation and differentiation of B cells (Bosnjak et al., 2011; Caraballo and Zakzuk, 2012; Fallon and Mangan, 2007; Georas et al., 2005; Kotsimbos and Hamid, 1997; Till et al., 1997; Viola et al., 1998). Th17 cells participate in the protection against extracellular bacterial and fungal

pathogens (Curtis and Way, 2009; Curtis et al., 2010; Ivanov et al., 2009; Leppkes et al., 2009; Lin et al., 2009; van de Veerdonk et al., 2009).

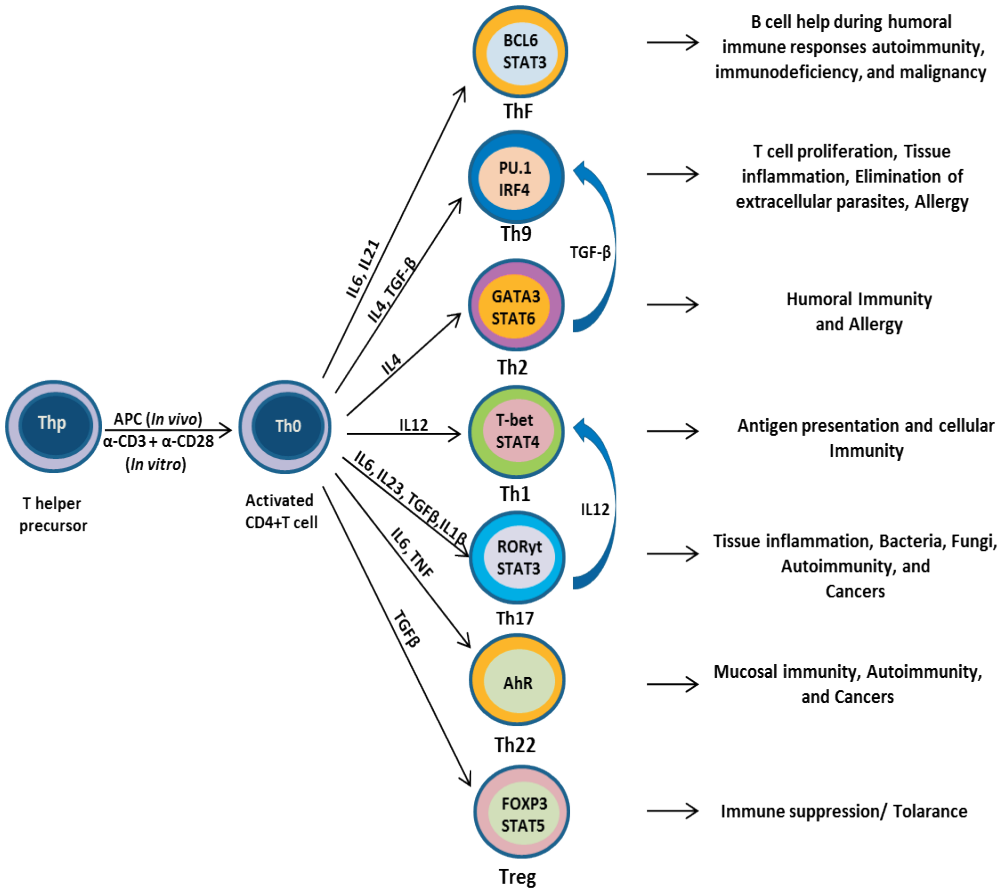

Figure 1: Schematic overview of the differentiation of naive CD4+ T helper cells into distinct effector and regulatory subsets and their immune function.

Controlled regulation of the molecular events occurring during differentiation of the Th cell subtypes is required for immune defense, and inappropriate execution of these mechanisms can result in various inflammatory and autoimmune diseases. For example, increased Th1 and Th17 cell responses are associated with various organ specific autoimmune diseases, such as T1D, RA, MS, crohn's disease (CD) (Damsker et al., 2010; Duhen et al., 2013; El-behi et al., 2010; Hemdan et al., 2010; Jäger et al., 2009; Kleinewietfeld and Hafler, 2013; Kleinewietfeld et al., 2013; Marwaha et al., 2012; Zielinski et al., 2012). However, enhanced Th2 responses result in the pathogenesis of allergic reactions, such as asthma (Bosnjak et al., 2011; Caraballo and Zakzuk, 2012; Fallon and Mangan, 2007; Georas et al., 2005; Kotsimbos and Hamid, 1997).

Table 1: Functional and regulatory characteristics of regulatory and effector CD4+ T helper cell subsets.

Cell Type Features	Th1	Th2	Th17	Treg	Tfh	Th9	Th22
Polarizing Cytokines	IFNγ,IL12, IL27	IL4	IL6, TGFβ, IL23, IL21, IL1-β	TGFβ	IL6, IL21, CXCL13	TGFβ, IL4	IFNα, IL6
Surfce Cytokine Expression	IL12RB2, IFNγR, TIM3	IL4R, CRTH2, TIM1	IL23R1, IL12RB2, IL1-R1, CCR6 (h), CD161(h)	CD25, CD39,CD73, CD101, CD127, FR4(m), GITR/AITR	CD84, CXCR5, IL6R, IL21R, GP130	---	PDGFR, CCR10
Unique Cytokine Secretion	IFNγ	IL4, IL5, IL13	IL17A,L17F,IL21, IL22	TGFβ	IL6	IL9	IL22, TNFα
Lineage-Specific Transcriptional Regulators	T-bet	GATA3	RORA, RORγt	FOXP3	BCL6	PU.1	AhR
STAT Regulators	STAT1, STAT4	STAT6	STAT3	STAT5	STAT3	STAT6	--
Other Transcription	JUN, ATF3	GFI1, SATB1	BATF, IRF4, AHR, IKZF3, FOSL2	-	c-MAF, BATF	IRF4	-

Th1 cells

Th1 cells are important for both the eradication of intracellular pathogens and for the pathogenesis of many autoimmune diseases. Th1 differentiation is initiated by interleukin-12 (IL12), which activates STAT4 (Signal Transducer and Activator of Transcription 4). Activated STAT4 subsequently translocates to the nucleus to bind regulatory regions of genes and induce their transcription, including the key Th1 cytokine *Ifnγ*. IL12 executes its signals through a receptor complex composed of two subunits, IL12Rβ1 and IL12Rβ2, the expression of which are induced upon TCR activation (Berenson et al., 2006; Germann et al., 1993; Schulz et al., 2009; Ylikoski et al., 2005). Th1 cell differentiation is further promoted by IFNγ, which activates the phosphorylation of STAT1. Upon activation STAT1 further induces the transcription of Th1 specific genes, such as *T-bet*. T-bet is a key TF for the regulation of Th1 differentiation and induces the expression of *Ifnγ* in a positive feedback loop mechanism (Djuretic et al., 2007; Lund et al., 2005, 2003, 2004, 2007). Additionally, Th1 cells express CXCR3 chemokine receptor (Langenkamp et al., 2003; Sallusto et

al., 1998; Yamamoto et al., 2000; Zhang et al., 2000), which has an affinity for three distinct interferon inducible proteins, CXCL9, CXCL10 and CXCL11, and guides the migration of Th1 cells to sites of inflammation that may arise due to wound or infection (Groom and Luster, 2011a, 2011b; Groom et al., 2012).

Th2 cells

Th2 cells play an important role in immune protection against extracellular parasites and critical for the induction and the development of several allergic states including asthma. IL4 initiates Th2 differentiation from naive CD4+ T cells and activates the phosphorylation of STAT6. Upon phosphorylation, STAT6 translocates to the nucleus and induces the transcription of Th2 lineage specific TF *Gata3* and multiple cytokines, including *Il4, Il5, Il13* and *Il25* (Chen et al., 2003; Jankovic et al., 2007; Kaplan et al., 1996, 1996; Lund et al., 2005, 2003, 2004, 2007; Shimoda et al., 1996; Takeda et al., 1996). These cytokines serve pleiotropic functions in specific immune responses. IL4 is the most important cytokine involved in allergic inflammation as it controls the immunoglobulin (Ig) class switching to IgE and secretion by B cells, as well as inducing the expression of FcεRI (low affinity IgE receptor) on the surface of B cells and mononuclear phagocytic cells. IL4 induces the expression of FcεRII (high-affinity IgE receptor) on mast cells and other cells and initiates their subsequent degranulation and secretion of various inflammatory mediators involved in the elimination of parasites (Bosnjak et al., 2011; Caraballo and Zakzuk, 2012; Fallon and Mangan, 2007; Georas et al., 2005; Kotsimbos and Hamid, 1997; Till et al., 1997; Viola et al., 1998). IL5 mainly acts on eosinophils through IL5R and induces their proliferation, survival, activation and differentiation. IL5 binds to IL5R on the surface of eosinophils which further leads to upregulation of *Cd11b* and blocking of apoptosis signals (Kotsimbos and Hamid, 1997). IL13 plays a significant role in the gastrointestinal infections due to parasites, such as helminths, and participates in the induction of asthma through increased mucosal secretion, as well as hypersensitivity. IL13 is also involved in the stimulation of tissue fibrosis at the site of inflammation (Till et al., 1997). IL25 promotes Th2 responses by upregulating the production of IL4, IL5 and IL13. It regulates key functions including IgE switching, enhanced Ig secretion, eosinophilia and production of mucus (Saenz et al., 2010; Wang et al., 2007). Th2 cells also express C-C type chemokine receptors on their surface, such as CCR4 and CRTH2 expressed on Th2 cells (Pappas et al., 2006; Sallusto et al., 1998). CCR4 binds its ligands, such as CCL17 and CCL22. These ligands are induced during an allergic response on the airway epithelial cells (Mariani et al., 2004; Perros et al., 2009; Sallusto et al., 1998). CRTH2 specifically crosslinks prostaglandin D2, which is produced by IgE activated mast cells, and stimulates cytokine production as well as proliferation and survival of Th2 cells during allergic inflammation (Cosmi et al., 2000; Gyles et al., 2006; Iwasaki et al., 2002; Pérez-Novo et al., 2010).

Th17 cells

Th17 cells play a key role in mounting host immune responses against extracellular bacteria and fungi, as well as in mediating tissue inflammation and autoimmune diseases (Damsker et al., 2010; Duhen et al., 2013; El-behi et al., 2010; Hemdan et al., 2010; Jäger et al., 2009; Kleinewietfeld et al., 2013; Marwaha et al., 2012; Zielinski et al., 2012). Defects in Th17 cell development are associated with hyper-immunoglobulin E syndrome (HIES, Job's syndrome), a group of immune disorders that lead to increased susceptibility to staphylococcal and candida infections (Ma et al., 2008; Milner et al., 2008, 2010). Th17 cells are named after their associated cytokine family - IL17 (IL17A and IL17F). Other Th17 specific cytokines include IL21, IL22, IL26, GM-CSF, MIP3α, and TNFα. IL17A and IL17F share a common receptor, IL17RA, which is expressed in multiple tissues, including joints, skin, lung, and intestine, where they induce proinflammatory cytokines, such as IL6, IL1, and TNFα. IL21 is a cytokine of pleiotropic functions, including amplification of Th17 development, activation of T cells and NK cells, and stimulation of B cell differentiation into long lived plasma cells and memory B cells (Annunziato et al., 2007; Ge et al., 2013; Graeber and Olsen, 2012; Ma et al., 2008). IL22 and IL26 are members of the IL10 family, which play a significant role in mucosal immunity, tissue repair and are overexpressed in chronic inflammatory diseases, for example psoriasis (PS), RA and CD (Corvaisier et al., 2012; Dambacher et al., 2009; Prans et al., 2013; Xu et al., 2013a). Th17 cells express C-C type chemokine receptors on their surface, such as CCR4 and CCR6 (Ge et al., 2013; Oo et al., 2012; Singh et al., 2008; Wang et al., 2009a). CCL20 is produced at mucosal surfaces and by several other types of immune cells (Baba et al., 1997; Hirata et al., 2010; Hirota et al., 2007), and regulates the migration and recruitment of CCR6 expressing Th17 cell to the mucosal associated lymphoid tissues (MALT) in the intestine, such as Peyer's patches (Annunziato et al., 2007; Hirota et al., 2007; Wang et al., 2009a). Additionally, they express IL23 receptor (IL23R) and CD161 (Cosmi et al., 2011; Kleinewietfeld et al., 2013; Miao et al., 2013a). However, the expression and role of CD161 in Th17 cells is disputed. Based on a recent study, CD161 is also expressed in a sub-population of human Treg cells that produces IL17 in STAT3-dependent manner when activated with IL1β only (Afzali et al., 2013; Pesenacker et al., 2013).

Th17 cell differentiation is initiated by a cocktail of cytokines, TGFβ1, IL6 and/or IL1β, which induce the expression of key TFs, such as *Stat3, Irf4, Batf*, and *Rorγt*, which are essential for the development of these cells (Annunziato et al., 2013; Ciofani et al., 2012; Durant et al., 2010; Ivanov et al., 2006; Schraml et al., 2009; Tuomela et al., 2012; Yang et al., 2008). Although this cytokine cocktail can produce Th17 cells, the addition of IL23 is needed for the stabilization of these cells, as well as their capacity to mediate autoimmune tissue inflammation in model of autoimmunity, experimental autoimmune encephalomyelitis (EAE) (Caruso et al., 2008; Maloy and

Kullberg, 2008). In developing Th17 cells, IL23 mediates these functions through binding to its receptor (IL23R), and blocks the expression of IL10, an anti-inflammatory cytokine, and make these cells pathogenic (Ghoreschi et al., 2010; Haines et al., 2013; Kobayashi et al., 2008; Lee et al., 2012; Morrison et al., 2011). Several SNPs have been associated with the IL23R, which have been linked to various autoimmune diseases, such as inflammatory bowel disease (IBD), PS, CD, MS, ulcerative colitis (UC), systemic lupus erythematosus (SLE) and ankylosis spondylitis (AS) (Davidson et al., 2013; Hazlett et al., 2012; Nair et al., 2009; Núñez et al., 2008; Pidasheva et al., 2011; Sánchez et al., 2007; Yu et al., 2012; Zhai et al., 2012). Although, IL23 signaling is associated with the pathogenic nature of Th17 cells in EAE model of autoimmunity as well as linked to various autoimmune diseases based on genome wide association studies (GWAS), It is still unclear whether IL23 signaling alone, or IL23 dependent cytokines and effector molecules make Th17 cells pathogenic in several autoimmune diseases. Studies have shown that GM-CSF is needed to make Th17 cells pathogenic, based on observations that GM-CSF-deficient mice were unable to induce EAE (Codarri et al., 2011; El-behi et al., 2010; McGeachy, 2011; Sonderegger et al., 2008). Additionally, recent studies showed that T-bet is crucial for the pathogenic property of Th17 cells. In developing Th17 cells, T-bet transactivates the expression of TGFβ3, which further induces the expression of both *T-bet* and *Il23r* (Gocke et al., 2007). *T-bet*−/− mice failed to induce EAE and other autoimmune diseases, suggesting a role of T-bet in the induction of pathogenic Th17 cells. However, a recent study presented evidence that appears to nullify the role of T-bet in pathogenic Th17 cells (Duhen et al., 2013). Interestingly, the role of salt concentration in modulating the generation and function of pathogenic Th17 cells and autoimmune response both in human and mouse has been discussed (Kleinewietfeld and Hafler, 2013; Wu et al., 2013). Apart from their role in autoimmune diseases, Th17 cells play a role in various cancers, including lymphoma, myeloma, colon cancer, gastric cancer, hepatocellular cancer, pancreatic cancer, prostate cancer, breast cancer, and ovarian cancer (Chugh et al., 2013; Galande et al., 2011; Greten et al., 2012; Kim et al., 2013; Prabhala et al., 2010; Qian et al., 2013; Su et al., 2010; Ye et al., 2013). It has also been suggested that Th17 are induced by stromal cells during *H. pylori* infections and in the gastric tumor microenvironment (Pinchuk et al., 2013; Su et al., 2010). Additionally, Th17 cells may promote tumor angiogenesis via activation of oncogenic STAT3 signaling and by inducing the expression of angiogenic factors, such as *Vegf*, *Peg2*, and various cytokines (Gu et al., 2011; Jiang et al., 2013). Th17 cells have stem cell-like properties and provide long term immunity (Muranski et al., 2011; Wei et al., 2012). However, despite the significant efforts made in describing involvement of Th17 cells in various cancers, the complete understanding of molecular mechanisms defining functional role of Th17 cells in tumor immunity are poorly understood.

Th9 cells

Nearly 20 years ago, a study suggested the emergence of an IL2 dependent IL9 producing CD4+ T cell population that was enhanced by cytokine cocktail of TGFβ and IL4 (Houssiau et al., 1992; Stassen et al., 2012). This finding was further confirmed by two major studies, after which these IL9 producing populations were named accordingly as Th9 cells (Dardalhon et al., 2008; Veldhoen et al., 2008). Th9 differentiation is induced by the cytokine cocktail of TGFβ and IL4 combined with activation of naïve CD4+ T cells either with a specific antigen or anti-CD3/CD28 antibodies, and induce the transcription of *Pu.1* (purine-rich box 1) and *Irf4 (*Interferon Regulatory Factor 4), which regulate the transcription of *Il9* gene (Kaplan, 2013; Perumal and Kaplan, 2011). Th9 cells express unique cytokine receptors, such as CXCR3, CCR3 and CCR6, which mediate the recruitment of Th9 cells to inflammatory sites (Kara et al., 2013). Functionally, Th9 cells are neither anergic nor suppressive, but can enhance T cell proliferation, tissue inflammation and, together with Th2 cells, take part in the eradication of extracellular parasites (Dardalhon et al., 2008; Lu et al., 2012; Veldhoen et al., 2008). Studies have linked IL9 production by Th9 cells with allergic diseases such as asthma and atopy (Soroosh and Doherty, 2009). Although IL9 is produced under specific conditions by other Th subtypes, including Th2, Th17 and induced Treg (iTreg), Th9 cells are a unique Th cell subset. For example, Th9 cells do not express TFs such as T-bet, Gata3, Rorγt, and Foxp3, which are the key transcription regulators of Th1, Th2, Th17, and iTreg cells. However, it has been shown that Th9 cells are progeny of Th2 cells, based on expression of *Il9* and *Il4* at the early stage of Th2 cell differentiation (Goswami et al., 2012; Veldhoen et al., 2008). Additionally, Th9 cells under TGFβ and IL4 culture conditions also co-express *Il10* gene, another cytokine produced by Th2 cells (Veldhoen et al., 2008). Other reports have shown that Th9 cells originate from Th2 cells as a result of induction of other TFs, such as PU.1 and IRF4, which silence *Il4* and activate *Il9* gene transcription (Chang et al., 2010; Staudt et al., 2010). This suggests that Th2 cells act as an intermediate for Th9 cells. However, another study has reported a novel pathway of Th9 stimulation, which occurs directly from naive CD4+ T cells, in the presence of a high concentration of TGFβ and IL4, only upon engagement with OX40 and upon induction of the non-canonical NF-KB (RelB– p52) pathway (Tamiya et al., 2013; Xiao et al., 2012). Furthermore, in some models for Th9 generation, IL1, IL25, IL21 or IL33 favor the induction of Th9 cells (Angkasekwinai et al., 2010; Blom et al., 2011; Wong et al., 2010). As a final point to note, Th9 cells are less well studied than other Th subtypes. However, there is still scope for further investigation targeting several questions, such as molecular mechanisms involved during the course of Th9 cell generation, their connections with other Th cell subsets, and their significance in immune defense and in pathology of several immune mediated diseases.

Th22 cells

The Th22 cell subset is also a relatively new CD4+ Th cell subtype, characterized by the secretion of IL22 (Duhen et al., 2013; Trifari and Spits, 2010). IL22 belongs to the family of IL10 cytokines, including IL19, IL20, IL24, IL26, IL28 and IL29 (Wolk et al., 2010). Th22 cell differentiation can be induced by IL6 and TNF (Duhen et al., 2013, 2009). Th22 cells are different from Th1 and Th17 cells as they do not secrete IFNγ and IL17, respectively. Th22 cells also express C-C type chemokine receptors, such as CCR6, CCR4, CCR10, and aryl hydrocarbon receptor (AhR) is the key Th22 specific TF (Duhen et al., 2009; Eyerich et al., 2009; Zhang et al., 2011). Th22 cells have been described to mediate epithelial innate immune responses, as well as to participate in the pathogenesis of inflammatory skin diseases, such as atopic eczema, PS and allergic contact dermatitis. (Boniface et al., 2007; Eyerich et al., 2009; Fujita et al., 2009; Nograles et al., 2009; Zhang et al., 2011). Recently, Th22 cells have been shown to provide mucosal immunity against enteropathogenic bacteria, as well as having a role in HIV-associated mucosal immunopathogenesis (Basu et al., 2012; Kim et al., 2012). Th22 cells have also been suggested to participate in the pathogenesis of autoimmune diseases, including RA, AS, T1D, SLE and MS (Kagami et al., 2010; Qin et al., 2011; Xu et al., 2013a, 2013a; Zhang et al., 2013b; Zhao et al., 2013). Th22 cells along with Th17 cells are associated to be involved in cancers (Tian et al., 2013).

Tfh cells

Tfh cells are recently defined specialized subsets of CD4+ Th cell that selectively provide help to B cells during humoral immune responses. They express molecules which induce the activation and differentiation of B cells into immunoglobulin (Ig) secreting cells, as well as generating immunological memory (Crotty, 2011; Deenick et al., 2011; Perreau et al., 2013). Tfh cell differentiation is initiated by a cytokine cocktail of IL6 and IL21, and are characterized by the expression of BCL6 as their key TF, surface molecules, such as CD40L, CXCR5, SAP, BTLA, ICOS, AND PD-1, and secrete cytokines such as IL21, IL6, IL10. Upon interaction with a ligand, surface receptors including CXCR5, SAP, and BTLA, facilitate Tfh cell trafficking to the germinal center (GC) of B cell zone (follicles) of the secondary lymphoid organs, such as the lymph node and tonsils (Nurieva et al., 2008). Other important surface molecules, such as CD40L, ICOS, and PD-1 are required for direct cell-cell contact during T cell help to B cells (Tellier and Nutt, 2013). The precise control of Tfh cell generation and function is vital for providing immunity and health. Inappropriate development and function of Tfh cells can be associated with several immunopathologies, such as autoimmunity, immunodeficiency, and malignancy (Deenick et al., 2011; Gómez-Martín et al., 2011; Hu et al., 2012; Zhang et al., 2013b). Overall, a complete understanding of differentiation and the regulation of Tfh cells is of central importance for rational design of improved vaccine development strategies.

Treg cells

Treg cells are critical in the establishment and maintenance of peripheral tolerance through limiting the effector T cell responses (Gavin and Rudensky, 2003; Lehtimäki and Lahesmaa, 2013; Ohkura et al., 2013). Treg cells express key TF FOXP3 and secrete immune-regulatory cytokines, such as IL10 and TGFβ (*Table 1*) (Goodman et al., 2012). There are two major groups of Treg cells; i.e. thymus derived natural Treg (nTreg) and extrathymically derived adaptive or iTreg (Abbas et al., 2013). nTreg cells are developed in the thymus and express IL2rα chain (CD25) and FOXP3, which is critical for their development and immunosuppressive activity (Fontenot et al., 2003; Hori et al., 2003; Williams and Rudensky, 2007). iTreg cells are induced in the periphery from mature conventional CD4+ T cells upon antigenic stimulation under tolerogenic conditions and mount an antigen-specific immunosuppressive response (Haribhai et al., 2011). Thus, both of these cells have an important contribution to the maintenance of immunological homeostasis (Bilate and Lafaille, 2012). Several studies in the mouse have demonstrated that dysregulation of Treg development and function can lead to autoimmunity and cancer, and presumably similar effect can be possible in humans (Itoh et al., 1999; Miyara et al., 2011; Sakaguchi et al., 2001). High proportions of FOXP3+ Treg cells are associated with tumors in several animal tumor models and human cancer (Facciabene et al., 2012; Nishikawa and Sakaguchi, 2010; Quezada et al., 2011; Wilke et al., 2010). Treg cells for example, promote tumorigenesis by turning down the antitumor immune response, as indicated when the dysregulation of Treg cell function resulted in enhanced survival and diminished metastases (Onizuka et al., 1999; Shimizu et al., 1999). Furthermore, iTreg cells have been induced in various models of inflammatory diseases, including autoimmune and allergic diseases, such as arthritis, colitis, diabetes, EAE, and asthma (Piccirillo, 2008; Yadav et al., 2013). iTreg cells stimulate immune tolerance by controlling damage caused by inflammatory action of Th1, Th17, and Th2 effector cells.

2.3 Regulation of gene expression during T lymphocyte lineage specification

A short overview of genetic and epigenetic basis of gene regulation

In higher eukaryotic organisms, lineage specification and commitment during development and cellular differentiation is governed by a network of regulatory mechanisms that drive temporal and cell-type-specific gene expression changes and determine diverse cellular fates from a single common genome (Berger, 2007; Farkas et al., 2000). The eukaryotic genome, such as in human and mouse, is very large and hierarchically packaged into a chromatin structure to fit compactly within a cell nucleus. The nucleosome is the basic repeating structural unit of chromatin, composed

of 146 base pairs (bp) of DNA super helix wrapped around a core of the histone octamer with two units of each H2A, H2B, H3, and H4 proteins. H1 histone protein interconnects two nucleosomes by linker DNA. This wrapping structure can be described to resemble "beads on a string" and forms the template for the higher order of packing into dense chromatin fibers (Luger et al., 1997; Richmond and Davey, 2003; Robinson and Rhodes, 2006; Robinson et al., 2006). The higher order chromatin structure which limits the accessibility of trans-acting elements (such RNA polymerases and other regulatory proteins, e.g. TFs) to cis-acting DNA elements is transcriptionally silent (Farkas et al., 2000; Francis et al., 2004; Mohd-Sarip and Verrijzer, 2004; Narlikar et al., 2002). However, it can undergo several structural rearrangements, which allows the nucleosome to restructure and reposition (nucleosome positioning), and the removal of histone octamers (by ATP), exposes DNA sites to transcription regulatory factors, such as TFs, co-activators and basal transcription machinery, for the regulation of gene transcription (Dilworth et al., 2000; Narlikar et al., 2002, 2013; Ringrose and Paro, 2004). Thus, chromatin directs gene regulation programs through distinct transcriptional and epigenetic mechanisms (Cantone and Fisher, 2013).

Gene regulatory programs are governed by the network of TFs and epigenetic mechanisms that mediate specific changes in the gene expression. TFs represent a class of regulatory sequence specific DNA binding proteins that bind to cis-regulatory elements in the genome, such as promoters, enhancers, insulators, and silencers to regulate the expression of target gene(s) (Farkas et al., 2000; Ng et al., 2008; Schimmang, 2013). Apart from specifically recognizing and preferentially binding to the DNA strand through the DNA binding domain, TFs contain other functional domains, such as the trans-activation domain (protein binding domain), which enable them to recruit other regulatory factors, chromatin-remodeling complexes and histone-modifying enzymes to these cis-regulatory sequences to create the chromatin landscape. TFs preferentially bind to their response elements located within nucleosome depleted regions (Bai and Morozov, 2010; Guenther, 2011). Hence, TFs specifically bind to open chromatin structures based on the enrichment of the DNase1 hypersensitive sites (DHS) on these nucleosome depleted regulatory DNA elements.

The advancement of microarray and sequencing technologies have enabled us to define the global patterns of gene expression or ''transcriptomes''. Additionally, coupling microarray or sequencing technologies with RNA interference (RNAi) and/or gene knockout and chromatin immunoprecipitation (ChIP) techniques has allowed the identification of direct and indirect targets of TFs on a genome-wide scale and to define their contribution to specific cellular transcriptomes (Hawkins and Ren, 2006; Sajan and Hawkins, 2012; van Steensel, 2005). Global mapping of TFs revealed preferential binding at thousands of specific DNA sites in the genome in a sequence-specific manner and regulated the transcription of their target genes in different cell types in response to differentiation

cues (Johnson et al., 2007). Moreover, a single TF alone can not determine cell fate during lineage specification, the coordinated action of a series of transcriptional regulators that form transcriptional regulatory networks govern the functional program of the cells (Amit et al., 2009; Teng et al., 2013). Several lines of evidences suggest that the response elements contain binding sites for more than one TF, indicating that these TFs co-regulate the expression of target genes by binding to co-motifs on response elements. Importantly, studies on integrative analysis of single nucleotide polymorphisms (SNPs) from various GWAS databases suggest the association of SNPs over TF motifs (Bryzgalov et al., 2013; Ciofani et al., 2012). Thus, the disruption of TF binding sites by disease-associated SNPs causes changes in the gene expression profile in relevant cell types.

A second level of gene regulation is controlled by epigenetic mechanisms that regulate the accessibility of TFs to the cis-regulatory regions of their target genes within the highly ordered chromatin structure (Berger, 2007; Cantone and Fisher, 2013; Dilworth et al., 2000; Farkas et al., 2000; Francis et al., 2004; Luger et al., 1997; Mohd-Sarip and Verrijzer, 2004; Narlikar et al., 2002, 2013; Richmond and Davey, 2003; Ringrose and Paro, 2004; Robinson et al., 2006). The term 'Epigenetics' was coined by Conrad H. Waddington, who referred to it as a display of genetic activity that lead to heritable changes in the phenotype or gene expression program occurred without modifying DNA sequence (Holliday, 2006; Iovino and Cavalli, 2011; Nicol-Benoit et al., 2013; Slack, 2002; Waddington, 1969, 2012). He proposed a model for an epigenetic landscape to show cellular fate during development (*Figure 2*).

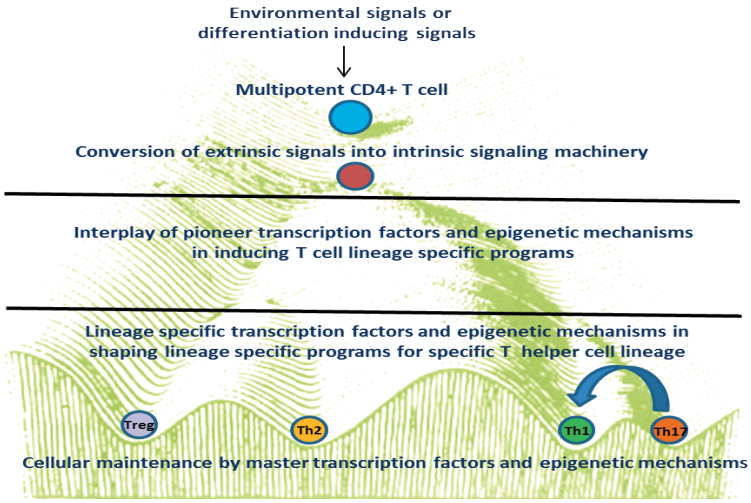

Figure 2: Simplified version of Waddington's model showing the epigenetic landscape for CD4+ T helper cells.

The epigenetic mechanisms participate in the regulation of gene expression include DNA methylation, post-translational modification of histone tails and histone variants, remodeling of nucleosome structure, chromatin interaction/chromosome confirmation, and non-coding RNAs (ncRNAs) (Auyeung et al., 2013; Barski et al., 2007, 2009; Bartel, 2009; Berger, 2007; Huang and Berger, 2008; Lee, 2012; Mendenhall et al., 2013; Mercer and Mattick, 2013; Roh et al., 2005, 2007; Ziller et al., 2013). These epigenetic changes open up the chromatin structure and expose DNA sites accessible to the TFs and other regulatory DNA binding proteins for the regulation of gene transcription. Additionally, these epigenetic changes are established during the development and differentiation of various cells and tissues, and are altered in response to intrinsic and environmental stimuli. The general principles of epigenetic modifications are discussed in following subsections (*Figure 3*).

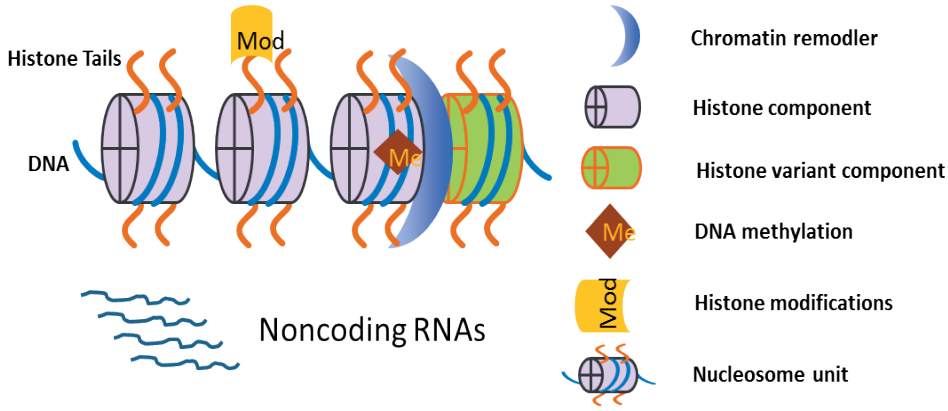

Figure 3: Epigenetic modifications associated with gene regulation.

DNA methylation

DNA methylation is central to epigenetic regulation of gene expression in eukaryotes. Generally, DNA methylation on CpG dinucleotide cytosine residues at gene promoters repress gene transcription by restricting the accessibility of TFs to the target DNA (Guibert et al., 2009; Koh and Rao, 2013; Li et al., 2013; Reddington et al., 2013; Smith and Meissner, 2013). In somatic cells, DNA methylation is maintained in successive generations by the action of DNMT1 and is considered as one of the most stable epigenetic mark with epigenetic memory. Global mapping of DNA methylation (DNA methylome) in plants and several organisms, have revealed DNA methylation both in the CpG and non CpG context (Akopian et al., 2012; Arand et al., 2012; Bock et al., 2012; Dowen et al., 2012; Gifford et al., 2013; Heyn and Esteller, 2012; Lister et al., 2013, 2008, 2008, 2009, 2011; Novakovic and Saffery, 2010; Sindhu et al., 2012; Smith and Meissner, 2013; Ziller et al., 2013). DNA methylation at CpGs is associated mainly with promoters and at non CpGs is associated with actively transcribed gene body regions in embryonic stem (ES) cells. Genome wide

comparative analysis of DNA methylation in ES cells and induced pluripotent stem (iPS) cells have identified differences in DNA methylation profiles between these cells types, which questions the efficacy of iPS cell reprogramming as an alternative to human ES cells (hESCs) (Bock et al., 2011; Meissner, 2010; Ohi et al., 2011). The function of DNMTs is well established, but the mechanisms of DNA methylation by these DNMTs are not fully characterized (Challen et al., 2012; Jost et al., 2013). However, there may be multiple mechanisms involved in accomplishing these tasks depending on the specific biological perspectives. For example, recent studies have shown that 5-hydroxymethylation (5hmC) of cytosine, a relatively new epigenetic modification, is found to be associated with pluripotent nature as well during differentiation of hematopoietic stem (HSc) and of ES cells (Ficz et al., 2011; Laird et al., 2013). A role of TET proteins have been linked to be involved in mediating DNA methylation reactions has been recently studied (Neri et al., 2013; Shen and Zhang, 2013; Shen et al., 2013). Interestingly, genome wide mapping of 5-hydroxymethylation revealed the deposition of this mark in actively transcribed gene bodies as well as over a subset of enhancers (Hackett et al., 2013; Pastor et al., 2011, 2012; Sérandour et al., 2012; Yamaguchi et al., 2013).

Post-translational modification of the histone tails

Post-translational modifications in the histone tails are associated with both active and repressive chromatin states, depending on the type of modification and location in the genome. Histone modification includes methylation, acetylation, phosphorylation, ubiquitylation and sumoylation. Differential combinatorial patterns of histone modification marks are used to profile the functionality of histone epigenome. Histone modification representing an active and silent chromatin state resulted by the action of trithorax group (TrxG) and polycomb group (PcG) protein complexes, respectively (Dilworth et al., 2000; Francis et al., 2004; Narlikar et al., 2002; Ringrose and Paro, 2004). Moreover, several genome-wide combinatorial analyses of these histone modification marks have revealed three distinct chromatin states: active, silent and poised (Barski et al., 2007, 2009; Berger, 2007; Bernstein et al., 2006; Ernst et al., 2011; Hawkins et al., 2010; Heintzman et al., 2009; Hon et al., 2009a, 2009b; Huang and Berger, 2008; Jiang et al., 2011a; Ram et al., 2011; Roh et al., 2007, 2005; Tan et al., 2011; Zhou et al., 2011; Zhu et al., 2013). For example, mono/di/trimethylation at the lysine 4 residue of H3 histone (H3K4me1/me2/me3) is associated with permissive transcription and H3K9me3 and H3K27me3 is associated with repressive transcription. However, opposing modifications, H3K4me3/ H3K27me3 co-localize to form a ‘bivalent domain’, representing poised chromatin states and have been reported to be present in several promoters in different cell types (Bernstein et al., 2006; Hawkins et al., 2010). H3K36 trimethylation is associated with actively transcribed gene body (Kolasinska-Zwierz et al., 2009). Acetylation of histone tails plays a critical role in chromatin function and gene expression. Histone acetylation is regulated by two antagonizing

enzymes, histone acetyltransferases (HATs) and deacetylases (HDACs), respectively that create and remove acetyl group in the histone tails. During the cellular development, gene knockout of HATs suggesting their importance in the proper execution of developmental programs (Wang et al., 2008, 2009b). H3K27ac occupancy in the genome is associated with active chromatin state and active gene transcription (Jenuwein, 2001; Rice and Allis, 2001). Genome wide studies have shown that colocalization of mono/di/tri methylation of lysine 4 of histone 3 (H3K4) with acetylated H3K27 is associated with chromatin decondensation and active gene transcription (Creyghton et al., 2010; Hawkins et al., 2010; Heintzman et al., 2009). Thus, these histone modifications are marked in distinct genomic locations, such as promoters, introns, exons, intergenic regions and associated with different functions in the context of gene regulation (Bernstein et al., 2006; Ernst et al., 2011; Hawkins et al., 2010; Heintzman et al., 2009; Jiang et al., 2011a; Ram et al., 2011; Zhou et al., 2011; Zhu et al., 2013).

Nucleosome positioning or chromatin remodeling

The nucleosome is the basic repeating structural unit of chromatin. It forms the higher order chromatin structure that regulate the accessibility of proteins to DNA and influences gene expression (Luger et al., 1997; Richmond and Davey, 2003; Robinson et al., 2006). However, nucleosomes can undergo restructuring and repositioning (nucleosome positioning), which includes the removal of histone octamers and facilitates the accessibility of transcription regulatory factors, such as TFs, coactivators and basal transcription machinery for the regulation of gene transcription, to DNA binding sites, (Berger, 2007; Cantone and Fisher, 2013; Dilworth et al., 2000; Farkas et al., 2000; Francis et al., 2004; Luger et al., 1997; Mohd-Sarip and Verrijzer, 2004; Narlikar et al., 2002, 2013; Richmond and Davey, 2003; Ringrose and Paro, 2004; Robinson et al., 2006).

Several studies have shown that nucleosome occupancy is reduced over the transcriptionally active regions, and these nucleosome free regions were flanked by two nucleosomes (Farkas et al., 2000; Francis et al., 2004; Mohd-Sarip and Verrijzer, 2004; Narlikar et al., 2002). Nucleosome positioning is an ATP-dependent process that regulates chromatin structure and nucleosome dynamics (Dilworth et al., 2000; Narlikar et al., 2013; Ringrose and Paro, 2004). Several enzymatic mechanisms can regulate histone-DNA interactions within the nucleosomes called chromatin or nucleosome remodelers. The chromatin remodelers are complexes of multiple proteins that drive ATP hydrolysis to slide or dissolve histone octamers. There are different types of nucleosome remodelers, such as SWI/SNF, SWR, INO80, ISWI, and Mi-2/CHD, each of these serves diverse functions to up or down regulate the gene transcription (Hauk and Bowman, 2011; Udugama et al., 2011; Yen et al., 2012; Zentner et al., 2013; Zofall et al., 2006).

Table 2: Chromatin modifications involved in gene regulation. (Adapted from Berger 2007, Wilson CB et al., 2009).

Chromatin modifications	Residues modified	Function of modification	Effect on transcription
Post-translational modifications (PTMs) of histone tails			
Acetylation	H3K(9,14,18,K27,56) H4K(5,8,12,16) H2aK5, H2bK(12,15)	Recruits/ binds bromodomain containing proteins (e.g. TAF1)	Permissive
Methylation (Lysine)	H3K4me1, me2, me3 H3K9 me1	Recruits chromodomain, PHD, tudor domian containing proteins (TFIID, CHDI, WDR5)	Permissive
	H4K27me3, H3K9me3	Recruits/ binds PRC1 complex, CBX5(HP1) and DNMTs	Repressive
Methylation (Arginine)	H3K(2,17,26), H4K3	Recruits chromodomain, PHD, tudor domian containing proteins (TFIID, CHDI, WDR5)	Permissive
Phosphorylation	H3S/T(3,10,28), H2A, H2B	Recruits chromodomain, PHD, tudor domian containing proteins (TFIID, CHDI, WDR5)	Permissive
Ubiquitylation	H2AKub120,	Unclear	Permissive
	H2BKub119	Unclear	Repressive
Sumoylation	H2AK126, H2BK(6/7)	Unclear	Repressive
DNA methylation			
Methylation at cytosine	5-methylcytosine (5mC) (CpG islands)	Recruits repressive complexes and DNMTs	Repressive
	5-hydroxymethylcytosine (5hmC) (Gene bodies, enhancers, low-intermediate CpG promoters)	Unclear	Permissive/Repressive

Cis-acting regulatory elements and chromatin interaction/chromosome confirmation

Cis-regulatory DNA elements regulate gene expression by controlling the on/off state of genes (Nelson and Wardle, 2013). Cis-regulatory elements are highly conserved among vertebrates and usually constitute binding sites for multiple TFs (Wittkopp and Kalay, 2012). These cis-regulatory modules are present at promoters, enhancers, insulators, silencer and locus control regions (LCRs) in the genome, and serve as site for epigenetic modifications (Riethoven, 2010). Different chromatin landscapes at these cis-regulatory regions regulate the accessibility of the transcription machinery, which further regulates the gene expression level in the development or lineage specific gene expression patterns (Ansel et al., 2003; Carey et al., 2012; Hardison and Taylor, 2012; Splinter and de Laat, 2011).

ncRNAs

ncRNAs represents another form of epigenetic regulation other than histone modifications and DNA methylation. Based on their transcript size, ncRNAs can be categorized into 2 major classes: long ncRNAs (>200 nucleotides) and small ncRNAs (<200 nucleotides) (Costa, 2005; Mattick, 2004). There are several classes of small ncRNAs, such as small interfering RNAs (siRNAs), micro RNAs (miRNAs) and PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snRNAs). These ncRNAs regulate gene expression via distinct mechanisms (Hauptman and Glavac, 2013; Karagiannis and El-Osta, 2004). For example, miRNAs are widely distributed throughout the genome and regulate gene expression by binding coding regions or untranslated regions (UTRs) of target mRNA transcripts, which results in either mRNA degradation or inhibition of translation. Long ncRNAs (lncRNAs) were reported to greatly regulate expression of genes (Shi et al., 2013; Wahlestedt, 2013). Studies have shown high tissue and species-specific expression patterns of lncRNA. Long intergenic ncRNA (lincRNA) is widely studied class of lncRNA (Guttman et al., 2011; Ulitsky and Bartel, 2013). Analyses of the miRNome and lincRNome in several models of cellular differentiation and development (including T cells) was recently reported and revealed cell-specific lincRNAs (Hu et al., 2013a, 2013b).

2.3.1 Transcriptional regulation of T-helper lineage specification

During the early stages of T lymphocyte development in the thymus, common lymphoid precursors (CLPs) go through sequential lineage specification to acquire essential characteristics for T cell fate commitment (CD4 or CD8 T cells), while suppressing the alternative lineage path. After their maturation in thymus, CD4+ or CD8+ T cells travel to peripheral lymphoid organs, such as spleen, lymph nodes, tonsils, and MALT tissues where they encounter antigenic signals from antigen presenting cells to perform distinct killer and helper function, respectively. The signaling pathways, transcriptional regulatory networks, and epigenetic regulatory mechanisms defining distinct the sequential stages from naive progenitors to mature CD8+ killer T or CD4+ Th cells have been studied in depth during the past two decades (David-Fung et al., 2009; Deftos et al., 2000; Naito and Taniuchi, 2010; Rothenberg, 2007; Siu, 2002; Tanaka and Taniuchi, 2014; Xu et al., 2013b). During early T cell development, TFs play a significant role in coordinating developmental events by priming the transcription of lineage specific regulatory genes that restrict the multi-lineage potential and drive the development potential towards their T cell lineage fate (Evans and Jenner, 2013; Kanno et al., 2012; Zhou et al., 2009). The TFs critical for lineage specification and commitment of T cells include Notch/CSL, GATA3, HEB, Bcl11b, HES-1, TCF1, PU.1, Th-POK, E proteins, amongst others, which play the key role in different stages of T cell development, as discussed earlier (Braunstein and

Anderson, 2012; Franco et al., 2006; Gimferrer et al., 2011; Kappes, 2010; Muroi et al., 2008; De Obaldia et al., 2013; Rui et al., 2012; Tanigaki and Honjo, 2007; You et al., 2013).

Lineage commitment during Th cell development and differentiation is determined by instruction from environmental cues (including cytokines), which are conveyed through cytokine receptors and converted into amplified intrinsic signals through activation of specific TFs, thus leading to changes in the gene expression profiles that drive specification towards specific Th lineage (Zhou et al., 2009). Thus, depending on the nature of signal and type of cytokines produced by APCs, multipotent naive CD4+ T cells differentiate into functionally distinct effector Th subsets, including Th1, Th2, Th17, Th9, Th22, Tfh, and Treg cells, thus making them a powerful model system for the study of lineage specification (Adamson et al., 2009; Evans and Jenner, 2013; Kanno et al., 2012; Zhou et al., 2009). The molecular basis for Th cell lineage specification and commitment relies on its unique gene expression patterns, which determine cell-type specific functions and properties while opposing the fate of alternative subsets. Thus, Th cell lineages can be distinguished from each other on the basis of their unique transcriptional profiles (Chen et al., 2003; Jankovic et al., 2007; Kaplan et al., 1996; Lund et al., 2005, 2003, 2004, 2007; Shimoda et al., 1996; Takeda et al., 1996; Tuomela et al., 2012). Moreover, T cell specific TFs drive gene expression profiles to determine specific T cell fates (Chen et al., 2003; Ciofani et al., 2012; Durant et al., 2010; Elo et al., 2010; Sadlon et al., 2010; Wei et al., 2011, 2010; Yu et al., 2009a; Zheng et al., 2007). There are major TFs that act as 'master regulators', which have been suggested to be essential and sufficient for driving unique cell fates. Although the concept of 'master regulator' TFs is useful in defining the key regulators, it undermines the complexity of Th cell function and is far from reality. However, it is becoming clear that integrated networks of regulatory TFs are needed to understand the complete differentiation program. In the past, efforts have been made to understand the complete picture of cellular specification during differentiation of specific phenotypes using system-wide approaches to construct and decode the gene regulatory networks of TFs co-expressed within the cell (Ciofani et al., 2012; Novershtern et al., 2011). With the advancement of new high throughput microarray and sequencing technologies, functional genomics approaches have played a key role in constructing TF mediated transcriptional regulatory networks of several cellular differentiation and development programs, including T cells. The current understanding of the TFs controlling lineage specification and commitment during Th cell differentiation is summarized below.

Transcriptional regulation of Th1 cell differentiation

Th1 cell differentiation is initiated by IL12, which signals through its receptors on activated CD4+ T cells. This induces phosphorylation of STAT4, which then

translocates to the nucleus and binds to regulatory elements in the DNA to regulate the transcription of its target genes, including *Ifnγ*. IFNγ further stimulates phosphorylation of STAT1, which induces the transcription of Th1 inducing genes, such as *Il12rβ* and *T-bet,* which further amplify the Th1 differentiation in a positive feedback loop (Schulz et al., 2009; Usui et al., 2003, 2006; Ylikoski et al., 2005; Zheng et al., 2011). Studies have demonstrated that STAT4 also promotes Th1 development by negatively regulating the Th2 cell differentiation (Lund et al., 2004; Murphy and Reiner, 2002; Ouyang et al., 1998, 2010). Additionally, role of IL2 dependent STAT5 has been implicated in Th1 differentiation (Liao et al., 2011). Thus, STAT4, and STAT1 bind at several genetic loci encoding lineage specifying cytokines and TFs and regulate their expression, and are therefore considered as pioneer factors based on their ability to initiate the process of lineage specification. Previous studies have revealed that STATs, including STAT1 and STAT4, help in establishing the landscape of global enhancers in differentiating Th1 cells (Afkarian et al., 2002; Murphy et al., 1999; Nishikomori et al., 2002). T-bet is the master regulator for Th1 differentiation, as it transactivates the expression of *Ifnγ* by forming a positive feedback loop and enhances the IL12 signaling by activating the expression of the *Il12rβ2* gene (Usui et al., 2003, 2006). Nevertheless, other studies have suggested that STAT4 is needed for T-bet to accomplish IL12-dependent Th1 lineage specification (Thieu et al., 2008). Furthermore, T-bet interacts with several other Th cell defining TFs, such as Hlx, Ets, BCL6 and RUNX3 family members, to repress the alternative cell fates by repressing the transcription of their lineage specific genes (Djuretic et al., 2007; Koch et al., 2009, 2012; Mullen et al., 2002). It was shown that T-bet co-interacts with BCL6 to inhibit the expression of subsets of lineage defining genes of the alternative T helper cell fates than Th1 cells. Interaction of RUNX3 with T-bet is required for repression of *Il4* transcription in mouse. Several studies have shown that T-bet interacts with GATA3 to repress Th2 cytokine gene expression and inhibits Th2 development (Hwang et al., 2005; Szabo et al., 2000). Moreover, further studies have shown that T-bet and GATA3 control the alternative lineage cell fate by targeting other pathways of T-cell differentiation (Jenner et al., 2009; Kanhere et al., 2012). Previously it has been shown that T-bet also inhibits Th17 development in a STAT1 independent manner, potentially by repression of RUNX1 mediated *Rorc* gene transcription (Lazarevic et al., 2011; Mathur et al., 2006; Rangachari et al., 2006; Villarino et al., 2010). Recent study reveals that T-bet suppresses IRF4 to negatively regulate Th17 lineage commitment (Gökmen et al., 2013).

Transcriptional regulation of Th2 cell differentiation

Th2 cell differentiation is programmed by IL4. Combined with TCR induced signals, IL4 phosphorylates and activates STAT6, which, in combination with the signals from NFAT, AP-1, NF-κB, activates the transcription of the signature cytokine *Il4*, and *Gata3*, a master TF required for Th2 cell lineage commitment. Upon

phosphorylation, STAT6 homodimerizes and translocates to the nucleus where it activates its target genes. It has been established that genetic inhibition of STAT6 expression severely impairs Th2 cell differentiation (Goenka and Kaplan, 2011). A study has shown that STAT6 enhances the expression of GATA3 by switching the PcG complex with the TrxG complex at the *Gata3* locus during Th2 cell differentiation (Onodera et al., 2010). Moreover, other studies on genetic deletion or using RNAi combined with genome-wide mapping of STAT6 binding both in human and mouse system revealed a large number of STAT6 regulated genes during Th2 cell differentiation (Elo et al., 2010; Wei et al., 2010). Recently in mouse, it has been shown that STAT6 and STAT4 are involved in shaping the global enhancer landscapes in Th2 and Th1 cells, respectively (Vahedi et al., 2012). Consistent with these results, our study presented in this thesis has shown that STAT6 binds at the enhancer regions even before they become active in differentiating human Th2 cells (Hawkins et al., 2013). Hence, STATs, combined with TCR-induced TFs, such as NFAT and AP1, work as pioneer factors to shape the global enhancer landscapes, which are exploited by master TFs to control chromatin remodeling and gene expression of the number of genes important for respective Th cell subsets. However, other studies have shown STAT6-independent induction of Th2 differentiation, suggesting the existence of alternative pathways (Finkelman et al., 2000; Jankovic et al., 2000; Min et al., 2004). Studies have also demonstrated that IL2-induced STAT5 is critical for Th2 cell differentiation (Kagami et al., 2001; Lin and Leonard, 2000; Moriggl et al., 1999; Zhu, 2010). Moreover, both GATA3 and STAT5 bind on different sites of the *Il4* locus and enhance the early production of IL4 (Zhu et al., 2003). In a recent study in *Stat3*- deficient Th2 cells, STAT6 failed to interact with its target loci, suggesting that STAT3 cooperates with STAT6 at several gene loci in developing Th2 cell differentiation, (Stritesky et al., 2011). Thus, these studies advocate the significance of multiple STATs in mediating Th2 differentiation.

GATA3 can auto regulate its own expression to further promote Th2 differentiation. Studies have shown that genetic deletion of *Gata3* gene completely abrogate Th2 differentiation, both *in vitro* and *in vivo*; however forced expression of *Gata3* is required for IL5 and IL13 production, but not IL4 (Pai et al., 2004; Zhu et al., 2004). Moreover, other studies have shown that GATA3 can operate its effect on Th2 differentiation via many different mechanisms, including transcriptional activation of Th2 specific cytokine genes (*Il4, Il5*, and *Il13* genes), interaction with other TFs, and through epigenetic modification (Kishikawa et al., 2001; Tanaka et al., 2011; Zhang et al., 1998). Recent global mapping of GATA3 revealed that it can directly regulate the expression of a large number of genes involved in Th2 differentiation (Horiuchi et al., 2011; Wei et al., 2011). Additionally, it has been shown that there are lineage specific GATA3 binding sites for different lineage cells, indicating the need of other cofactors to determine global GATA3-binding in different lineages (Wei et al., 2011). Thus, GATA3 adopts different mechanisms to activate Th2 differentiation as well as to

maintain the cellular identity. However, studies have also shown that GATA3 is involved in repression of expression or function of several signaling pathways and transcriptions factors of the alternative Th cell lineages. For example, GATA3 suppresses Th1 differentiation by physically interacting with T-bet or directly inhibiting the expression of *Stat4* and *Il12rβ* (Hwang et al., 2005; Jenner et al., 2009; Usui et al., 2003). Additionally, GATA3 also inhibits Th1 differentiation by interacting with RUNX3. RUNX3 cooperates with T-bet for binding the *Ifng* promoter and *Il4* silencer regions to induce IFNγ production, and suppress IL4 production (Ansel et al., 2004). Furthermore, several lines of evidence suggested the role of GATA3 in chromatin remodeling of Th2 cytokine gene loci of *Il4, Il13* and *Il5*. Studies showing that enforced expression of *Gata3* induces DHS at these Th2 specific cytokine gene locus in Th1 cells (Ouyang et al., 2000). GATA3 activates or represses the cytokine gene locus, either through binding with the co-activator or the co-repressor, for example the interaction of GATA3 with Chd (a key component of NuRD chromatin remodeling complex) complex with HATs induces chromatin remodeling at Th2 cytokine locus and interaction with HDACs repress the *T-bet* transcription.

Several reports suggest the role of various other TFs in the regulation of Th2 differentiation. For example, JUNB and c-MAF cooperate to selectively stimulate *Il4* expression (Kim et al., 1999; Li et al., 1999). IRF4 cooperates with NFATc2 to activate the *Il4* promoter and *Il4* gene expression in Th2 cells (Rengarajan et al., 2002). STAT6 induces TF *Gfi-1*, selectively enhances *Gata3* expression and promotes Th2 cell expansion (Zhu et al., 2002). Notch signaling regulates *Gata3* and *Il4* expression by binding to the *Gata3* promoter and the HSV enhancer of *Il4* (Amsen et al., 2004, 2007; Fang et al., 2007). Dec2 induces the expression of *JunB* and *Gata3* to induced Th2 cytokine gene expression (Yang et al., 2009). In Th2 cells, TCF-1 cooperates with β-catenin to induce the expression of *Gata3* and repress the transcription of *Ifnγ* gene, thus promoting IL4-independent Th2 differentiation (Yu et al., 2009b). Furthermore, studies have shown that SATB1 expression is upregulated in Th2 cells (Chen et al., 2003; Lund et al., 2005). SATB1 recruits β-catenin to the *Gata3* promoter and regulate the transcription of of *Gata3* and *Il5* genes (Ahlfors et al., 2010; Notani et al., 2010). Ikaros TF supports Th2 cell differentiation by repressing *T-bet* and *Ifnγ* transcription (Quirion et al., 2009; Thomas et al., 2010). Thus, these studies demonstrated the involvement of different TFs and signaling pathways to drive Th2 differentiation.

Transcriptional regulation of Th17 cell differentiation

Th 17 cells are a relatively new Th subset as compared to classical Th1 and Th2 cells. Unlike Th1 and Th2 cells, Th17 differentiation can be induced by a cocktail of more than one cytokine (Korn et al., 2009). In the mouse, Th17 differentiation from

naive CD4+ T cells is induced by a cytokine cocktail of TGFβ + IL6/IL21 or IL23 or IL23+IL1β. However, Th17 differentiation in humans can be induced by different cytokine cocktails, such as TGFβ + IL21, TGFβ + IL23 (IL1β/IL6/TNF) or TGFβ + IL1β (IL6/IL21/IL23) in human cord blood and IL1β + IL6 (serum-containing medium) or IL1β + IL23 (serum-containing medium) in human PBMCs (Annunziato et al., 2013; Bettelli et al., 2006; Tuomela et al., 2012). These cytokines combined with TCR activation induce various TF mediated regulatory mechanisms that control the specification and commitment of developing Th17 cells during Th17 differentiation. The role of several TFs has been reported to perform either positive or negative regulatory functions during Th17 cell development.

During the early stages of Th17 differentiation, combined TCR and cytokine signaling activates STAT3, which translocates to the nucleus to initiate early gene expression by binding to its target genes including lineage specific TFs, *Rorα* and *Rorγt* (Laurence et al., 2007; Yang et al., 2007a, 2008). In mouse, using genome-wide transcriptional profiling and ChIP-Seq of STAT3, suggest that STAT3 directly regulates the transcription of several target genes implicated in Th17 cell differentiation, including key TFs, such as *Batf, Rorα, Rorγt, Runx1, Ahr, Irf4* and c-Maf, and signature cytokine genes, such as *Il17α, Il17f* and *Il21* (Ciofani et al., 2012; Durant et al., 2010). STAT3 is also important for the intrinsic expansion of Th17 cells, mainly in the context of inflammation (Durant et al., 2010).

RORγt is the key TF of Th17 cells. It has been observed that enforced expression of RORγt in *Stat3* knockout mice is able to induce the expression of *Il17a*. While genetic deletion of *Rorγt* gene completely abolished the Th17 differentiation. Thus, these studies suggest that RORγt is essential and sufficient for generation of Th17 cells and can act as the master regulator (Huh and Littman, 2012; Huh et al., 2011; Ivanov et al., 2006; Ueda et al., 2012; Yang et al., 2008; Yu et al., 2013; Zhang et al., 2013a). Apart from STAT3 and RORγt, several other TFs have been reported to be expressed (though their expression is not restricted to Th17) and involved in promoting Th17 cell differentiation, including RORa, RUNX1, BATF, JUN, IRF4, AHR, NOTCH1, and c-MAF, AIOLOS, IKAROS, IkappaBzeta, HIF1α (Bauquet et al., 2009; Brüstle et al., 2007; Dang et al., 2011; Ivanov et al., 2006; Keerthivasan et al., 2011; Kim et al., 2013; Li et al., 2012; Nakahama et al., 2013; Okamoto et al., 2010; Quintana et al., 2013; Rutz et al., 2011; Schraml et al., 2009; Shi et al., 2011; Veldhoen et al., 2008; Wong et al., 2013; Yang et al., 2008; Zhang et al., 2013a). However, a number of TFs that negatively regulate Th17 development have been reported, such as T-bet, FOXP3, GFI1, ETS1, TCF1, EGR2, Th-POK, Jagged-1-Hes-1, TWIST1, PPARγ, KLF4, and ID3, in addition to other potential TFs (An et al., 2011; Chalmin et al., 2012; Engel et al., 2012; Gökmen et al., 2013; Joshi et al., 2011; Klotz et al., 2009; Lebson et al., 2010; Maruyama et al., 2011; Miao et al., 2013b; Moisan et al., 2007; Pham et al., 2013; You et al., 2013; Yu et al., 2011). Overall, these studies demonstrated that these

TFs utilize distinct mechanisms to modulate Th17 differentiation program, as discussed in the given references.

Transcriptional regulation of Tfh cell differentiation

Tfh cells are a new subtype of effector CD4+ T cells and are different from other established CD4+ T-cell lineages on the basis of their location at the follicular areas of germinal centers and specialized function to help B cell immunity (Crotty, 2011; Vinuesa and Cyster, 2011). Tfh cells also show distinct gene expression profiles in comparison to other effector Th cells including Th1, Th2, Th17, and Treg cells. Tfh cells are produced by a cytokine cocktail of IL6 and IL21, and are characterized by the expression of chemokine receptor CXCR5 (Heissmeyer and Vogel, 2013; Liu et al., 2013; Poholek et al., 2010; Tangye et al., 2013; Tellier and Nutt, 2013; Weinmann, 2013). BCL6 is a master regulator of the Tfh differentiation program. Studies have shown that genetic deletion of *Bcl6* in CD4+ T cells results in the failure to generate Tfh cells and proper GC B cell responses in vivo, while enforced ectopic *Bcl6* expression drives Tfh cell development (Baumjohann et al., 2011; Johnston et al., 2009; Nurieva et al., 2008, 2009; Yu et al., 2009a). Additionally, BCL6 is a transcriptional repressor and inhibits the expression of other Th cell lineage specifying factors, such T-bet, RORγt, and GATA3 (Lüthje et al., 2012). The above discussed studies suggest that BCL6 is essential and sufficient for the generation of Tfh cells. However, BCL6 serves different functions in other Th cell lineages (Huang et al., 2013). Additionally, BCL6 repressor, B-lymphocyte-induced maturation protein-1 (Blimp1) can suppress BCL6 expression. Overexpression of *Blimp 1* blocks Tfh differentiation and genetic deletion of *Blimp1* in CD4 T cells showed enhanced Tfh differentiation (Choi et al., 2013; Johnston et al., 2009). Tfh cells also express several other key TFs, including IRF4, c-Maf, BATF, and STAT3/5. STAT3 signaling is also critical in Tfh cell development. Recent studies show that STAT3 deficiency greatly reduced the number of CXCR5+ Tfh cells and led to defective GC responses and B-cell function to produce antibodies both in human and mouse (Ma et al., 2012; Nurieva et al., 2008). However, another STAT protein, STAT5, suppress Tfh cell generation and function (Johnston et al., 2012; Nurieva et al., 2012). Furthermore, it has been shown that c-MAF cooperates with BCL6 to promote Tfh cell differentiation (Kroenke et al., 2012). Recent studies have shown that BATF regulates Tfh differentiation by controlling the *Bcl6* and *c-Msf* gene expression through direct binding of BATF-JUN complex (Ellyard and Vinuesa, 2011; Ise et al., 2011). Another TF which plays the role in Tfh differentiation is IRF4. Studies have shown that IFR4 promotes Tfh cell differentiation by cooperating either with STAT3 or BATF-JUN complex to regulate IL21-induced genes including *Blimp-1*. *Irf4* deficient CD4+ T cells showed reduced STAT3 binding and failed to generate Tfh cells comprehensively due to an intrinsic defect in T cells (Bollig et al., 2012; Kwon et al., 2009; Li et al., 2012).

Transcriptional regulation of Th9 and Th22 cell differentiation

Differentiation of Th9 cells is induced by a cocktail of TGFβ and IL4 during activation of naive CD4+ T cells, either with a specific antigen or anti-CD3/CD28 antibodies and stimulates the expression of TFs *Pu.1* and *Irf4*, which regulates the transcription of the *Il9* gene (Veldhoen et al., 2008). Th9 cells are a unique subset of CD4+ Th cells that are different from Th2 and Treg cells. In Th9 cells, IL4 induces the activation of STAT6 and IRF4, whereas TGFβ induces the expression of *Pu.1*, which represses the transcription of both *T-bet* and *Gata3*. Collectively these events drive *Il9* expression (Angkasekwinai et al., 2010; Murphy and Reiner, 2002; Perumal and Kaplan, 2011). Ectopic expression of *Pu.1* in CD4+ T cells enhanced Th9 production by TGFβ and IL4, whereas *Pu.1* deficiency lead to suppression of Th9 cells (Chang et al., 2010). Additionally, similar experimental approach on IRF4 resulted in the same effect on Th9 development as PU.1 (Staudt et al., 2010). Mechanistically, PU.1 and IRF4 bind to the promoter region of *Il9* to induce *Il9* gene expression (Chang et al., 2010; Staudt et al., 2010). Sequence analysis of *Il9* regulatory region identified binding sites for other TFs, such as AP1, NF-Kb, NFAT, STATs, GATA1, GATA3, SMADs, and NOTCH (Perumal and Kaplan, 2011). Additionally, recent studies suggest a role for NF-KB, Notch receptors, BATF, SMAD2/3, TFs in the regulation of Th9 response (Elyaman et al., 2012; Jabeen et al., 2013; Xiao et al., 2012). However, the role of these TFs needs to be further considered.

The Th22 cell subset is similarly a relatively new CD4+ Th subtype, characterized by the secretion of IL22 (Duhen et al., 2009; Trifari and Spits, 2010; Trifari et al., 2009). Th22 cell differentiation can be induced by IL6 and TNFα (Duhen et al., 2009) and AHR has been suggested to serve as key TF (Duhen et al., 2009; Eyerich et al., 2009; Zhang et al., 2011). However, the molecular mechanisms and TFs controlling Th22 cells and differentiation are poorly characterized and need more attention.

Transcriptional regulation of Treg cell differentiation

It has been established that TF FOXP3 is a critical player in Treg cell development and homeostasis. Although, there is a general consensus that FOXP3 is essential for tolerance both in mice and humans, as documented by several studies where deficiency of FOXP3 and Treg has been associated with the severe autoimmune disease states (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Lin et al., 2007; Williams and Rudensky, 2007), studies on global analysis of FOXP3 occupancy in Treg cells suggest that FOXP3 is only partially responsible for Treg signatures (Birzele et al., 2011; Fu et al., 2012; Gavin et al., 2007; Hill et al., 2007; Marson et al., 2007; Pfoertner et al., 2006; Rudra et al., 2012; Zheng et al., 2007). This raises the possibility of the involvement of other TFs in Treg cell development (Delgoffe et al., 2012; Fu et al., 2012; Rudra et al., 2012). Studies have shown that

FOXP3 cooperates with other nuclear factors to establish the Treg signature and functions (Bettelli et al., 2005; Kim and Leonard, 2007). A recent study demonstrated that an array of TFs, including Eos, IRF4, SATB1, Lef1, and GATA1, can synergize with FOXP3 to form a transcriptional network controlling Treg cell differentiation (Fu et al., 2012). However, during the initial stage of Treg development, TFs activated by TCR signaling, such as NF-KB, NFAT, AP1 and FOXO1, have been shown to modulate Treg development and function (Kim and Leonard, 2007). Another study has suggested that FOXP3 cooperates with NF-KB to deter the gene expression program in effector Th cells (Bettelli et al., 2005). Additionally, studies have demonstrated that c-Rel, a NF-KB TF family member, regulates *Foxp3* expression through directly binding to cis-regulatory elements, including known non-coding DNA regulatory region at the *Foxp3* locus upon TCR/CD28 stimulation (Long et al., 2009; Visekruna et al., 2010). Apart from NF-KB, a Ca^{2+} activated transcription TF NFAT is suggested to regulate the Treg cells (Bopp et al., 2005). In human Treg cells, NFAT is directly bound to the *FOXP3* promoter to regulate its expression but does not have influence on its suppressive activity of Treg cells (Mantel et al., 2006). Additionally, studies have suggested that FOXP3 physically interacts with NFAT to control the Treg function (Vaeth et al., 2012; Wu et al., 2006). TF, AP1 controls *Foxp3* transcription through directly recruitment at the *Foxp3* promoter (Mantel et al., 2006). CREB regulates *Foxp3* expression by binding to the CNS2 element of *Foxp3* non-coding DNA elements (Kim and Leonard, 2007). However, the molecular mechanisms through which NF-KB, NFAT, AP1 and CREB participate in the modulation of Treg differentiation and function has remained poorly understood and are open for future investigation. Another TF family, FOXO positively regulates Treg cell differentiation and function through distinct mechanisms (Kerdiles et al., 2010; Ouyang et al., 2010). Additionally, studies investigating the role of Eos TF in Treg cell differentiation suggest that Eos controls and takes part in FOXP3-dependent gene repression in regulatory CD4+ T cells (Pan et al., 2009a; Sharma et al., 2013). Helios regulates Treg cells by inducing epigenetic silencing of *Il2* gene expression (Baine et al., 2013). Moreover, RUNX family members, such as RUNX1 and RUNX3 have been suggested to promote Treg cell differentiation and function either through regulating *Foxp3* expression by direct binding at *Foxp3* promoter region or by physical interaction with FOXP3 (Kitoh et al., 2009; Klunker et al., 2009; Ono et al., 2007). Furthermore, recent studies have suggested that IL2 mediated STAT5 signaling promotes Treg differentiation via distinct mechanisms both *in vivo* and *in vitro* (Burchill et al., 2007; Chen et al., 2011; Guo et al., 2013; Mahmud et al., 2013).

2.3.2 Epigenetic mechanisms underlying T-helper lineage specification

Emerging evidence has established that TF networks and signaling pathways coordinate with epigenetic mechanisms, including histone modifications, DNA methylation and chromatin remodeling, to modulate gene expression programs in various cellular differentiation systems (Auyeung et al., 2013; Barski et al., 2007, 2009; Bartel, 2009; Lee, 2012; Mendenhall et al., 2013; Roh et al., 2007, 2005; Shi et al., 2013; Ziller et al., 2013). It has been shown that most of the developmental genes in pluripotent/multipotent progenitor cells are either inactivated or expressed at very low levels and maintained in specific a bivalent chromatin structure, however, during cell differentiation, these bivalent chromatin structures change into monovalent structures resulting in either a gain or loss of gene activity, suggesting the role of chromatin regulation in determining the cell fate (Bernstein et al., 2006). These epigenetic changes are guided by various epigenetic modifying factors. For example, an undifferentiated ES cell state is marked by the expression of pluripotency genes, such as OCT4 or NANOG, and their promoter and or enhancer regions are enriched with H3K4me3 (promoters) and H3K4me1(enhancers) respectively (Gifford et al., 2013; Hawkins et al., 2010; Jiang et al., 2011a; Lister et al., 2011; Mendenhall et al., 2013). Furthermore, CD4+ T cells are multipotent cells and have the ability to differentiate into distinct cell fates that are determined by the expression of lineage specific TFs and signature cytokines and controlled by various layers of epigenetic mechanisms. For example, in naive CD4+ T cells, these TFs and cytokines are either inactive or minimally expressed and their gene loci are marked by bivalent or repressive monovalent chromatin structures. However, during differentiation, these epigenetic marks are erased or gained by active epigenetic marks enabling the transcription of these TFs and cytokine genes (Ansel et al., 2003; Zhou et al., 2009). In the recent past, studies on examining the novel epigenetic mechanisms as well as mapping the global profiles of the epigenetic modifications including histone modifications, DNA methylation, DHS and ncRNAs in Th cells have provided understanding of the epigenetic mechanisms regulating Th cell development and commitment (Chong et al., 2008; ENCODE Project Consortium et al., 2012; Liston et al., 2008; Monticelli, 2013; Pagani et al., 2013; Roh et al., 2007, 2005; Schones et al., 2008; Thurman et al., 2012; Vahedi et al., 2012; Wei et al., 2009, 2010; Zhou et al., 2008; Zou et al., 2001). Interestingly, very recently studies have started to focus on linking epigenetic regulation with several disease states originating from uncontrolled Th cell activity (Ciofani et al., 2012; Hawkins et al., 2013; Lovinsky-Desir and Miller, 2012; Ngalamika et al., 2012). Studies based on genetic deletion of the genes encoding for the microRNA-processing endonucleases *Drosha* and *Dicer* show that regulatory ncRNAs play an important role in T cell differentiation (Liston et al., 2008; Monticelli, 2013; Pagani et al., 2013; Zhou et al., 2008).

Epigenetic regulation of Th1 and Th2 differentiation

The first evidence of the role of epigenetic mechanisms in Th cells came from studies using drugs. Treatment with 5-azacytidine, a DNA methylation inhibitor, resulted in enhanced production of IL2 and IFNγ, and HDAC inhibitors caused increased production of IFNy and IL4, the signature cytokines for Th1 and Th2 cells, respectively (Valapour et al., 2002; Young et al., 1994). These findings were further supported by studies on genetic deletion of genes encoding DNA methyl transferase 1(*DnmtI*) and methyl-CpG-binding domain protein 2 (*Mbd2*) in Th1 and Th2 cells (proteins mediate gene silencing through recruitment of HDACs and chromatin remodeling complexes to the DNA methylation sites). Genetic deletion of *Dnmt1* and *Mbd2* genes in mice showed increased expression of *Ifng* and *Il4* in Th1 and Th2 cells and ability of these cells to inhibit the expression of cytokine gene associated with opposing lineage suggesting the role of epigenetic mechanisms (Makar et al., 2003). Furthermore, Brahma related gene 1 (BRG1), a chromatin remodeling complex gene is part of chromatin remodeling complex with STAT4 (Th1 specific TF) involved in nucleosome displacement and chromatin remodeling at *Ifng* gene promoter and required for expression of *Ifng* gene in Th1 cells (Zhang and Boothby, 2006). Additionally, deletion of *Mll* gene (a histone methyltransferase) is required for maintenance not for induction of the expression of *Gata3* and cytokines *Il4* and *Il13* in Th2 cells (Kozuka et al., 2011; Yamashita et al., 2006). However, *Mel18* (H3K27me3 binding poly comb repressor complex 1 protein) knockout mice depleted *Gata3* expression in Th2 cells (Kimura et al., 2001). Recently, SUV39H1 a methyl transferase that mediate methylation of H3K9me3 mark, associated with repressive HP1 protein to maintain the transcriptional silencing of Th1 gene loci, thus provide stability to Th2 cells (Allan et al., 2012).

Until recently, most studies defining epigenetic regulation in Th cells were focused on the changes in the chromatin structures and accessibility at cytokine gene loci of Th1 and Th2 cells. These cytokine gene loci are controlled through their promoters and many other cis-regulatory elements, such as enhancers, silencers, and insulators that have been experimentally determined by mapping of DHS, DNA methylation, various histone modifications and presence of several conserved non-coding sequences (CNS). The epigenetic mechanisms responsible for the regulation of gene expression through these cytokine loci are discussed in detail by several studies (Ansel et al., 2003; Rowell et al., 2008; Wilson et al., 2009). Studies have shown that undifferentiated CD4+ T cells express background levels of the *T-bet* and *Gata3* TFs, and detectable amounts of cytokine gene expression for *Ifng*, *Il4* and *Il13* (Grogan et al., 2001), and gene loci are characterized by inactive or poised state of DHS, histone modifications and a high degree of CpG methylation (Baguet and Bix, 2004; Collins et al., 2010; Fields et al., 2004; Jones and Chen, 2006; Lee and Rao, 2004; Makar et al., 2003; Schoenborn et al., 2007; Tykocinski et al., 2005; Yamashita et al., 2006). However, in differentiated Th1 and Th2 cells, the chromatin state at

these gene loci are either associated with the acquisition of DHS, marked increase in permissive histone modifications, loss of repressive H3K27me3 and DNA demethylation which maintain active gene repertoire of lineage specific TFs and signature cytokines for specific lineage in cell specific manner and vice versa for opposing lineage (Baguet and Bix, 2004; Chang and Aune, 2007; Collins et al., 2010; Fields et al., 2004; Jones and Chen, 2006; Kim et al., 2007; Lee and Rao, 2004; Lee et al., 2000, 2001; Makar et al., 2003; Miller et al., 2008; Naoe et al., 2007; Ouyang et al., 2000; Schoenborn et al., 2007; Soutto et al., 2002; Yang et al., 2007b). Thus, from these studies, it is evident that the genetic loci for genes encoding for lineage specific TFs and cytokines are linked with permissive epigenetic state in selective lineages, while repressive or bivalent epigenetic state in the alternative lineages. In the recent years, several genome wide studies have profiled DHS state, nucleosome positioning, histone modifications, DNA methylation to understand the epigenetic changes associated with naive and differentiated Th cells involving both in human and mouse (ENCODE Project Consortium et al., 2012; Roh et al., 2007, 2005; Schones et al., 2008; Thurman et al., 2012; Vahedi et al., 2012; Wang et al., 2008; Wei et al., 2009, 2010). These studies suggest that changes in epigenetic profiles are correlated with gene transcription in T cells. For example, permissive histone modification, such as H3K4me1, H3K4me2, H3K4me3, H3K79me3, H3K27me1, H3K9me1, H4K20me1 and H3K27ac, are associated with gene activation, whereas H3K9me3, H3K27me2 and H3K27me are correlated with gene repression. Two opposing marks, H3K27me3 and H3K4me3, co-localized at various promoters to form a bivalent domain and associated with low gene expression state, thus associated genes are poised for either activation or repression during development and differentiation in ES cells as well as in T cells (Bernstein et al., 2006; Roh et al., 2006; Wei et al., 2009). Remarkably, *T-bet* and *Gata3* gene promoters were marked with bivalent domains, suggesting that the *T-bet* and *Gata3* genes are "poised" for expression upon specific signals in these cells (Wei et al., 2009). Recent studies including a study presented in this thesis (report III) investigated global mapping of enhancer elements during Th1 and Th2 differentiation that identified several lineage specific enhancers correlated with lineage specific gene expression profiles suggesting their role in determining cellular fate. Furthermore, lineage specific enhancer motif analysis and ChIP, followed by the next generation sequencing for pioneering TFs suggested the role of STATs in shaping the chromatin landscapes during Th cell differentiation (Wei L et al., 2010, Vadehi Get al., 2012, Hawkins RD et al., 2013).

Epigenetic regulation of Th17 and Treg Differentiation

Th17 and Treg cells are relatively new compared to classical Th1 and Th2 cells, and so far the epigenetic and regulatory mechanisms governing their differentiation and development have not yet been as extensively investigated. Th17 cells are marked by the secretion of signature cytokines, IL17A and IL17F, and it has been shown that

these cytokine loci are marked with H3 acetylation and H3K4me3, which are regulated in STAT3 dependent manner (Akimzhanov et al., 2007; Wei et al., 2007). Additionally, a regulatory CNS (CNS2) region localized upstream of *Il17a* cytokine loci is occupied by lineage specific TF RORγt. Moreover, global mapping of histone modification patterns showed that promoters of Th17 cytokines genes, *Il21*, *Il17a* & *Il17f*, *Il1r1*, *Il17re*, and key TF *Rorγt* were marked with active H3K4me3 mark and well correlated with the permissive expression pattern. On the other hand, *Il17* and *Il21* and *Roryt* promoter regions were marked with repressive H3K27me3 mark in other T helper lineages including, Th1, Th2, iTreg, and nTreg cells. Interestingly, *Gata3* and *T-bet* gene loci are associated with bivalent epigenetic profiles, suggesting that these cells are poised to be redirected towards Th1 and Th2 cells (Wei et al., 2009).This suggest that these epigenetic marks mediate selective gene expression patterns during Th cell differentiation. Moreover, other studies confirm the previous finding that lineage specific TFs, such as STAT3, IRF4, RORC controls chromatin accessibility at gene loci for Th17 genes including *Il17a*, *Il17f*, *Il23r*, *Ccl20*, *Il1r1*, *Ltb4r1* genes by regulating histone modifications in Th17 cells (Ciofani et al., 2012; Durant et al., 2010). However, there is still scope for integrative analysis of TFs and epigenetic modification mediated regulation of Th17 differentiation and development to understand the complete scenario.

nTreg and iTreg express FOXP3, and suppress various effector Th cell subsets (Th1, Th2, Th17), and Tfh cells (Bilate and Lafaille, 2012; Chaudhry et al., 2009; Chung et al., 2011; Curotto de Lafaille and Lafaille, 2009; Linterman et al., 2011; Rudra et al., 2012). It was shown that the difference in propensity of nTreg and iTreg cells for reprograming also depends on their epigenetic status, based on the histone modification and DNA methylation profile of the *Foxp3* and *Roryt* locus, respectively. Induced Treg cells express RORγt in a TGF-β dependent manner, but *Il17a* expression is suppressed. This is consistent with the observation that the *Il17a* locus is marked by H3K27me3, but the *Roryt* locus is marked by H3K4me3, suggesting that *Il17a* expression is inhibited by FOXP3. However, in nTreg cells (Wei et al., 2009; Zhou et al., 2008) it has been established that FOXP3 is a key lineage specific regulator of Treg differentiation and development. Additionally, in the recent past, various studies have focused on understanding epigenetic modifications, including methylation and acetylation of histone, and methylation of cytosine residue at CpG dinucleotides, at the *Foxp3* locus. It has been established that *Foxp3* locus is methylated at CpG dinucleotides in naive CD4+ T cells, stimulated CD4+ T cells, and iTregs, while nTregs show demethylation of the *Foxp3* locus and this process is governed by the DNA methyltransferases DNMT1 and DNMT3b (Baron et al., 2007; Janson et al., 2008; Lee et al., 2001; Liang et al., 2002; Nagar et al., 2008; Wieczorek et al., 2009). Likely, DNMT inhibitors induced *Foxp3* expression and increased the number of Treg cells (Floess et al., 2007; Zorn et al., 2006). Additionally, studies on comparative global analysis of DNA methylation profiles in conventional Tcells (conv T) and Treg

cells displayed a Treg-specific DNA hypomethylation signature that was correlated with the expression of genes important for Treg function, such as *Foxp3*, *Ctal4*, *Il2ra*, *Cd40lg*, *Ikzf2* (Helios), *Ikzf4* (Eos), and *Tnfrsf18* (GITR) (Ohkura et al., 2013; Schmidl et al., 2009; Wieczorek et al., 2009). In contrast, ChIP of H3K4me1 and H3K4me3 combined with the next generation sequencing identified lineage specific histone methylation patterns in human Treg cells (Tian et al., 2011). In this study, ChIPseq analysis demonstrated that enrichment of H3K4me3 at proximal promoter regions were similar both in CD4+CD25+FOXP3+ Treg and activated conventional CD4+CD25+FOXP3- T cells, including *CTLA4*, *IL2Ra*, and *TNFRSF18* gene promoters except for few promoters including *FOXP3* and *CCR7* were also Treg lineage genes. In contrast, H3K4me1, a mark for enhancer elements, was enriched over non-promoter distal regions and exhibited a high degree of lineage specific binding pattern, including Treg specific genes, such as *IL2RA*, *FOXP3*, *CTLA4* and *TNFRSF18*. These findings suggest that enhancer elements are important for driving lineage specific gene expression patterns in Treg cells, which is consistent with findings in other cellular systems, including various cancer cells, human ES and recently on Th1 and Th2 cells (Hawkins et al., 2010, 2013; Heintzman et al., 2009).

Role of ncRNAs in Th cell differentiation and development

Genetic deletion of the genes encoding for the miRNA-processing endonucleases, such as *Drosha* and *Dicer* showed disruption of machinery responsible for generation of miRNAs that regulate the stability and function of Th cells, suggesting that regulatory ncRNAs play an important role in Th cell differentiation and associated immune diseases (Chong et al., 2008; Liston et al., 2008; Monticelli, 2013; Pagani et al., 2013; Zhou et al., 2008). Several studies have attempted to build 'miRNome' or 'lincRNome' to identify the miRNAs and lincRNAs that drive Th cell differentiation and lineage commitment in mouse and human lymphocytes (Hu et al., 2013a, 2013b).

Two pioneer studies on genome wide profiling of miRNAs, using high-throughput RNA-seq, identified lineage specific miRNAs in nearly 50 immune cell types, suggesting that they have unique roles in regulating lineage specificity (Basso et al., 2009; Landgraf et al., 2007). Several studies have identified miRNAs that influence the development and function of the Th cells. For example, miR-125b is responsible for maintaining the naive state of precursor Th cells, miR-182 is involved in clonal expansion, miR-326 promotes Th17 differentiation, miR-146a has been shown to be involved in mediating Treg cell suppressor function (Lu et al., 2010; Pagani et al., 2013; Rossi et al., 2011; Stittrich et al., 2010), miR-155 regulates development of Treg cells and Th17 cells (Hu et al., 2013b; Kohlhaas et al., 2009; Yao et al., 2012), miR-10a, inhibits *Bcl6* expression and regulates the plasticity of Th cells (Takahashi et al., 2012), and miR‑17~92 cluster regulates Th1 cell

differentiation (Jiang et al., 2011b). Recently, miRNAs miR‑301a miR-21 and miR-146b were shown to regulate Th-17 differentiation (Liu et al., 2013; Mycko et al., 2012).

In contrast to miRNAs, lncRNAs utilize distinct molecular mechanisms to regulate gene expression and are regulated during normal physiological and disease states. Furthermore, lncRNAs have been shown to regulate T cell differentiation and function. For example, NRON lncRNA regulates trafficking and functional repression of NFAT (Sharma et al., 2011). Growth-arrest-specific transcript 5 (*Gas5*) arrests T cell growth (Mourtada-Maarabouni et al., 2008). LncRNA from the T early α promoter (*Tea*), Nettoie Theiler's Pas Salmonella (NeST), also called Theiler's Murine Encephalitis Virus Possible Gene1 (*Tmevpg1*), is selectively expressed in Th1 cells compared with Th2 and Th17cells and regulate the expression of *Ifnγ* (Collier et al., 2012; Gomez et al., 2013; Vigneau et al., 2003). Genome wide expression profiling of lncRNAs in mouse CD8+ T cells using custom array has indicated lncRNA mediated regulation of differentiation and activation of lymphocytes (Pang et al., 2009). Recently, genome-wide expression profiling of lincRNAs in various mouse T cell lineages has identified several lincRNAs showing highly dynamic and lineage-specific expression profiles (Hu et al., 2013a). Additionally, Pagani et. al. have claimed to have identified several lncRNAs obtained from a comprehensive transcriptome analysis of human lymphocytes subsets isolated from both peripheral blood and lymphoid tissues (Pagani et al., 2013). However, most of our current understanding relies on the studies regarding lncRNA and miRNA function in Th cells from mouse system, and relatively little is known in human Th cells and thus needs further attention. Additionally, the use of systems biology approaches in diseased patient samples will help to identify mutations associated with altered ncRNA expression and their correlation with disease state.

2.4 Inferring the significance of disease-associated SNPs in the gene regulation

GWASs have identified several disease-associated SNPs, and have provided insight into understanding the etiological process of complex diseases. However, many of these studies have failed to determine the functional phenotypic relevance of the SNPs. This is probably because many of the SNPs are located far from the gene-coding regions. Thus, to understand how the SNPs affect complex traits relies on the functionality of the genomic elements harboring them. It has been observed that 45% and 43% of these disease-associated SNPs are located in the intronic and intergenic regions respectively (Hindorff et al., 2009). Based on analyses of the expression quantitative trait loci (eQTLs), regulatory SNPs (rSNPs) have been shown to alter the expression of associated genes (Cheung and Spielman, 2009). Large projects, such as the NIH Road Map project, ENCODE Project and other important studies on genome-

wide assessment of epigenetic features, have shown that the genomic distribution of the SNP-associated complex traits were enriched in the genomic regions similar to gene regulatory regions, such as promoters and enhancers, (Boyle et al., 2012; Ciofani et al., 2012; ENCODE Project Consortium et al., 2012; Kasowski et al., 2013; Maurano et al., 2012; Schaub et al., 2012). Thus, SNPs potentially affect expression of the genes associated with disease phenotypes through regulating the chromatin accessibility and epigenetic features of these regions. Although, many of the functional SNPs are not lead SNPs but constitute other variants in linkage disequilibrium (LD) with the lead SNP. All these SNPs in the LD are regarded as functional and trait associated. Furthermore, few recent studies including our own have integrated SNPs from publicly available GWASs catalogs with epigenetic landscapes in distinct Th cell lineages to determine whether the disease-associated SNPs are potential regulatory SNPs (Ciofani et al., 2012; Hawkins et al., 2013). It has been observed that several SNPs are located within the binding motifs of biologically relevant TFs on cis-regulatroy modules (CRMs), such as enhancers and promoters. Further, experiments to determine whether the regulatory SNPs disrupt the TF binding sites over CRMs suggest their regulatory significance in modulating gene expression of associated phenotypic trait (Hawkins et al., 2013).

2.5 Future prospects in T helper cell differentiation and development

Recent studies have expanded our understanding on known and novel Th cell lineages and molecular mechanisms involved in their differentiation and lineage commitment. The advent of new experimental techniques and genome-wide approaches using next generation sequencing technologies has allowed us to understand the complex cellular systems at the level of "-ome"s, such as the transcriptome, proteome, miRNAome, epigenome and interactome. However, the integration of information obtained from these different "omic" datasets and the extraction of something meaningful from these data remains a challenge. Although, researcher have begun linking this information with GWASs studies on complex human disease traits, including inflammatory and autoimmune diseases, providing clues regarding the molecular mechanisms involved in pathophysiology of immune mediated diseases associated with Th cells, future studies will continue to characterize the functional significance of cis-regulatory modules on non-coding regions of the genome and characterize the functional relevance of regulatory SNPs in the context of associated human diseases. Additionally, over the next few years, studies will focus on systematically understanding the dynamic interactions among the genome, proteome and epigenome, identifying factors and mechanisms involved in shaping or regulating the epigenome. These questions will hopefully provide novel insight into the system level understanding of Th cell differentiation and lineage commitment and their implications in human health and diseases.

AIMS OF THE STUDY

The overall goal of the thesis was to study the transcriptional and epigenetic regulation of human Th cell differentiation - with a special focus on transcriptional regulatory mechanisms responsible for Th17 cell differentiation and epigenetic regulation of human Th1/Th2 cell differentiation.

The specific aims of this PhD thesis were to:

I Profile the gene expression changes during early stage of human Th17 cell differentiation.

II Characterize the role of STAT3 mediated transcription regulation during early human Th17 cell differentiation.

III Perform genome wide analysis of histone modification marks revealing the lineage-specific enhancer landscapes during the early stage of human Th1 and Th2 cell differentiation.

MATERIALS AND METHODS

4.1 $CD4^+$ T cell isolation from human cord blood

Naïve CD4+ T cells were isolated from umbilical cord blood of healthy neonates (Turku University Central Hospital, Turku, Finland) or peripheral blood (buffy coats) from healthy blood donors (Finnish Red Cross, Helsinki, Finland). Ficoll-Paque gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden) was performed to isolate mononuclear cells followed by CD4+ cell purification using α-CD4-coated magnetic beads (Dynal CD4+ Cell Isolation Kit, Invitrogen, UK). Purified $CD4^+$ cells from several individuals were pooled. The Molecular Immunology Laboratory at Turku Centre for Biotechnology has approval from the Finnish Ethics Committee for usage of blood of unknown donors. (*Publication I, II, and III*)

4.2 Culture and polarization of CD4+ T cells to Th1, Th2, Th17 and iTreg cells

For culturing Th1 and Th2 cells, naive CD4+ T cells were cultured in Yssel's medium (Yessels et al., 1984) supplemented with 100 U/ml penicillin and streptomycin plus 1% human AB-serum (Finnish Red Cross, Helsinki, Finland). Cells were stimulated with plate-coated anti-CD3 (Immunotech, Marseille, France) and soluble anti-CD28 (Immunotech, Marseille, France). Th1 cell polarization was initiated with IL12 and anti-IL4 was added to oppose Th2 polarization, and Th2 cell polarization was initiated IL4 plus anti-IFNγ to block Th1 polarization. To promote cellular expansion, IL2 was added to the cultures at 48h (for details see materials and method section in *Publication III*).

For culturing Th17 cells, plate-bound anti-CD3 (Immunotech, France) and soluble anti-CD28 (Immunotech, France) were used to activate CD4+ cells in X-vivo serum free medium (Lonza, Walkersville, USA) supplemented with L-glutamine (Sigma, Dorset, UK), and 100 U/ml of penicillin and streptomycin (Sigma, Dorset, UK). A cytokine cocktail of IL6 (Roche, Basel, Switzerland), IL1β and TGFβ in the presence of neutralizing antibodies (anti-IL4 and anti-IFNγ) for Th1 and Th2 cell polarization was used to initiate Th17 polarization. iTreg differentiation was initiated with TGFβ with neutralizing antibodies (anti-IL4 and anti-IFNγ) to oppose Th1 and Th2 cell polarization (for details see materials and method section in *Publication I and II*).

Activated Th0 cells were cultured only in the presence of neutralizing antibody (anti-IL4 and anti-IFNγ). All antibodies were purchased from R&D Systems (Minneapolis, USA) unless otherwise stated along with cytokines. The details of the protocol and

quantity of antibodies used in the studies can be found from the materials and methods and supplementary methods section of the original Publications presented in this thesis (*Publication I, II, and III*).

4.3 Gene expression and analyses

4.3.1 Microarray studies

4.3.1.1 Illumina gene expression analyses (Publication I)

Total RNA from three cultures was isolated with RNeasy Kit according to the guidelines from the kit (Qiagen, Valencia, CA, USA). Total RNA from the samples collected from ten different time points of culture from Thp, Th0 and Th17 cells (100-250ng) was processed and hybridized on Illumina Sentrix Human HT-12 Expression BeadChip, version 3 (#BD-103-0603, Illumina, Inc. San Diego, USA).

R package Limma (Wettenhall and Smyth, 2004) was used for quantile normalization and to identify differentially expressed genes with moderate t-statistics with false discovery rate (FDR) <0.1 from all time points. The whole time-series of gene expression data was further filtered for the probes with p-values <0.05 at least in one time point and one cell type. Moreover, the probes with a standard deviation >0.15 across all the samples were considered in the analysis. Gene annotations and pathway analysis was performed with Ingenuity Pathways Analysis software package (www.ingenuity.com). K-means clustering mathematical method was performed to obtain clusters from all over the gene expression data.

4.3.1.2 Affymetrix gene expression analyses (Publication II)

Total RNA from samples collected at 0, 2, 12, 24, and 72 hours time-points of culture, with control siRNA and STAT3 siRNAs for Thp, Th0 and Th17 cells from three independent cultures was extracted using the RNeasy Kit (Qiagen, Valencia, CA) and hybridized on Affymetrix GeneChip Human Genome U219 arrays.

The Affymetrix microarray data processing was performed as described earlier (Irizarry et al., 2003). Affy package from Bioconductor was used for quantile-normalization and log2-transformation of raw data (Gautier et al., 2004). Th17- and Th0-measurements in the control-siRNA data were used to identify Th17 specific genes using paired and moderated t-statistic with a false discovery rate (FDR) < 0.1 (Benjamini and Hochberg, 1995; Wettenhall and Smyth, 2004). The effect of STAT3 knockdown on the gene expression was examined using the Th17 measurements from STAT3 specific siRNA and control (scramble) siRNA samples. Genes were

considered as differentially expressed when they showed consistent change in three biological experiments.

4.3.2 Helicos sequencing for digital gene expression analyses (*Publication III*)

Total RNA (100-250ng) from naive CD4+, activated Th0, and Th1 and Th2 cells was isolated (Trizol, Invitrogen, California, USA) and processed following the recommendations of Helicos sample preparation guidelines for digital gene expression sequencing on Heliscope sequencing platform (Helicos BioSciences, Cambridge, USA). The raw RNA-seq data was filtered and aligned to RefSeq hg18 reference genome followed by transcript count with Helicos Helisphere (http://open.helicosbio.com/mwiki/index.php/Main_Page). DESeq was used to combine duplicate values and identification of differentially expressed genes (Anders and Huber, 2010). Genes were considered as differentially expressed when they showed consistent change in three biological experiments.

4.3.3 Gene expression analysis by quantitative real time-PCR (*Publication I*)

Total RNA isolation and cDNA preparation is described in the materials & methods section of the original communications (*Publication I and II*). Universal Probe Library probes (Roche Applied Science, Basel, Switzerland) or custom made double labeled probes for FAM (reporter), TAMRA (quencher) were used. The qPCR reaction mixture (primers, probes, DNA template, and ROX Mix {Thermo Scientific, Foster City , California, USA }) was amplified and run on 7900HT Real-Time PCR System (Applied Biosystems, Foster City, California 94404, USA) (enzyme activation for 15 min and 40 cycles of 15s at 95ºC, 1min at 60ºC). The primers and probes were designed using Universal Probe Library for Human (Roche Applied Science) or primer 3 (NCBI) software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The real time PCR method gives a quantitative value for each sample run as a cycle threshold (ct). The housekeeping gene EF1α was used as reference transcript to normalize the relative levels of gene expression of target mRNA (Hamalainen et al., 2001). The primers and probes (Oligomer Oy, Helsinki, Finland, or Roche Applied Science) used in thesis studies are listed in *Table 3*.

4.4 Protein expression analyses

4.4.1 Western blotting (Publication I, II, and III)

Cells were harvested, washed with PBS, boiled at 95 ºC and lysed in Triton-X lysis buffer (TXLB) supplemented with proteinase and phosphatase inhibitors respectively (Roche, Mannheim, Germany). The samples were then subjected to sonication with

mechanical shearing (Bioruptor UCD-200, Diagenode, Seraing Belgium) and centrifuged. The supernatant was collected and the protein concentration quantified (DC Protein Assay, Bio-Rad, Hercules, CA, USA). The required amount of protein was loaded on SDS-PAGE gels for protein separation and transferred to nitrocellulose or PVDF (polyvinylidene difluoride) membranes. After protein transfer, membranes were blocked with 5% nonfat dried milk or BSA (Bovine serum albumin, Sigma, UK) and 0.05-0.1% Tween 20/ TBS. The Immobilon™ Western (Millipore Corporation, Billerica, USA) and Super Signal West Pico (Thermo Scientific, Rockford, USA) chemiluminescent HRP substrate was used for visualization of proteins.

4.4.2 Flow cytometry (*Publication I, II, and III*)

Surface Staining of Chemokine receptors

For surface staining, cells were washed and stained in FACS I buffer (0.5 % FBS/0.1 % atzide/PBS) at 4ºC for 15 to 30min followed by two times washing with FACS I buffer. After staining, cells were dissolved in 1% formalin-PBS. The staining was controlled with isotype specific antibodies. Cells were run on the LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using the Flowing software (Cell Imaging Core, Turku Centre for Biotechnology, Finland) or CyflogicTM software (CyFlo Ltd, Turku, Finland).

Intracellular staining of cytokines and protein

Preparation, processing, staining of intracellular proteins and their subsequent analysis by flow cytometer is described in the materials and methods section of the original communications presented in this thesis (*Publication I, II and III*).

The detailed information about antibodies used for the protein expression analysis is listed in *Table 4*.

4.4.3 Cytokine secretion analyses (*Publication I and II*)

The secretion of IL17A in cell culture supernatant was detected using Millipore MILLIPLEX® MAP Kit (Human Cytokine / Chemokine, 96-Well Plate Assay, Darmstadt, Germany) on day 3. The assays were performed in triplicate according to the manufacturer's recommendations. The number of living cells detected, based on forward and side scattering, in flow cytometric analysis (LSRII flow cytometer), was used to normalize the cytokine secretion for each sample.

4.5 Human CD4+ T cell nucleofection with siRNAs (*Publication II*)

Naïve CD4+ T cells were nucleofected (pulse electroporation) with control scramble siRNA and three individual siRNAs for STAT3 according to the previously described protocols (Tahvanainen et al., 2006). In short, CD4+ T cells (4-5 x 10^6) were nucleofected with three individual STAT3 siRNAs for STAT3 or non-targeting control siRNA (5µg siRNA, all from Sigma, Dorset, UK) followed by a resting period of 48 hours before culturing. The nucleofection was performed in 100µl Opti-MEM (Invitrogen, Carlsbad, CA, USA) with a Nucleofector™ device using the U-014 program (Amaxa, Cologne, Germany). After nucleofection, cells were kept in complete RPMI 1640 medium (RPMI 1640, 10% FBS, L-glutamine, and penicillin/ streptomycin antibiotics) in a 37 ºC incubator for 48 hours resting period before activation.

4.6 DNA binding assays (*Publication II and III*)

4.6.1 ChIP and ChIP-seq

Chromatin immunoprecipitation is a powerful method used to identify and characterize DNA- protein interaction involved in gene regulation in living cells. ChIP was carried out as previously described (Hawkins et al., 2010). In short, cells were fixed with 1/10 volume of formaldehyde cross-linking solution for 10 min, and after that quenched with 1/20 volume of 2.5 mM glycine solution for 10 min followed by washing with ice-cold PBS. Cells were pelleted and chromatin was prepared as described in the original article (*Publication II, III*). Sonication was performed using a bioruptor for chromatin fragmentation. The DNA fragments were analyzed with agarose gel electrophoresis. Most of the DNA fragments were found to be in range of 500–100 bps. Samples were spun at maximum speed for 10 min to remove cell debris as a pellet, and the supernatant was used for ChIP. A small amount (20-25ul) of chromatin supernatant was saved for the DNA input control. Chromatin supernatants were then immunoprecipitated with specific antibodies and processed further as previously described (Hawkins et al., 2010).

For ChIP-seq, DNA libraries were generated according to the Illumina recommendations, and sequencing was performed using either an Illumina Genome Analyzer GAII or Illumina Hiseq 2000 (Illumina, Inc., San Diego, USA), generating 10-25 x 10^6 reads per sample. Sequenced reads were aligned to the human reference genome (version hg18 or 19) using Bowtie (Langmead, 2010; Langmead et al., 2009a, 2009b). Only uniquely mapped single read/genomic location were taken for further analysis. Background read density was corrected from Input measurements. TF binding regions were recovered with MACS software (Zhang et al., 2008b).

Based on the ChIP-seq peaks on UCSC genome browser (UCSC Genome Informatics Group, University of California, Santa Cruz, CA, USA) representing protein- DNA binding regions, DNA binding sites were selected for further validation by quantitative real time PCR analysis using Universal Probe Library probes (Roche Applied Science) with custom ordered oligonucleotides. The ChIP-qPCR results were represented as a percentage of the input values according to the following calculation: $100 * 2^{(\text{Input Ct} - [\text{ChIP}]),}$ where input values were set to 100% (Elo et al., 2010). The primers and probes used in the study are listed in *Table 5 and 6*.

4.6.2 DNA affinity precipitation assay (*Publication II and III*)

DNA affinity precipitation assay (DAPA) experiments were conducted as described earlier (Cesi et al., 2005; Lo et al., 2008; Pan et al., 2009b) with minor modifications. Biotinylated and antisense bait oligonucleotides were purchased from Oligomer (Helsinki, Finland), as listed in *Table 7 and 8*. TFs specific oligo baits and mutant oligos, where mutation is incorporated in TF-specific sites, were selected based on the DNA binding region from TF-specific peaks of ChIPseq data visualized on the UCSC browser. In the oligonucleotides, only sense strands were subjected to label with biotin and antisense strands were unlabeled. Detailed protocol of the method is described in the *publications II and III*.

4.7 TF binding prediction analysis (*Publication II and III*)

TF binding prediction analysis was performed using ProbTF (Lähdesmäki et al., 2008) and TRANSFAC (Matys et al., 2006) software pipelines.

4.8 Disease association analysis.

The dbGaP catalog was used to collect disease-associated SNPs and disease annotations of genes were obtained from the National Human Genome Research Institute Genome wide Association Catalog (NHGRI GWAS, NIH at Bethesda, Maryland, USA). The method is described in detail in the materials and methods sections of *publication II and III*.

Table 3: Primer list and their respective probes for RT-qPCR assays (*Publication I*)

Gene	**1) 5' -PROBE- 3'**
	2) 5' -PRIMER 1- 3'
	3) 5' -PRIMER 2 -3'
BTBD11	1) Universal Probelibrary probe #64
	2) 5'- CCCCAGGGTGATATGAACTC -3'
	3) 5'- TTGCGGAACACATTCCTGT -3'
COL6A3	**1) Probe #31**
	2) 5'- TGTGCAAGATTCTGGTATGGA -3'
	3) 5'- TGATGACTCCGGGTTTGG -3'
EF1a	1) 5' -AGCGCCGGCTATGCCCCTG- 3'
	2) 5' -CTGAACCATCCAGGCCAAAT- 3'
	3) 5' -GCCGTGTGGCAATCCAAT- 3'
IL17F	1) Universal Probelibrary probe #10
	2) 5'- GGCATCATCAATGAAAACCA -3'
	3) 5'- TGGGGTCCCAAGTGACAG -3'
MIAT	**1) Probe #75**
	2) 5'- GAGTGTGTGTGCATCTTGACAAT-3'
	3) 5'- GAGGGGTCGAAGAGAATGTG -3'
RORC2	1) Universal Probelibrary probe #87
	2) 5'- AGACTCATCGCCAAAGCATC -3'
	3) 5'- TCCACATGCTGGCTACACA -3'

Table 4: Primary antibodies used in the immunodetection and flow cytometry studies (*Publication I, II, and III*)

Product	Catalog number	Company
ATP1B1	GTX113390	GeneTex, CA, USA
BATF	pab4003	Brookwood Biomedical, AL, USA
Cathepsin L (33/1)	sc-65393	Santa Cruz, CA, USA
FOXP3	ab2481	Abcam, UK
FVT1 (KDSR)	sc-100589	Santa Cruz
GATA3	558686	BD Pharmingen^{Tm} USA
Histone H2B	sc-10808	Santa Cruz
ITM2A	18306-1-AP	ProteinTech Group, IL, USA
Nap-22 (H-100) BASP1	sc-66994	Santa Cruz
NAPSIN A	ab9868	Abcam, UK
NOTCH1	sc-56014	Santa Cruz
RORγ (H-190)	sc-28559	Santa Cruz
Runx1	sc-28679	Santa Cruz
STAT3	9132L	Cell Signaling Technology MA, USA
TBX21	561262	BD Pharmingen^{Tm} USA
Vitamin D Receptor	MA1-710	Thermo scientific, MA, USA
CCR6-PE	559562	BD, USA
CD122 (IL2RB) Mouse Anti-Human PE	554525	BD
CD52 anti-human APC	316008	Biolegend, CA, USA
CXCR5 Alexa Fluor® 488 Rat Anti-Human	558112	BD Pharmingen^{Tm} USA
FAK (H-1) (PTK2) -PE	sc-1688-PE	Santa Cruz
IL-17A -FITC	11-7179	eBioscience, CA, USA
Lamin A/C (LMNA) PE	sc-7292-PE	Santa Cruz
phosphoSTAT3(pY705)	9131L	Cell Signaling Technology MA, USA
STAT6	MA-120	Santa Cruz

Table 5: Primer and probe sequences used in ChIP-qPCR assays for validation of predicted STAT6 binding sites over selected Th2 specific enhancers (*Publication III*).

			Probe sequence
	Oligo name	**5 `- oligo sequence -3 `**	**5 `- 6(FAM) probe sequence (TAMRA)-3 `**
1	**SEPTB1_1**	CAAAAGGAGCAGCTAGAAGAG	GGTGGGAGAAAGAGGAAGCT
	SEPTB1_2	TATAAAGGGCTCCCAGGACA	
2	**GAB2_1**	CTCCCTGACATCCGAACATT	CCCCAATGCTCAGTTCTGAG
	GAB2_2	TCACAGCAATTCGATGGTTT	
3	**RNF125_1**	CTGATATCCCATCCCCAGAA	CAACAATGAAGTGTTCTGTGTGC
	RNF125_2	TTATAAAAATGCAGTATTTGGTTTTC	
4	**IL10RA_1**	CTCAGCCTCCTTCCCTGAA	CCTAGGGAAAGAGCTCAGGC
	IL10RA_2	GCGAGTTCCCCTCAGAGTTA	
5	**FOXP1_1_1**	TTCCTTTATGCGACACTGGA	TGCCTGAAAAAGCAGAGACC
	FOXP1_1_2	GGTGACGCCTGACACCAT	
6	**ABHD6_1**	GAGCTGTCAGCAATCCTGTG	ATGCACAGACTGCTTCCTCC
	ABHD6_2	CAACCACTGTGTAAATGTCACTTG	

Table 6: Primer list and their respective probes for STAT3 targets by ChIP-qPCR validation (*Publication II*)

	Oligo name	5 `- oligo sequence -3 `	ProbeLibrary probe#
1	**BATF_pro_1_11_**	TGA AGT TTC CGC CCA TGT	11
	BATF_pro_2_11	GCA CGC TCT CTC TCT CTC TTG	
2	**STAT3_pro_1_52**	CTG GCT GTT CCG ACA GTT	52
	STAT3_pro_2_52	CCC AAG TCC TCG GCT CTT	
3	**MYD88_pro_1_50**	CCT CGA GAC CTC AAG GGT AGA	50
	MYD88_pro_2_50	GCG CTT CCT CTT TCT CCT G	
4	**JAK3_pro_1_12**	AGG CAG CGA GAG GAA AGT C	12
	JAK3_pro_2_12	GCC CTG ACT TTC GGT AAA TG	
5	**ZNF460_pro_1_75**	GAA ATG GGA GTA CTG GGC TTC	75
	ZNF460_pro_2_75	GAC TTC CCG TAC CCT GAG GT	
6	**RELB_pro_1_14**	AAA CGG CAG GTT CAA GTC C	14
	RELB_pro_2_14	ATC ACG CCT TAC CCA TTG AG	
7	**IRF7_pro_1_49**	GGA CGG GAA GTT TCG TCT C	49
	IRF7_pro_2_49	TGG TCG CAT CCA ATA ATA AGA A	
8	**JUNB_pro_1_12**	GAA ACC CCT CAC TCA TGT GC	12
	JUNB_pro_2_12	AGG GGC TCA AAG GAC CTC	
9	**ICAM1_pro_1_88**	CTT GGA AAT TCC GGA G	88
	ICAM1_pro_2_88	TGC AGT TAT TTC CGG ACT GA	
10	**BATF_in_1_12**	AGA GGG GGC GAA AAG	12
	BATF_in_2_12	GTT GGT AAG ACG GGA ACT GG	
11	**PTPRCAP_in_1_52**	TCT GGC CCT GTG AGA TCA G	52
	PTPRCAP_in_2_52	TGT TGG GGG AGG TGA GTG	
12	**HNRNPH2_in_1_52**	TCC GCC TCT TTC GTT CTC T	52
	HNRNPH2_in_2_52	CTT CCC GGC ACT GAG ATG	
13	**NOTCH1_in_1_75**	ACC GGG TGA CAG GAG CTA	75
	NOTCH1_in_2_75	AAA GAA GAG GAA GGA GGC TCA	
14	**MED16_pro_1_3**	TTG CAT ACG ACC ATT TCC AG	3
	MED16_pro_2_3	GAA AGT GCT CGT TGT TCT ACC	
15	**RUNX3_inter_1_81**	TGG CTT CCA CTT CTT AGA ATC C	81
	RUNX3_inter_2_81	CCC CCT TCC CGT AAA TGA	
16	**SOCS1_inter_1_54**	CTG ACG TTG GTC CCC ATC	54
	SOCS1_inter_2_54	CGA TAA CGC TTG TTG AAA CCT	

Table 7: Bait Oligonucleotides used to validate SNPs on transcription TF motif over identified enhancer elements (*Publication III*)

	Oligo name	5 `- oligo sequence -3 `	Modification
1	**STAT6_sense**	5`-Biotin-GGATCCGAGAGGTTTCCGGTGAATGTTAGA-3`	Biotinylation
	STAT6_antisense	5`-TCTAACATTCACCGGAAACCTCTCGGATCC-3`	
2	**STAT1_sense**	5`-Biot- TGGACAAACGGTTTACGGAAGGTGAGGCTG-3`	Biotinylation
	STAT1_antisense	5`- CAGCCTCACCTTCCGTAAACCGTTTGTCCA-3`	
3	**STAT1_ (m) sense**	5`-Biot- TGGACAAACGGTTTACAGAAGGTGAGGCTG-3`	Biotinylation
	STAT1_(m) antisense	3`- CAGCCTCACCTTCTGTAAACCGTTTGTCCA-5`	
4	**CREB_sense**	5`-Biot-GTTATAAGGACTAACTTGTTCCATGGCTAT-3`	Biotinylation
	CREB_antisense	5`- ATAGCCATGGAACAAGTTAGTCCTTATAAC -3`	
5	**CREB_ (m)sense**	5`-Biot-GTTATAAGGACTAATTTGTTCCATGGCTAT-3`	Biotinylation
	CREB_(m) antisense	5` -ATAGCCATGGAACAAATTAGTCCTTATAAC-3`	
6	**PPARG_sense**	5`-Biot- ACCAGCAAACAGCGTCACCACCACCCTCTC-3`	Biotinylation
	PPARG_antisense	5` -GAGAGGGTGGTGGTGACGCTGTTTGCTGGT-3`	
7	**PPARG_ (m)sense**	5`- Biot-ACCAGCAAACAGTGTCACCACCACCCTCTC-3`	Biotinylation
	PPARG_(m) antisense	5` -GAGAGGGTGGTGGTGACACTGTTTGCTGGT-3`	

Table 8: Bait oligonucleotides used to validate SNPs on STAT3 binding sites from ChIP-seq study (*Publication II*).

	Oligo name	5 `- oligo sequence -3 `	Modification
1	**IL10_sense**	5`-Biotin-GCA GAG CGT GAG GGG GAC TAG TGT TTA CT-3`	Biotinylation
	IL10_antisense	5`-AGT AAA CAC TAG TCC CCC TCA CGC TCT GC-3`	
2	**IL10_(m)sense**	5`-Biotin-GCA GAG CGT GAG GGA GAC TAG TGT TTA CT-3`	Biotinylation
	IL10_(m)antisense	5`-AGT AAA CAC TAG TCT CCC TCA CGC TCT GC-3`	
3	**PFKFB3_sense**	5`-Biot- ACC CTT GGT CTC TCG GAA TGC TAT TTT TT-3`	Biotinylation
	PFKFB3_antisense	5`- AAA AAA TAG CAT TCC GAG AGA CCA AGG GT-3`	
4	**PFKFB3_ (m) sense**	5`-Biot- ACC CTT GGT CTC TCA GAA TGC TAT TTT TT-3`	Biotinylation
	PFKFB3_(m) antisense	5`- AAA AAA TAG CAT TCT GAG AGA CCA AGG GT-3`	
5	**IL7R_sense**	5`-Biot-CAA ATA TTT CCT GAG TTT TTT TAT GAA-3`	Biotinylation
	IL7R_antisense	5`- TTC ATA AAA AAA CTC AGG AAA TAT TTG -3`	
6	**IL7R_ (m)sense**	5`-Biot-CAA ATA TTT CCT GAA TTT TTT TAT GAA-3`	Biotinylation
	IL7R_(m) antisense	5`- TTC ATA AAA AAA TTC AGG AAA TAT TTG -3`	
7	**NDFIP1_sense**	5`-Biot- AGG AAA TGT ATA GGA AAT GAT TGG ATC AT-3`	Biotinylation
	NDFIP1_antisense	5` -ATG ATC CAA TCA TTT CCT ATA CAT TTC CT-3`	
8	**NDFIP1_ (m)sense**	5`-Biot- AGG AAA TGT ATA GGG AAT GAT TGG ATC AT-3`	Biotinylation
	NDFIP1_(m) antisense	5` -ATG ATC CAA TCA TTC CCT ATA CAT TTC CT-3`	
9	**SETD1A_sense**	5`-Biot- AGC TCT GCC CTC CCC GCA AAC GCC AGC CT-3`	Biotinylation
	SETD1A_antisense	5`- AGG CTG GCG TTT GCG GGG AGG GCA GAG CT-3`	
10	**SETD1A_ (m1)sense**	5`-Biot- AGC TCT GCC CTC CCG GCA AAC GCC AGC CT-3`	Biotinylation
	SETD1A_(m1) antisense	5`- AGG CTG GCG TTT GCC GGG AGG GCA GAG CT-3`	
11	**TERC_ sense**	5`-Biot- TTC ACA AGC CCC CAT TGC CGG CGA GGG GT-3`	Biotinylation
	TERC_antisense	5`- ACC CCT CGC CGG CAA TGG GGG CTT GTG AA-3`	
12	**TERC_(m)sense**	5`-Biot- TTC ACA AGC CCC CAC TGC CGG CGA GGG GT-3`	Biotinylation
	TERC_(m)antisense	5`- ACC CCT CGC CGG CAG TGG GGG CTT GTG AA-3`	
13	**STAT3_1_ sense**	5'- TCC CCC CAC CAC TTC CCG GAA TAG CCC CAC -3'	Biotinylation
	STAT3_1_antisense	5'- GTG GGG CTA TTC CGG GAA GTG GTG GGG GGA -3'	
14	**STAT3_1_m1_ sense**	5'- TCC CCC CAC CAC TAT CCG TCA TAG CCC CAC -3'	Biotinylation
	STAT3_1_m1_antisense	5'- GTG GGG CTA TGA CGG ATA GTG GTG GGG GGA -3'	

RESULTS AND DISCUSSION

5.1 Profiling of the gene expression changes during early stage of human Th17 cell differentiation (*Publication I*)

5.1.1 Global transcriptional profiles at the early stage of human Th17 differentiation

During recent years, most of the studies of Th17 cells were involved in understanding and establishing their role in the pathogenesis of inflammation, autoimmunity, and cancer. However, for a precise understanding of these processes, it is essential to characterize the key signaling pathways and molecular mechanisms involved in the fate decision during the differentiation of Th17 cell lineage. To address this question, we performed global transcriptional profiling of *in vitro* cultured human CD4+ cells during the early stages of Th17 polarization. As a first step, we set up and established Th17 polarization conditions suitable for these measurements. For Th17 polarization, umbilical cord blood-derived naïve CD4+ T cells were activated with anti-CD3 and anti-CD28 in the presence of a cytokine cocktail of IL1β, IL6, and TGFβ, as well as neutralizing antibodies against IL4 and IFNγ. Th17 polarization was confirmed by analyzing the expression of marker genes such as *IL17F*, *RORC*, and *CCR6*, and by analyzing the secretion of IL17A (*Publication I- Figure 1A-C*). The selective upregulation of these marker genes under Th17 conditions showed successful polarization of the naive CD4+ T cells toward the Th17 cell lineage. Global transcriptional profiling during early stages of human Th17 polarization was then performed using Illumina Sentrix Human HT-12 Expression Version 3 Bead Chips. The samples for microarray analysis were collected at nine time points during the culture, starting from 0.5 hours until 72 hours (*Publication I- Figure 1D*). Microarray gene expression analysis revealed two waves of transcriptional changes in the cells polarized towards Th17 stimulation compared with TCR activation; the first wave falls within the first 4 hours of culture, and the second wave peaked after 6 hours (*Publication I-Figure 2*). The selected time points covered the early gene expression changes in the transcriptome, as very few genes were differentially expressed at the earliest 0.5 hours time point after which the number of differential expression increased (*Publication - Table S3*). The first wave of transcriptional changes peaked at 2 hours of polarization, where 256 probes representing 228 genes were differentially expressed. Additionally, most of the differentially expressed genes were up-regulated, suggesting stimulation of several signaling pathways in response to the Th17 inducing cytokine cocktail. In the second wave of transcriptional changes, which ranged from 6 hours to 72 hours, there was a large increase in the number of differentially expressed

genes, which peaked at 72 hours with 799 differentially expressed probes (*Publication I-Figure 2*). Out of 799 probes, 416 and 383 probes were up-regulated and down-regulated, respectively, indicating the regulation of Th17 polarization both by enhancing the signaling pathways that positively drive the differentiation and by shutting down the signaling pathways which negatively regulate the differentiation.

5.1.2 Dynamics of transcriptional changes during the early stage of human Th17 cell polarization

Gene annotation analysis revealed that the wide range of the differentially expressed genes were enzymes, kinases and transcription regulators. Additionally, the expression of cytokine and chemokine receptors (*Publication I-Table 1*) and their localization on the cell surface or in the extracellular space (*Publication I-Table 2*) suggest that the polarization process was initiated at the early stages with activation of the pathways required to mediate signaling with the neighboring cells. However, the appearance of new differentially expressed genes at each time point during the course of differentiation suggested that the process of lineage commitment to mature phenotype continues even after the first three days of polarization (*Publication I-Figure 2*). Gene expression pattern during the early polarization towards Th17 differentiation was highly dynamic in nature, as revealed by the vigorous changes in the gene expression throughout the selected time frame. For example, at 2 hour time point, most of the genes were differentially expressed and regulated only at this time point. Equally, several genes were differentially expressed and regulated at the 72 hour time point (*Publication I- Table S3*). As an illustration of these differences, the gene expression pattern showing log fold change ratio between Th17 and Th0 condition at 2 and 72 hours are shown as heat maps (*Publication I- Figure 3A and 3B*). Among these differentially expressed genes, upregulation of several known Th17 cell specific genes, such as *RORC*, *RORA*, *AHR*, *BATF3*, *VDR*, *IL17F*, *IL9*, *CCR6*, and *IL23R*, support the authenticity of the cultures and analyses. Importantly, our microarray results also revealed several novel genes potentially important for the Th17 differentiation process. We could not, however, detect the IL17A probe as differentially expressed because it was not functional on the microarray Chip used in the study. Furthermore, based on the overall gene expression pattern throughout the selected time points, we performed clustering of the differentially expressed genes (Th17/ Th0) to learn their dynamic expression patterns (*Publication I-Figure 4, Table S4*). The clustering analysis suggested three basic expression patterns throughout selected time points: upregulation, downregulation and rather steady expression. Additionally, there were only small differences in differential expression during the earliest time points, which were then followed by gene expression profiles biased towards the selected direction, suggesting that the counter regulation to the stimulus

due to TCR activation and the differential expression at later stages is due to both either active up or downregulation of the differentially expressed genes.

5.1.3 Validation of selected Th17 regulated genes and their expression in other T helper subtypes

The simplest way by which differential mRNA expression can affect the phenotype is when it regulates the corresponding protein level in the cell. We validated a number of genes with the biggest and long-lasting differential gene expression between Th17 and Th0 conditions at protein level (*Publication I- Figure S1*) by immunoblotting and flow cytometry. We used genes both with known and unknown functions as selection criterion for validation. Immunoblotting results validated the protein level expression of BASP1, CTSL1, RUNX1, BATF, FVT1, NOTCH1, VDR and ATP1B1 in cells polarized towards Th17 cells compared to their expression in activated Th0 cells at 24, 48 and 72 hours time points (*Publication I-Figure 5A*). And in keeping with the observations from the microarray data, flow cytometric analysis showed the upregulation of CXCR5, IL2RB and LMNA, and down regulation of CD2 and ITMA2 under Th17 polarizing conditions compared to Th0 cells (*Publication I-Figure 5B*). However, the microarray data also revealed the upregulation of *COL6A3* and *MIAT* genes, and downregulation of *BTBD11* gene in Th17 polarizing cells. These genes were validated with quantitative RT-PCR detection at 24, 48 and 72 hours (*Publication I-Figure 5C*).

In order to further characterize the selectivity of the above validated genes in Th17 polarizing conditions, we cultured cord blood-derived naive CD4+ T cells under Th0, Th1, Th2, iTreg or Th17 polarizing conditions for 72 hours and analyzed the expression of selected genes at the protein level with immunoblotting or flow cytometry. In confirmation of the specificity of differentiation for the Th cell subset cultures, the protein expression of TBX21, GATA3, and FOXP3, the key TFs for Th1, Th2 and iTreg cells, respectively, and the secretion of IL17A, which is the marker cytokine for Th17 cells, were found to be preferentially expressed in corresponding Th cell subsets (*Publication I-Figure 6A-B*). Consistent with the results obtained from microarray analysis and the protein validation, the expression of ATPB1, KDSR, IL2RB and CXCR5 were selectively increased under Th17 polarizing condition compared with Th1, Th2 or iTreg induction (*Publication I-Figure 6C and 6D*). However, even though the expression of CSTL and VDR were higher in cells under Th17 polarizing conditions than in Th0 cells, which confirmed the results from the microarray study, the highest expression of these genes were detected in the cells cultured under iTreg polarizing condition (*Publication I-Figure 6C*), suggesting that TGFβ induced expression of these genes. Furthermore, the expression of CD52 was observed to be downregulated in Th17 polarizing condition as compared to other T

helper subset polarizing conditions (*Publication I-Figure 6D*). To summarize, these results revealed a number of genes selectively regulated under Th17 polarizing condition, as well as other genes non-specific for Th17 conditions that are also differentially expressed in polarizing conditions for other Th cell subsets.

In summary, the data from this study describe in detail kinetic changes in the transcription profiles during early stages of Th17 cell differentiation and provide a valuable resource for elucidating molecular mechanisms and gene regulatory network governing Th17 differentiation in human. Additionally, further characterization of the candidate genes possibly playing key role in Th17 differentiation process and function, may prove to be potential pharmaceutical targets for the diagnostics or treatment of Th17 cell-mediated immune diseases.

5.2 Regulation of early human Th17 cell differentiation by STAT3 (*Publication II*, unpublished)

TFs play key role in driving Th cells lineage specification and commitment. STAT3, an early key TF for Th17 cells, critical for Th17 cell differentiation (Ciofani et al., 2012; Durant et al., 2010; Iida et al., 2011; Miyahara et al., 2008; Wang et al., 2009a; Yang et al., 2007a). In spite of its importance, our current understanding of STAT3 and its role in Th17 differentiation is based on studies carried out in mouse, whereas very little is known in human. Moreover, since STAT3 is a key upstream regulator of Th17 differentiation, characterization of downstream targets of STAT3 in human is of particular interest. In this study, we combined RNAi, ChIP, genome-wide methods and computational data integration to identify STAT3 targets during the initiation of human Th17 differentiation. In addition, we used data from GWAS catalog to detect the overlap of STAT3 binding sites with a large number of SNPs associated with various autoimmune and other immune disorders, including CD, RA, T1D, PS and allergic diseases such as asthma. The findings suggested that alterations in TF binding site motifs by SNPs can influence transcriptional regulation and cell fate, and thereby contribute to immunopathogenesis. Collectively, the results present new insight into human Th17 differentiation and how it may be modulated in diseased states.

5.2.1 STAT3 affects the expression of Th17-associated genes

To investigate the contribution of STAT3 in the Th17 cell differentiation process, we perturbed its expression in human CD4+ T cells using RNAi and investigated the effect on gene expression during early stages of Th17 differentiation. STAT3-RNAi-mediated downregulation in the expression of STAT3 and its known target BATF, compared with nontargeting scramble siRNA, demonstrated the validity of the three

distinct siRNAs used (*Publication II-Figure 1A*). Moreover, downregulation of STAT3 resulted in reduced expression of CCR6 and decreased secretion of IL17A (*Publication II-Figure 1B, C*). These results revealed that STAT3 is also critical for human Th17 cell differentiation, which is consistent with the reports in murine T cells (Ciofani et al., 2012; Durant et al., 2010).

5.2.2 Identification of STAT3 regulated genes during early stage of Th17 differentiation

After validating the role of STAT3 in the initiation of human Th17 polarization, we next aimed to identify the genome wide targets of STAT3 during the early stages of this process. In order to achieve this, we analyzed and compared the effect of Th17 polarizing cytokine stimulation between cells nucleofected with STAT3-specific siRNA or non-targeting control-siRNA. Because we focused on the initiation of Th17 differentiation process, the genome wide gene expression changes were analyzed within three days in response to Th17 polarizing signals under STAT3-RNAi treated cultures and compared with cultures treated with non-targeting scramble siRNA. The results obtained were further compared with results from activated T cells nucleofected with STAT3-specific siRNA and control non-targeting scramble siRNA (Th0; *Publication II- Figure 2A*). Previously we have shown that the maximal changes in gene expression took place already at 2 hours in Th17 polarizing cells, compared to cells cultured in Th0 condition (Tuomela et al., 2012). Consistent with this previous finding, under control non-targeting scramble siRNA treatment, in total 2528 genes showed a change in gene expression under Th17 culturing conditions, compared with Th0 culturing conditions (FDR<0.1). Out of 2528 genes, 1091 genes were upregulated and 1437 genes were downregulated. Additionally, the cells cultured for 72 hours showed changes in the expression of 1713 genes, of which 879 genes were upregulated and 833 genes were down regulated (*Publication II- Table S1*). Among the upregulated genes were several well-known lineage specific genes, such as *RORC, RORA, BATF, IRF4, FOSL2, AHR, IL23R, CCR6, IL21, IL17F* and *IL17A*, suggesting that siRNA treated T cells were able to successfully polarize towards the Th17 phenotype.

To identify the global STAT3 targets at the early stages of Th17 differentiation, we analyzed and compared the gene transcription profiles from human CD4+ T cells treated with different siRNAs specific for STAT3 together with those with non-targeting scramble siRNA and polarized towards Th17 conditions at 2, 12, 24, and 72 hours time points. We observed that altogether 1654 genes were regulated in a STAT3 dependent manner. Out of these 1654 genes, STAT3 upregulated the expression of 727 genes and negatively regulated the expression of 927 genes. The down regulation of genes promoting Th17 polarization and upregulation of genes for other key lineages suggests the importance of STAT3 both in lineage specification and commitment

during the differentiation of Th17 cells by promoting Th17 polarization as well as via opposing alternative lineages. Interestingly, already at 2 hours in cells differentiating towards Th17 condition, downregulation of STAT3 influenced the expression of 301 genes. Out of these 301 genes, the expression of 185 genes was reduced; including CXCR5 and SOCS3, indicating STAT3 positively regulates the expression of these genes. Conversely, the expression of 116 genes were upregulated upon STAT3 knockdown, including *STAT1, STAT2* and *SMAD7*, suggesting STAT3 suppresses the expression of these genes (*Publication II-Figure 2B, Table S2*). However, the maximum number of genes regulated STAT3 were observed at 72 hours in differentiating Th17 cells, where it influenced the expression of 929 genes. (*Publication II-Figure 2B*).

To investigate the proportion of the Th17 polarizing cytokine-regulated genes that were under the control of STAT3, we compared the genes differentially expressed in Th17 conditions (*Publication II-Figure 2A*) with STAT3 regulated genes (*Publication II-Figure 2B*). We observed that already at the 2 hour time point around 7% of the STAT3 regulated genes were differentially expressed in Th17 conditions as compared to Th0 cells (*Publication II -Figure 2C*). Further, we observed that the proportion of STAT3 regulated genes was increased following differentiation time points. The highest numbers of genes were regulated by STAT3 at 72h with 36% of the total differentially expressed gene (*Publication II - Figure 2C*). Overall, these observations suggest that STAT3 is an early transcriptional regulator of the Th17 differentiation program. Interestingly, we observed that the *FOSL2, NTRK3, IL16, DUSP10* and *CASP1* genes were differentially expressed during the Th17 differentiation process as well as regulated by STAT3 at all the time points studied (*Publication II -Figure 2D*). Altogether, we detected 44 genes with at least 2.8 fold differences in gene expression that were regulated in Th17 differentiating conditions as well as influenced by STAT3 down-regulation (*Publication II-Figure 2E*). The gene expression patterns of these 44 genes were dynamic showing early and late regulation; for example *GBP4, COL6A3,* and *CXCR5* were regulated at the earliest time points that we studied during Th17 polarizing stimulation as well as STAT3 knockdown, while *CCR6, HOPX* and *CSF2* were regulated at the later stages. Additionally, several genes, including *PALLD, COL6A3, CXCR5, FLT1, RORA, IL23R* and *PHLDA1*, were expressed at multiple time points and regulated in STAT3 dependent manner (*Publication II -Figure 2E*). Interestingly, results suggest that STAT3 regulates the expression of genes involved in different cellular functions. For example, PALLD (Palladin) is a cytoskeletal protein involved in the control of cell shape, adhesion, contraction, migration and invasion (Brentnall, 2012; Mykkänen et al., 2001; Pogue-Geile et al., 2006). The role of PALLD in T cells is poorly characterized. A vascular endothelial growth factor receptor (VEGFR) family protein, FLT1 (Fms-Related Tyrosine Kinase 1, also known as VEGFR1) participates in the regulation of angiogenesis and vasculogenesis. It has been shown that CD45RO+ CD4+ memory T lymphocytes express FLT1, which

regulates IFNγ and IL17 production in memory T cells (Basu et al., 2010; Kim et al., 2010), suggesting that FLT1 plays a role in immune responses. PHLDA1 (pleckstrin homology-like domain, family A, member 1) is a proline-histidine rich nuclear protein shown to play a role in various tumors (Johnson et al., 2011; Neef et al., 2002). Interestingly, the expression of CXCR5, a chemokine receptor, was induced during Th17 differentiation and its expression was negatively regulated upon STAT3 downregulation by siRNAs specific for STAT3 (*Publication II -Figure 2E*). Previously, it has been observed that CXCR5 expression is induced by both IL6 and IL21 in a STAT3-dependent manner in Tfh cells (Nurieva et al., 2008). In line with microarray results, we validated the expression of CXCR5 in Th17 polarizing conditions and its regulation by STAT3 (*Publication II -Figure 2F*). Our findings confirm the STAT3 mediated regulation of CXCR5 and suggesting its role in Th17 cells.

5.2.3 Identification of direct targets of STAT3 by genome wide ChIP sequencing

After the identification of genes regulated by STAT3 during the early stages of Th17 polarization, we next investigated the direct targets of STAT3 using ChIP-seq. To define an optimal early time point for ChIP-sequencing, we first studied the kinetics of STAT3 phosphorylation after induction of human Th17 differentiation (*Publication II -Figure 3A*). The phospho-STAT3 kinetics results revealed three waves of phosphorylation patterns, with two major peaks at 0.5 hour and 48 hour after Th17 polarization. We observed a maximum phosphorylation signal of STAT3 (67% of pSTAT3 positive cells) at 0.5 hour post Th17 polarization. STAT3 phosphorylation is further increased and peaks at 48 hour. However, although the first 0.5 hour peak was Th17 specific, the second highest peak at 48 hours was also observed in TCR/CD28 activated cells, suggesting a TCR/CD28 activation dependent increase in STAT3 phosphorylation at this time point. Since, we were interested in identifying immediate direct targets of STAT3 in Th17 polarized cells by ChIPseq; we chose the 0.5 hour time point because at this time point the level of phospho-STAT3 peaked maximum in Th17 polarized cells compared with Th0 cells. Altogether, we identified 2981 STAT3 binding sites (*Publication II – Table S3*). Further analysis revealed that around 21% STAT3 binding sites were located in the immediate promoter region or transcription starting sites (TSS), which is consistent with the importance of STAT3, mediated regulation of transcription. Consistent with other reports on genome wide binding analysis of STATs proteins, we also observed that around 70% of the STAT3 binding sites were found in either introns (35%) or intergenic regions (36%), suggesting that STAT3 may regulate gene expression through binding to distal regulatory elements (*Publication II -Figure 3B*), (Elo et al., 2010; Wei et al., 2010). Further analysis revealed that around 50% of the STAT3 binding sites were localized within 10kb up-

and down-stream of TSS (*Publication II -Figure 3C*). Furthermore, in silico DNA motif analysis using TRANSFAC confirmed the enrichment of STAT3 motif as the strongest motif among the identified STAT3 binding sites (*Publication II -Figure 6B*).

We next extended our analysis by combining STAT3 ChIP-seq analysis with gene expression analysis of STAT3 RNAi to reveal direct targets of the Th17 polarization process. The results revealed that panels of genes regulated by STAT3 were in fact bound by it to their regulatory regions at 30 min after induction of Th17 polarization. Our results showed that STAT3 directly regulates 22% of the STAT3-regulated differentially expressed genes in polarizing Th17 cells at the 2 hour time point. Moreover, the proportions of direct STAT3 targets increased to 29% at 12h and 24h, and further decreased to 17% at 72h (*Publication II -Figure 3D*). Significantly, these analyses identified several previously reported direct targets of STAT3, including SOCS3, BATF and RORA (Chen et al., 2007). It has been established that STAT molecules are critical in driving Th subset differentiation, for example the roles of STAT1 and STAT4, STAT6, and STAT3 are well documented for Th1, Th2 and Th17 differentiation, respectively. Interestingly we observed that STAT3 regulated the expression of several STATs (STAT1, STAT2, and STAT4) including itself by direct binding to their promoters. Moreover, STAT3 binding sites were observed in the intergenic regions of the *FOSL2* and *DUSP10* genes, which were regulated upon STAT3 downregulation at all the measured time points. (*Publication II –Table S3*).

To further investigate the kinetics of STAT3 binding to its target genes, we selected 15 STAT3 ChIPseq binding sites annotated for various genes and validated these with ChIP-qPCR at the time points of 0h, 0.5h, 2h, 12h and 72h in cells differentiating towards Th17 and in Th0 cells that were activated only (*Publication II-Figure S2)* . ChIP-qPCR analysis revealed that STAT3 preferentially bound to the selected regions only the at 0.5h time point and under Th17 polarizing conditions (*Publication II - Figure 4*). Interestingly, STAT3 binding to these loci was lost at the later time points during Th17 polarization, suggesting temporal binding for STAT3 in regulating expression of associated genes. Thus, we were able to identify the early direct STAT3 targets involved in the initiation of Th17 polarization.

5.2.4 STAT3 mediated transcriptional network in Th17 cell polarization

Combined analysis of STAT3 ChIP-sequencing and gene expression allowed us to identify TFs regulated during early polarization of Th17 cells and regulated by STAT3. The maximum number of regulated TFs was found to be at 2h under Th17 polarizing conditions, where 279 and 185 TFs were upregulated and downregulated, respectively (*Publication II -Figure 5A*). Further overlap with gene expression upon STAT3 RNAi revealed that out of these, 20 TFs were regulated by STAT3, including *FOSL2* and *STAT3*, which were upregulated in response to Th17 polarization and

positively regulated by STAT3 (*Publication II -Figure 5B)*. In contrast, the expressions of TFs, such as *NPAT, ZNF211* and *NKAP* were downregulated in Th17 polarizing cells and negatively regulated by STAT3, as STAT3 downregulation increased their expression. Additionally, although the expression of *SMAD7, STAT1* and *STAT2* were upregulated during Th17 polarization, STAT3 knockdown further enhanced their expression, indicating that STAT3 suppresses their expressions (*Publication II –Figure 5C*). The largest number of TFs regulated by STAT3 was observed at 72h (*Publication II -Figure 5B*), suggesting the rationale behind maximum regulation of overall gene expression by STAT3 (*Publication II -Figure 2B*). Among these STAT3 regulated TFs, including *RORA, MAF, PPARG, RUNX1, HOPX,* and *RBPJ* were upregulated during Th17 polarization in a STAT3-dependent manner. In contrast to this, several TFs, such as *KLF3, GFI1, STAT4, GATA3* and *IKZF2,* were downregulated during early Th17 differentiation in a STAT3-dependent manner (*Publication II-Figure 5D*). Interestingly, a number of TFs, including *BATF, RORA, FOSL2, IKZF2, HOPX* and *AFF3*, were differentially expressed throughout multiple time points, implicating their role in determining the cellular fate of developing Th17 cells.

Combined analysis of the data from gene expression profiling with or without STAT3 downregulation by siRNA and STAT3 ChIP-Seq experiments, we divided the TFs into four categories. The first category of TFs encodes direct STAT3 targets, which were differentially expressed during Th17 polarization (Th17 vs Th0), regulated by STAT3 (STAT3 siRNA vs non-targeting scramble siRNA) as well as bound by STAT3 (STAT3 ChIP). The first category of TFs includes STAT1, STAT3, BATF, IKZF3, RUNX1, FOSL2, BCL6 and IRF9 (*Publication II -Figure 5E*). Notably, previous studies have shown the role of each of these TFs in the regulation of Th17 differentiation (Amadi-Obi et al., 2007; Ciofani et al., 2012; Hofer et al., 2012; Laurence et al., 2007; Quintana et al., 2013; Schraml et al., 2009; Zhang et al., 2008a). Each TF forms a highly coordinated network to further regulate Th17 cell differentiation (*Publication II – Figure S3*). The second category of TFs encodes indirect targets of STAT3, which are not bound by STAT3 but their expression was changed during Th17 differentiation and regulated by STAT3, suggesting that STAT3 indirectly regulates their expression through other factors. The third category encodes putative STAT3 targets that are bound by STAT3 and their expression regulated during Th17 differentiation but not regulated by STAT3. Interestingly, several TFs of this category, such as IRF4, NOTCH, RUNX3, IKZF4 and JUN, have previously been shown to be involved in the differentiation of Th17 cells or Treg cells (Brüstle et al., 2007; Fu et al., 2012; Keerthivasan et al., 2011; Klunker et al., 2009; Li et al., 2012; Sharma et al., 2013). The fourth category of TFs includes STAT3-independent TFs that were differentially expressed during Th17 differentiation but neither regulated nor bound by STAT3 (*Publication II –Table S6*). This category of TFs may cooperate with STAT3 or STAT3 regulated TFs to form the combinatorial

transcription regulation network, which regulates Th17 lineage specification via a highly coordinated transcriptional network (*Publication II -Figure S3*).

5.2.5 Association of disease-associated SNPs with STAT3 binding sites

Because Th17 cells were shown to participate in the development of various autoimmune diseases (Chen and O'Shea, 2008; Korn et al., 2009) and STAT3 is the major regulator of Th17 development, we used a SNP data base to examine whether disease-associated SNPs overlap with the STAT3 binding sites identified by STAT3 ChIP-seq analysis. We found that SNPs associated with PS, MS and CD significantly overlap with STAT3 binding sites (*Publication II – Table S7 and Figure 6A*). Equally, Immuno-Chip analysis showed that SNPs for 9 autoimmune diseases overlapped with ChIPseq STAT3 binding sites (*Publication II -Table S8*). We then searched for variant positions within the STAT3 motifs of STAT3 binding sites identified as overlapping with disease-associated SNPs (*Publication II - Figure 6B*). We observed multiple SNPs associated with autoimmune diseases within the STAT3 motifs (*Publication II - Table S8*). For example, a SNP (rs947474) associated with T1D and overlapping with a STAT3 motif was identified upstream of *PFKFB3* (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3) and *PRKCQ* (Protein kinase C, theta). The reference nucleotide for this SNP is G and the mutant is A. The G to A transition overlapped nucleotide 12 on the STAT3 motif, i.e., nucleotide A. Importantly, the *PRKCQ* gene regulate the expression of *Ifng* and *Il17*, and modulate immune response in an autoimmune disease model (Tan et al., 2006).

We further examined whether SNPs at the STAT3 binding motif of these predicted target genes could alter STAT3 binding. For these analysis, we performed DAPA assay using reference oligonucleotide sequences from the Hg19 harboring the predicted STAT3 binding site identified in our ChIP-seq data or point mutation (associated SNP) for selected from six disease-associated SNPs that overlap STAT3 binding sites (*Publication II – Table S4 and S8*). These were as follows: the CD, T1D and UC-associated SNP rs3024505, which is in a close vicinity of the IL10 gene; the T1D-associated SNP rs947474 nearby *PFKFB3, LOC399715* and *PRKCQ* genes; the PCB-associated rs17313508 nearby *IL7R, CAPSL* and *UGT3A1* genes; the MS associated SNP rs6580224 with nearest neighboring genes *NDFIP1, GNPDA1* and *SPRY4*; the PS-associated SNP rs12443808 located near *SETD1A, HSD3B7* and *STX1B*; and the Celiac disease (CeD) and MS associated SNP rs2293607 with location closest to *TERC* and *ACTRT3* genes. Consistent with our previous finding that SNPs can change the TF binding affinity, the binding of STAT3 was reduced when variant oligonucleotides harboring disease associated SNPs rs3024505, rs947474, rs6580224 and rs2293607 were used (*Publication II- Figure 6C*). However, STAT3 binding was increased when variant oligonucleotide for PS-associated SNP

rs12443808 was used. These results suggest how a disease-associated SNP may regulate STAT3 mediated transcription by affecting the expression of its targets important for early human Th17 cell differentiation in a disease setting.

5.3 Global mapping of lineage specific enhancer landscapes in early differentiating Th1 and Th2 cell lineages

The precise control of lineage specification and commitment and proper balance between these effector and regulatory lineages are critical to avoid various autoimmune disorders. To understand the process of lineage specification and commitment, we analyzed global chromatin state maps of histone modifications in an attempt to identify lineage specific enhancers in cells polarizing towards Th1 and Th2 lineages after 72 hours. ChIP-seq with antibodies specific for histone modification marks that distinguish between distal enhancer and proximal promoter regions (H3K4me1, H3K4me3 and H3K27ac) were used. To our surprise, even at this initial stage of differentiation, our analysis revealed several lineages specific enhancer elements correlating with cell specific gene expression changes. Further examination of TF binding on lineage specific enhancers revealed enrichment of known and novel T cell-specific TFs important for driving lineage-specific gene expression. Finally, we integrated enhancer analysis with immunopathogenic associated SNPs from a GWASs database, indicating the significance of these distal cis-regulatory elements in the disease etiology.

5.3.1 Global enhancer chromatin states identified lineage-specific enhancer elements in Th1 and Th2 differentiating cells

To investigate the process of lineage specification and commitment, we analyzed and compared global chromatin state maps of histone modifications in order to identify lineage specific enhancers in cells differentiated towards Th1 and Th2 lineages after 72 hours. ChIP-seq with antibodies specific for histone modifications (H3K4me1, H3K27ac, and H3K4me3), which discriminate enhancer and proximal promoter elements, respectively, were used (*Publication III - Figure S1A*). At 72 hours, cells were already directed to polarize towards Th1 and Th2 cells, based on the protein expression of lineage specific TFs, T-bet and GATA3 (*Publication III - Figure S1A*). After seven days, the expression of key lineage markers confirmed that the cells had correctly differentiated to Th1 and Th2 cell lineages in the polarization settings used in the study (*Publication III - Figure S1B and C*).

Depending on the genomic location of H3K4me1 compared to H3K4me3 (Heintzman et al., 2009), our analysis identified 16,507, and 13,466 enhancers in the Th1 and Th2 cell lineages respectively, compared with 16,552 enhancers in activated Th0 cell

lineage (*Publication III - Table S1A*). Further analysis suggested that at this initial stage of polarization, the enhancer patterns were relatively similar among activated and differentiating Th1 and Th2 cells (*Publication III - Figure 1B*). However, lineage specific enhancers can be determined using a recent analytical method based on nucleosome displacement (or nucleosome free regions (NFRs)) when comparing histone modification profiles between two samples (He et al., 2010) (*Publication III - Figure 1E-F, Figure 2, Figure S2, Table S1B*). Based on this method, our analysis resulted in subsequent number of lineage-specific enhancers: 1636 (Th0), 2144 (Th1), and 2654 (Th2) (*Table S2a*).

To determine whether identified lineage-specific enhancers are correlated with lineage specific genes during Th1 and Th2 differentiation, we analyzed gene expression profiles from control activated Th0 and Th1, Th2 differentiating cells using the Heliscope platform. Gene expression analysis identified significant number of differentially expressed genes between Th1/ Th0 or Th2/ Th0 (*Publication III - Table S4B*). Further, to correlate expression of these genes with lineage specific-enhancers, we determined lineage-specific gene upregulated in one culture condition relative to the other culture conditions. We identified 121and 292 genes expressed in Th1 and Th2 culture condition respectively, as well as 116 were upregulated in Th0 culture condition (*Publication III - Figure 1G; Table S4A; see Table S5 for all genes*). In support of these observations, the genes identified in this study were consistent with our previous findings (Elo et al., 2010; Lund et al., 2005, 2003, 2007) suggesting that these genes are crucial for the cellular specification and commitment to attain the desired cell fate. The observation that only a limited number of lineage-specific enhancers were correlated with lineage-specific genes emphasized the role of enhancers in guiding the lineage specific gene expression program. In summary, we have used enhancer maps to establish the first understanding of lineage-specific gene regulation in a population of cells that were not yet fully committed and largely similar, thereby suggesting the role of enhancers in driving gene expression changes critical for each lineage commitment.

5.3.2 Fate of lineage-specific enhancers during differentiation

Previous studies have reported that acetylation of H3K27 is associated with enhancer activity (Heintzman et al., 2009; Roh et al., 2007, 2005). However, there are subsets of enhancers that lack this modification and are deemed as “poised enhancers” (Creyghton et al., 2010; Hawkins et al., 2010; Rada-Iglesias et al., 2011). For example, more than 2,000 enhancers lacked H3K27ac in human ES cells (Hawkins et al., 2010; Rada-Iglesias et al., 2011), while, less than 50% enhancers overlapped with H3K27ac in mouse ES cells (Creyghton et al., 2010). Likewise, we also observed that this modification was absent in a large fraction of enhancers in all of the Th cell

subsets that we analyzed. Consistent with this finding, lineage-specific enhancers lacking acetylation were identified (*Publication III - Figure 2*), suggesting that these enhancers are in a 'poised state' and waiting for signals that enable loading of TFs to drive transcription. The identification of poised enhancers at this initial stage of cellular differentiation reflects that the cells are poised for commitment into distinct lineages.

The presence of DHS is indicative of an open chromatin structure for TFs binding and is reflect active regulatory elements. Thus, in order to investigate the fate of the Th1 and Th2 cell specific enhancers during the later stage of differentiation, we made use of DHS data from ENCODE for peripheral blood-derived Th1 and Th2 cells at day 7 (ENCODE Project Consortium et al., 2012; Thurman et al., 2012) to compare with our data at 72 hours. The results for the Th1 lineage indicated that 489 out of 653 enhancers active at 72 hours (K27ac) remain active based on their DHS status at day seven, suggesting that these enhancers are required throughout cellular differentiation. The remaining 155 lose DHS, suggesting that these enhancers are active and required for lineage commitment rather than maintenance and are not used any more at the later stage of differentiation. Additionally, around 300 enhancers poised at 72 hours instead become active (gain DHS later) at day 7 indicating that these enhancers are required to maintain cellular state at the later stage. However, majority, of the enhancers (~1200) are marked by H3K4me1 only and they have neither the K27ac mark nor gain DHS, suggesting that these enhancers might be used either at some point during differentiation or remain poised to alter the gene expression in response to other external stimuli. The Th2 results demonstrated that 835 of altogether 847 enhancers were active at 72 hours (K27ac) and remained active based on their DHS status at day seven. The remaining enhancers (~9) were observed to lose DHS. Approximately 1400 enhancers of the 1800 enhancers poised at 72 hours become active (gain DHS later) later at day seven. In contrast to the Th1 linage, only ~400 of the enhancers are marked by H3K4me1 alone and remained inactive in Th2 cells (*Publication III - Figure 2*). The above observations suggest that the epigenetic state of the cells has already been instructed to modulate future cell fate commitment.

5.3.3 Analysis of putative enhancer binders

TFs are key players in driving the enhancer activity through binding to these distal regulatory sites and facilitating their looping to promoter regions of the target gene and therefore in determining upregulation of the lineage-specific gene. Enrichment of TF motifs for lineage specific TFs over regulatory site of enhancers has been used to predict cell type-specific active enhancers that reflect the biology of that cell type. Previous studies have shown strong correlations between enrichment of binding motifs for expressed lineage specific TFs within active enhancers, suggesting that

lineage-specific TFs are key drivers of enhancer activity (Ernst et al., 2011; Hawkins et al., 2010; Heinz et al., 2010; Hoffman et al., 2010; Robertson et al., 2008). Motivated by these findings, we examined the enrichment of known TF motifs over enhancer sequences with ProbTF (Lähdesmäki et al., 2008) and TRANSFAC (Matys et al., 2006) tools and combined these with statistical analysis. The identified TF motifs were then filtered for the TFs expressed in each cell lineage, thus revealing both novel and known TFs regulating the expression of lineage specific genes during Th cell differentiation (*Publication III - Table S2*).

Further analysis showed that lineage-specific enhancer sequences were in fact enriched for known TF motifs specific for T cell (*Publication III - Table S3, Methods*). For example, activated Th0 cell specific enhancer sequences contain TF motifs for AP-1, NFAT, and NF-KB TFs, which play a key role in T cell activation (Ansel et al., 2006). TF motifs enriched for Th1 enhancers include key Th1 regulators, such as STAT1, STAT4, ATF3 and JUN. The role of these TFs in regulating Th1 polarization have been well documented (Afkarian et al., 2002; Filén et al., 2010; Jenner et al., 2009; Szabo et al., 2000). Lastly, Th2 cell-specific enhancer motifs were overrepresented for TFs, including STAT6, GATA3, NFIL3, GFI1, PPARG and BACH. The expression of all of these TFs were shown to be enhanced in differentiating Th2 cells and suggested to control several Th2-specific genes (Elo et al., 2010; Horiuchi et al., 2011; Kaplan et al., 1996; Kashiwada et al., 2011; Zhu, 2010). Thus, the integration of lineage-specific enhancer maps and computational tools for analysis of TF motifs, which were further filtered for TF expression, has revealed how key lineage specific TFs are likely exploit enhancer binding sites to drive lineage specification and commitment during Th cell differentiation and development. Additionally, we also observed that several lineage specific enhancers were selectively enriched for TFs motifs for expressed TFs, such as OCT, HNF, FOX family members whose role in T cell differentiation is not clear (*Publication III - Figure 3B-E; Table S3*). Further investigations into these novel regulators could help fill in the gaps in constructing the early regulatory networks controlling Th cell differentiation.

Additionally, we have validated a subset of enhancers and their cognate predicted TF motifs, using Th2 specific enhancers with predicted motifs for STAT6 TF. Chromatin-immunoprecipitation for H3K4me1, H3K27ac and STAT6 at 0h (Thp), 4 hours and 72 hours was performed for six Th2 specific enhancers containing STAT6 motif and quantitated using RT- PCR (*Publication III- Figure 4*). Our analysis identified that these enhancers are located nearby genes which are specifically expressed in Th2 cells, including the genes with known and novel function such as *RUNX1, IL10RA, FOXP1, SETBP1, GAB2 and ABHD6*. We showed that these enhancers, enriched with H3K4me1 and H3K27ac, are specifically bound by STAT6 in cells differentiated to Th2 cells relative to Thp and stimulated Th0 cells, indicating that STAT6 regulate

these genes in Th2 cell lineage in an enhancer-specific manner. Altogether, these data provide insight into the mechanistic aspects of both TF binding and chromatin state modifications in driving lineage-specific gene expression. Additionally, our results suggest the early marking of some enhancers loaded with TFs, yet unable to gain functionality until a later state based on acquisition of H3K27ac (*Publication III - Figure 4*).

5.3.4 Enhancers overlap with disease associated SNPs

SNPs from GWASs have been associated with several complex diseases. However, many of the studies are unable to determine the functional phenotypic relevance of the SNPs, probably because many of the SNPs are located outside of the gene-coding regions. From an evaluation of GWAS catalogued, it has been observed that 45% and 43% of these disease- associated SNPs are located in the intronic and intergenic regions, respectively (Hindorff et al., 2009). Regulatory SNPs are shown to alter the expression of genes associated with the expression of quantitative traits (Cheung and Spielman, 2009). Large projects, such as the NIH Road Map project, ENCODE Project and other important studies on genome-wide assessment of epigenetic features have shown that the genomic distribution of SNP- associated complex traits were enriched in the genomic regions similar to gene regulatory regions, such as promoters and enhancers, (Boyle et al., 2012; Ciofani et al., 2012; ENCODE Project Consortium et al., 2012; Maurano et al., 2012; Ni et al., 2012; Schaub et al., 2012). Thus, the studies suggest that SNPs potentially affect the expression of genes associated with disease phenotypes through regulating the chromatin accessibility and epigenetic features of regions implicated in gene regulation. To investigate whether disease-associated SNPs overlap with the Th enhancer elements detected in our research are regulatory SNPs, we used SNPs from the National Human Genome Research Institute-Genome wide Association Catalog (www.genome.gov/gwastudies) and overlapped these with enhancers identified for Th1 or Th2 differentiated or activated cells at 72 hours. We performed integrative investigation of the mapped disease-associated SNPs, which revealed that 1,281 SNPs associated with multiple autoimmune disease were distributed among all disease categories (*Publication III - Table S8; Table 1*), including MS, UC, CD, PS, RA, T1D, and asthma (Anderson et al., 2011; Barrett et al., 2009; Cargill et al., 2007; Duerr et al., 2006; Hafler and De Jager, 2005; International Multiple Sclerosis Genetics Consortium et al., 2007; Moffatt et al., 2010; Nair et al., 2009; Rioux et al., 2007; Stahl et al., 2010). The highest overlap was observed for RA and UC with each more than four hundred SNPs. CD and MS were among the minimally enriched SNPs (~40 SNPs). GWAS analysis for age-related macular degeneration (AMD) and age-Related Eye Disease Study (AREDS) served as a control for our analysis because this diseases have little or no relationship with Th cells and the observed enhancers overlap was insignificant.

5.3.5 Inferring functional significance of rSNPs on enhancer elements

We next examined that SNPs overlapped with enhancers detected specifically for cells polarized for Th1 and Th2 lineages as well activated Th0 cells. Our analysis revealed 76 associated SNPs (*Publication III - Table S10*) directly overlapping with lineage-specific enhancer predictions based on lineage-specific NFR. Out of 76 SNPs, a maximum of 27 and 23 SNPs were associated with RA and UC , nine with T1D, one with CD, two with MS, three with PS, and 11 for asthma (*Publication III - See Table S11 for lineage distributions*). These SNPs were correlate or associate with all Th cell lineages studied. We further tested whether these SNPs could modulate the transcriptional networks through assessing if SNPs located over enhancers were actually falling within identified TF motifs. We did in fact identify several SNPs falling within TF motifs of biologically relevant TFs. For example, we found that eleven SNPs associated with asthma were overlapped with identified lineage-specific enhancers. For example, rs2604931 SNP, positioned within an AhR/ARNT motif in Th0 lineage specific enhancer resulted in G/A switch. Studies have suggested that AhR is critical in regulating Th17 cell fates (Quintana et al., 2008). However, the role of AHR in Th1 or Th2 lineage specification is largely unclear. The predicted gene for this enhancer is *MAML3*, a transcriptional co-activator for Notch receptors. The role of the Notch pathway in determining cell fate during development as well as for Th cell differentiation has been documented (Amsen et al., 2004). In another example, we found five associated SNPs for T1D within TF motifs of Th1-specific enhancer sites. Interestingly, the rs13101295 SNP lies within the GR motif of one such Th1-specific enhancer. The predicted enhancer target gene was *RHOH*, a Rho GTPase important for Th1 differentiation (Gu et al., 2006; Li et al., 2002). Additionally, there were several other predicted gene targets of enhancers overlapping with SNPs including well-known genes specific for T cells (*Publication III - Table S2*). For example, predicted target genes, such as *AHRR, IER3* and *DUSP16*, were targets for enhancer SNPs in T1D. *BATF, IL2RA, PFKFB3, PRKCQ* and *ITPR3* were predicted targets of enhancers harboring SNPs associated with RA. Interestingly, we found that a SNP, rs7904311, is falling within the STAT1 motif of an intronic enhancer of the *IL2RA* gene associated with RA. IL2RA and STAT1 are known to take part in T helper cell proliferation and polarization. Moreover, predicted targets for enhancers overlapping SNPs, including *JAK3, PRM1, TNFRSF6B, IL6, NFKB, GATA3,* and *IL10* were associated with UC. It was notable that a SNP, rs406103, lies within the PPARG motif of a Th2- specific enhancer and *GATA3* as its predicted target gene. GATA3 is known as master transcriptional regulator of Th2 cell differentiation. PPARG TF was highly expressed in Th2-specific manner in our gene expression data (*Publication III - Figure 6*).

SNPs in the TF motifs on Th-specific enhancers could alter the TF binding, and hence presumably cause changes in the target gene expression potentially regulating Th cell differentiation and eventually contribute to the diseases etiology, because enhancers

are central for guiding the expression levels of their target genes during early Th cell differentiation. Thus, in order to determine if an rSNP associated with disease affect TF binding within enhancer elements, and can cause functional changes in the enhancer mediated gene expression, we validated subsets of SNPs enriched within predicted TF motifs at selected enhancers using DAPA assays. As the bait for DAPA, we used *in silico* designed oligo sequences derived either from the hg18 genome reference sequence across the only predicted motif or predicted motif carrying SNPs as single base-pair variants for selected lineage-specific enhancers. We tested three SNPs in the TF motif region at predicted lineage-specific enhancers, including the rs7904311 SNP-associated with RA at the STAT1 binding site on predicted enhancer targeting *IL-2RA* in Th0 cells; the T1D-associated SNP rs604388 at the CREB binding site on predicted enhancer targeting *SIRPG* in Th0 cells, and UC-associated SNP rs406103 at the PPARG binding site on predicted enhancer targeting *GATA3* in Th2 cells. As expected on the basis of the prediction analysis, CREB, PPARG, and STAT1 bound to reference bait sequence, while TF binding was reduced with oligonucleotides containing disease variant corresponding to the disease-associated SNPs (*Publication III - Figure 6D*). The results indicated that a SNP within TF motif at enhancers modulates TF binding, further indicating a likely mechanism through which SNPs could possibly modulate enhancer action and affect the expression of target genes.

SUMMARY

Selective specification of CD4+ T cells to differentiate into functionally distinct effector Th and Treg cell lineages is required to protect the body from pathogenic infections while defective differentiation program can result in various inflammatory and autoimmune diseases. Thus, elucidating the signaling pathways and regulatory mechanisms instructing the specification of Th cell lineages during differentiation and development is essential to understand the development of immune mediated diseases. Moreover, with technological advancement and increased knowledge of genomes, high-throughput analysis methods enable the identification of participating molecules and regulatory sites shaping the differentiation of Th cell lineages. In addition, most of our current knowledge on Th cell differentiation depends on studies performed in mouse, while the signaling pathways and molecular mechanisms guiding human Th17 cell differentiation are not well defined. The present study characterized molecules involved in human Th17 cell differentiation as well as the role of STAT3 in driving Th17 cell polarization by using genome-wide measurements. In addition, global chromatin state maps of histone modifications were analyzed to identify lineage-specific enhancer in cells polarizing towards Th1 and Th2 lineages.

Genome-wide gene expression profiling of *in vitro*–cultured human CD4+ T cells during the early stages of Th17 polarization identified known and novel genes involved in the process. In addition, our analysis revealed the dynamic regulation of these genes with respect to time throughout the early stages of Th17 differentiation. The differential expression of a few candidate genes was confirmed at the protein level by western blot and flow cytometry analyses. The selectivity of these candidate genes was further analyzed in other Th subsets, specifically in Th1, Th2 and iTreg cells. The dataset described provides a detailed resource for constructing the gene regulatory networks regulating the Th17 differentiation in human.

The role of STAT3 in regulating Th17 differentiation was investigated by using siRNA-mediated down-regulation of gene expression, genome-wide transcriptome profiling and ChIP- sequencing. Analysis of the data revealed several primary and secondary targets of STAT3 during the early stage of differentiation, including TFs, signaling molecules, enzymes, cytokines and chemokines. Further, a large number of SNPs associated with various immune disorders, including autoimmune diseases, such as CD, RA, T1D and asthma were found to be located at STAT3 binding sites. Finally, the integrated dataset on STAT3-mediated transcriptional network presented in this study will provide a basis for understanding and modulating Th17-mediated pathogenic immune responses in human.

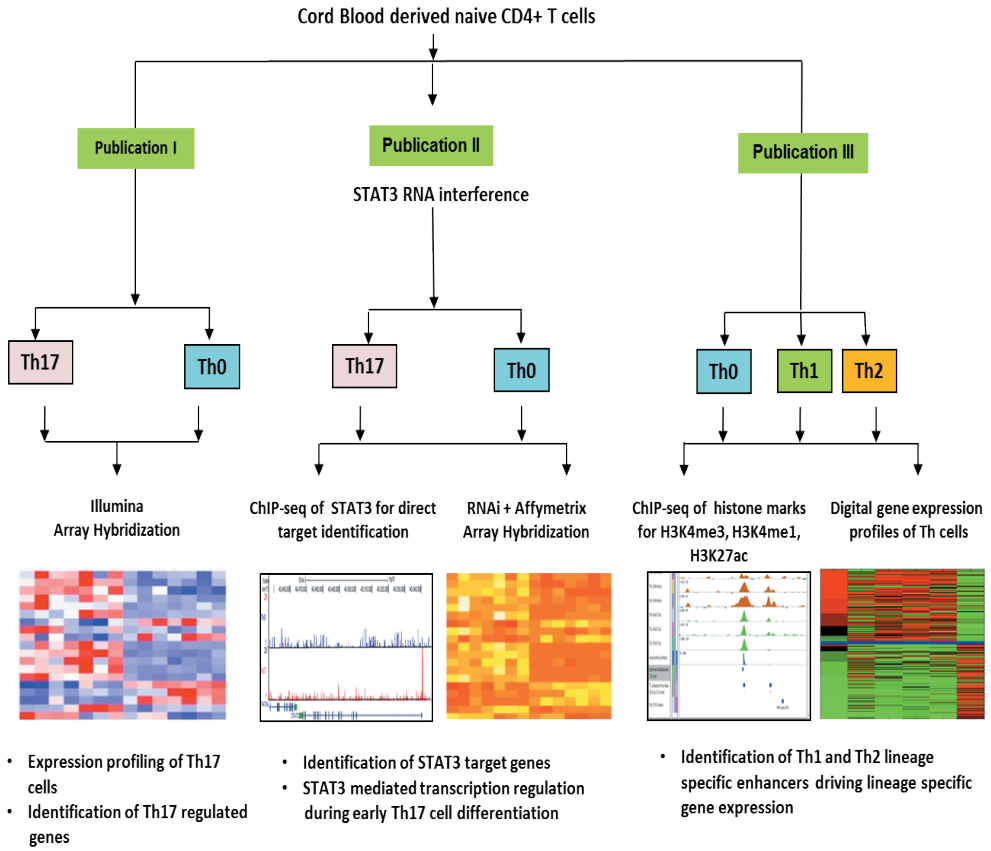

Figure 4: Schematic diagram showing the summary of the all studies presented in this thesis.

ChIP-seq with antibodies recognizing histone modifications was used to generate global chromatin state maps of histone modifications to identify lineage-specific enhancers in cells polarizing towards Th1 and Th2 lineages after 72 hours. Our analysis revealed that several lineages-specific enhancers are at work directing gene expression even at this early stage of Th1 and Th2 differentiation. Further examination of TF binding on lineage-specific enhancers revealed enrichment of known and novel T cell specific TFs important for driving of lineage-specific gene expression. Last but not least, integration of lineage-specific enhancers with immunopathogenic associated SNPs indicated significance of these distal cis-regulatory elements in the disease etiology.

In summary, the work presented in this thesis provides a detailed analysis of the transcriptional and epigenetic control of human Th cell differentiation using multiple approaches, including high-throughput analysis methods. It has revealed new information of how disease-associated SNPs in the gene regulatory elements can modulate transcriptional programs in Th-mediated pathogenic immune responses in human.

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