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# INTERLEUKIN-4 INDUCED LEUKOCYTE DIFFERENTIATION

by

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*Think where mans glory most begins and ends,*
*and say my glory was I had such friends.*

*William Butler Yeats (1865 – 1939)*

# ABSTRACT

**Helena Ahlfors**
**Interleukin-4 induced leukocyte differentiation**
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National Graduate School of Informational and Structural Biology, 2009

Monocytes, macrophages and dendritic cells (DCs) are important mediators of innate immune system, whereas T lymphocytes are the effector cells of adaptive immune responses. DCs play a crucial role in bridging innate and adaptive immunity. Naïve CD4+ Th progenitors (Thp) differentiate to functionally distinct effector T cell subsets including Th1, Th2 and Th17 cells, which while being responsible for specific immune functions have also been implicated in pathological responses, such as autoimmunity, asthma and allergy. The main objective of this thesis is to dissect the signaling networks involved in the IL-4 induced differentiation of two important leukocyte subtypes, Th2 cells and DCs. Gene expression profiling lead to identification of over 200 genes which are differentially expressed during cytokine induced differentiation of human monocytes to DCs or macrophages and which are likely to be essential for the proper biological functions of these cell types. Transcriptome analysis demonstrated the dynamic regulation of gene expression by IL-12 and IL-4 during the initiation of Th cell differentiation, which was partly counteracted by an immunosuppressive cytokine, TGFβ, present in the culture media. Results from RNAi mediated gene knockdown experiments and global gene expression analysis elucidated that SATB1 regulates multiple genes important for Th cell polarization or function as well as may compete with GATA3 for the reciprocal regulation of *IL-5* transcription. In conclusion, the results obtained have extended our system-level understanding of the immune cell differentiation processes and provide an excellent basis for the further functional studies which could lead to development of improved therapeutic approaches for a range of immunological conditions.

Keywords: Dendritic cell, T helper cell differentiation, asthma, cytokine, transcription factor, gene regulation, RNA interference

# TIIVISTELMÄ

**Helena Ahlfors**

**Interleukiini-4:n säätelemä valkosolujen erilaistuminen**

Turun Biotekniikan keskus, Turun Yliopisto ja Åbo Akademi; Lääketieteellisen biokemian ja genetiikan laitos, Turun yliopisto

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Monosyytit, makrofagit ja dendriittisolut ovat tärkeitä synnynnäisessä immuunivasteessa, kun taas T-lymfosyyteillä on keskeisiä tehtäviä hankitussa immuunivasteessa. Dendriittisolut esittelevät vieraat mikrobiantigeenit lymfosyyteille ja näin yhdistävät synnynnäisen ja hankitun immuniteetin. Th-solut kehittyvät yhteisestä esiasteesta toiminnallisesti erilaisiksi alatyypeiksi, joista parhaiten tunnetaan Th1-, Th2- ja Th17-solut. Näillä Th-soluilla on spesifiset tehtävänsä immuunivasteessa, kun taas Th-solujen säätelemän immuunivasteen epätasapaino voi johtaa vakavien häiriötilojen syntyyn elimistössä, kuten autoimmuunitauteihin ja atooppisiin sairauksiin. Tämän väitöskirjatutkimuksen päätavoitteena on selvittää IL-4-sytokiinin säätelemät Th2- ja dendriittisolujen erilaistumiseen tarvittavat signalointireitit ja säätelyverkostot. Geenienkspression profiloinnilla löydettiin yli 200 geeniä, jotka ilmenevät eri tavalla sytokiinien avulla monosyyteistä erilaistetuissa dendriittisoluissa ja makrofageissa ja joiden voidaan olettaa olevan tärkeitä näiden solutyyppien biologisille toiminnoille. Th-solujen varhaisen erilaistumisen aikana IL-12 ja IL-4 -sytokiinien vaikutus osoitettiin olevan dynaamista, ja tämä pystyttiin osittain estämään immunosupressiivisella TGFβ -sytokiinilla. RNA-interferenssillä aikaansaatu geenin hiljeneminen yhdistettynä geeniexpressioanalyysiin osoitti, että SATB1 säätelee useita Th-solujen erilaistumiselle ja toiminnalle tärkeitä geenejä sekä lisäksi saattaa kilpailla GATA3-transkriptiotekijän kanssa IL-5-sytokiinin vastavuoroisesta säätelystä. Tässä tutkimuksessa esitetyt tulokset lisäävät ymmärrystämme immuunisolujen erilaistumisprosesseista sekä tarjoavat erinomaisen perustan toiminnallisiin jatkotutkimuksiin, jotka mahdollisesti johtavat immuunivälitteisten sairauksien hoitomenetelmien kehitykseen.

Avainsanat: dendriittisolu, T-auttajasolujen erilaistuminen, astma, välittäjäaine, transkriptiotekijä, geeninsäätely, RNA-interferenssi

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| 3C | chromosome conformation capture assay |
| Acetyl-CoA | acetyl coenzyme A |
| AP-1 | activator protein 1 |
| APC | antigen presenting cell |
| ATF | activating transcription factor |
| ATP | adenosine 5'-triphosphate |
| BAC | bacterial artificial chromosome |
| bp | base pair |
| BSA | bovine serum albumin |
| C/EBP | CCAAT/enhancer binding protein |
| CaI | calcium ionophore |
| cAMP | cyclic adenosine 3',5'-monophosphate |
| CBP | CREB binding protein |
| CD | cluster of differentiation |
| CBF | core binding factor |
| CCR | chemokine (C-C motif) receptor |
| cDNA | complementary DNA |
| CGRE | conserved GATA3 response element |
| ChIP | Chromatin immunoprecipitation |
| CHX | cyclohexamide |
| CLE0 | conserved lymphokine element 0 |
| CNS | conserved noncoding sequence |
| ConA | concanavalin A |
| CREB | cyclic AMP response element binding protein |
| CsA | cyclosporine A |
| Ct | threshold cycle |
| CTCF | CCCTCbinding factor |
| CXCR | chemokine (C-X-C motif) receptor |
| DC | dendritic cell |
| DNA | deoxyribonucleic acid |
| Dnmt | DNA methyltransferase |
| DTT | dithiothreitol |
| EAE | experimental autoimmune encephalomyelitis |
| ECM | extracellular matrix |
| EF | elongation factor |
| EMSA | electrophoresis mobility shift assay |
| ETS | E twenty-six transcription factor |
| FACS | fluorescence activated cell sorter |
| FCS | fetal calf serum |
| FITC | fluorescein isothiocyanate |
| FISH | fluorescent in situ hybridization |
| GATA | GATA binding protein |
| GEO | Gene Expression Omnibus |

| | |
|---|---|
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| GO | gene ontology |
| GR | glucocorticoid receptor |
| GST | glutathione S-transferase |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase |
| HG | human genome |
| HIV | human immunodeficiency virus |
| HRP | horse radish peroxidase |
| HS(S) | DNase I hypersensitive site |
| IE | intronic enhancer |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| IPA | Ingenuity Pathways Analysis |
| IRF | interferon regulatory factor |
| JAK | janus tyrosine kinase |
| kb | kilobase |
| kDa | kilodalton |
| KO | knockout |
| LCR | locus control region |
| LPS | lipopolysaccharide |
| MAR | matrix attachment region |
| MHC | major histocompatibility complex |
| miRNA | microRNA |
| MNase | Micrococcal nuclease |
| mRNA | messenger RNA |
| NFAT | nuclear activator of activated T cells |
| NF-κB | nuclear factor of kappa light polypeptide gene enhancer in B cells |
| NK(T) | natural killer (T) cell |
| NRE | negative regulatory element |
| NuRD | nucleosome remodeling and deacetylase |
| Oct | octamer factor |
| PAGE | polyacrylamide gel electrophoresis |
| pb | plate bound |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PHA | phytohaemagglutinin |
| PIAS | protein inhibitor of activated STATs |
| PMA | phorbol 12-myristate 12-acetate |
| PT | pertussis toxin |
| RA | rheumatoid arthritis |
| RAN-GTP | RAS-related nuclear protein-guanosine triphosphate |
| RE | response element |
| RISC | RNA-induced silencing complex |

| | |
|---|---|
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| ROR | retinoic-acid-receptor-related orphan nuclear hormone receptor |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| RUNX | Runt related transcription factor |
| SATB1 | special AT-rich binding protein 1 |
| SBS | SATB1 binding site |
| SDS | sodium dodecyl sulphate |
| SH2 | src homology region 2 |
| shRNA | short hairpin RNA |
| siRNA | short interfering RNA |
| SLR | signal $\log_2$ ratio |
| SOCS | suppressor of cytokine signaling |
| STAT | signal transducer and activator of transcription |
| T1D | Type 1 diabetes |
| Tc | cytotoxic T cell |
| TCF | T cell factor |
| TCR | T cell receptor |
| TGF | transforming growth factor |
| Th(p) | T helper cell (progenitor) |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |
| Treg | T regulatory cell |
| TSA | Trichostatin A |
| TSS | transcription start site |
| UTR | untranslated region |
| WT | wild type |

# 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). Additional unpublished data is presented.

I Lehtonen A.*, Ahlfors H.*, Veckman V., Miettinen M., Lahesmaa R.* & Julkunen I.* (2007). Gene expression profiling during differentiation of human monocytes to macrophages or dendritic cells. *J. Leukoc. Biol.* **82**:710-20 (*Equal contribution)

II Lund R., Ahlfors H., Kainonen E., Lahesmaa A.-M., Dixon C. & Lahesmaa R. (2005) Identification of genes involved in the initiation of human type 1 or 2 T helper cell commitment. *Eur. J. Immunol.* **35**: 3307-19

III Ahlfors H., Limaye A., Elo L.L., Tuomela S., Burute M., Gottimukkala K., Notani D., Rasool O., Galande S.* & Lahesmaa R.* SATB1 dictates expression of multiple genes including IL-5 involved in human T helper cell differentiation. Manuscript submitted (*Equal contribution)

# 1 INTRODUCTION

We are daily exposed to various intra and extracellular pathogens, and therefore our immune system has evolved to specifically respond to these attacks and to eradicate the foreign bodies. The front line of host defense is provided by the nonspecific innate immune response that includes monocytes, macrophages, dendritic cells (DCs), NK cells, mast cells, eosinophils, basophils and neutrophils. These cells are immediately available to combat a wide range of pathogens for not growing freely in the body. However, innate immunity does not lead to long-lasting memory of specific pathogen, which is the most important feature of the adaptive immunity. DCs are the key players bridging the innate and adaptive immune responses as they present captured and processed antigens to naïve T and B lymphocytes. The activation and function of all these various white blood cells, leukocytes, depends on the nature of the pathogen.

Many different cell types of the body, including leukocytes, produce cytokines, such as interleukins, interferons, tumor necrosis factors, chemokines and colony stimulating factors, which mediate the communication between cells and regulate inflammatory reactions. Cytokine signaling controls cell fate; i.e. cell differentiation, growth, proliferation and survival. Interleukin-4 (IL-4), an important immunomodulatory cytokine secreted by a highly restricted repertoire of cell types, plays an essential role in the cellular differentiation processes, including polarization of Th2 cells from common naïve lymphoid CD4+ Th progenitor (Thp) and *in vitro* generation of immature DCs from monocytes.

The T cell population is divided into two groups, the CD8+ cytotoxic killer T (Tc) cells and the CD4+ T helper (Th) cells. Depending on the antigen, cytokine environment and costimulatory signals naïve Th cells differentiate to functionally distinct effector T cell subsets including Th1, Th2 and Th17 cells, defined by their characteristic profiles of cytokine production. These subsets of Th cells are responsible for specific immune functions; Th1 cells contribute to cell mediated immunity, while Th2 cells are responsible for humoral responses. Their tasks are complemented by the more recently identified Th17 cells, which provide immunity against extracellular bacterial and fungal pathogens. Th cells have also been implicated in pathological responses; Th1 and Th17 cells contribute to the autoimmune diseases and Th2 cells are involved in the pathogenesis of asthma and allergy. Disorders of the immune system occur when the immune response is inappropriate, excessive or lacking and thus, dissecting the pathways and regulatory networks controlling the differentiation of Th subsets or any immune cell type is of great importance to be able to develop better ways of diagnosing, providing treatment and even preventing many immunological conditions.

The objective of this thesis is to elucidate the signaling networks regulated by IL-4 during differentiation of two important leukocyte subtypes, Th2 cells and DCs. The aim is to identify novel GM-CSF/IL-4 controlled genes in monocyte-derived macrophages and DCs and key regulators of IL-4 induced DC differentiation, to capture the early changes in gene expression profiles induced by IL-12 and IL-4 during Th cell differentiation, to characterize the functional importance of SATB1 in Th cell polarization and to elucidate the regulation of *IL-5* by SATB1 and GATA3.

# 2 REVIEW OF THE LITERATURE

## 2.1 Interleukin-4 in the immune system

### 2.1.1 Hematopoiesis and interleukin-4

All blood cells derive ultimately from the common pluripotent hematopoietic stem cells (HSC) in the bone marrow (Figure 1). The formation of blood cells, termed hematopoiesis, involves extensive cell proliferation and differentiation through several progenitor cell stages and is regulated by various cytokines secreted by cells of the bone marrow stroma and the immune system (Ogawa 1993, Janeway *et al.* 2001). IL-4 is an important immunomodulatory cytokine expressed by a highly restricted pattern of cells of distinct lineages located in diverse locations in the body (e.g. circulating T cells vs. tissue mast cells). IL-4 plays an essential role in protective immunity as well as in inflammatory diseases (Weiss & Brown 2001).

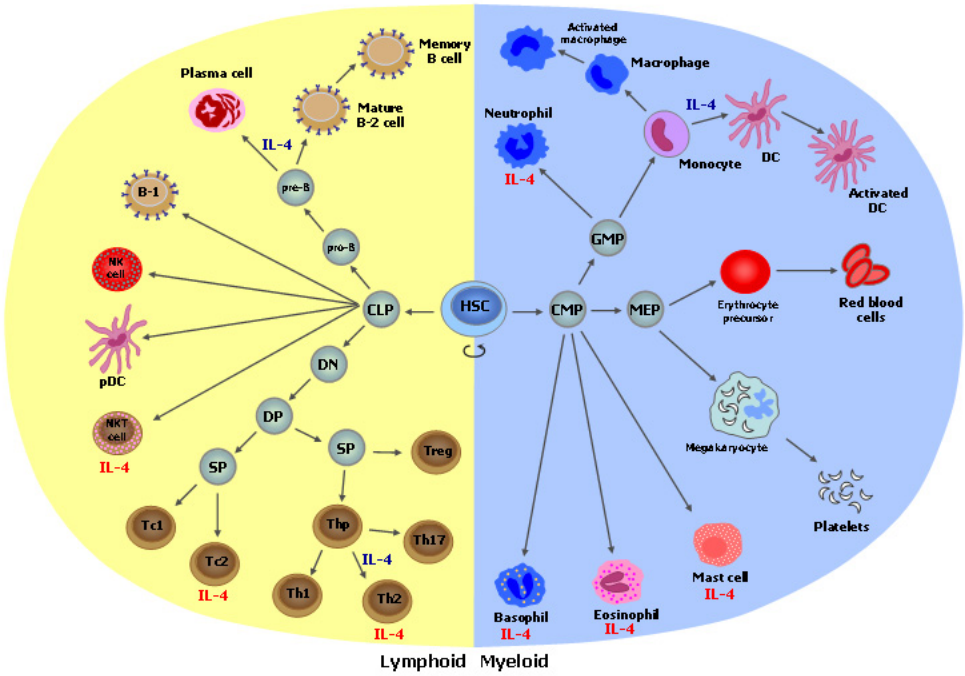


**Figure 1. Involvement of IL-4 in hematopoiesis and immune system function.** The development of different lineages of blood cells schematically illustrated. ÍL-4 below a given cell type marked in red refers to cells producing IL-4. IL-4 near arrows and marked in blue is involved in the developmental transition indicated by the arrow. B-1, B-1 type B cell; B-2, B-2 type 'conventional' B cell; pre-B, pre–B cell; pro-B, pro–B cell; CLP, common lymphoid progenitor; DN, double-negative T cell; DP, double-positive T cell; SP, single-positive T cell; NK, natural killer; pDC, plasmacytoid dendritic cell; NKT, natural killer T; Th1, T helper type 1 cell; Th2, T helper type 2 cell; Th-17, T helper type 17 cell; Treg, regulatory T cell; DC, dendritic cell; GMP, granulocyte-macrophage progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor. Modified from Baltimore *et al.* 2008.

### 2.1.2 JAK-STAT signaling

Cytokines mediate their effects on all cell types involved in inflammation via JAK-STAT signaling (recently reviewed by O'Shea & Murray (2008)). Cytokine binding to its cognate receptor activates receptor-associated tyrosine kinases of the Janus kinase family (JAKs) that in turn phosphorylate tyrosine residues in the receptor cytoplasmic domain. This provides a docking site for signal transducers and activators of transcription (STATs). These cytosolic proteins with Src homology 2 (SH2) domains are phosphorylated by JAKs and homo- and heterodimerized before being translocated to the nucleus where they regulate transcription of various genes (Janeway *et al.* 2001). Four JAKs (JAK1, JAK2, JAK3 and TYK1) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) are known and they are variably activated by different cytokines (Schindler & Darnell 1995, O'Shea *et al.* 2002) (Figure 2). Nuclear proteins that inhibit activated STATs (PIAS) as well as cytokine-inducible cytoplasmic tyrosine phosphatases, suppressors of cytokine signaling (SOCS) proteins, block the STAT activity and thereby negatively regulate cytokine signaling (Levy & Darnell 2002, Alexander & Hilton 2004, Yoshimura *et al.* 2007).

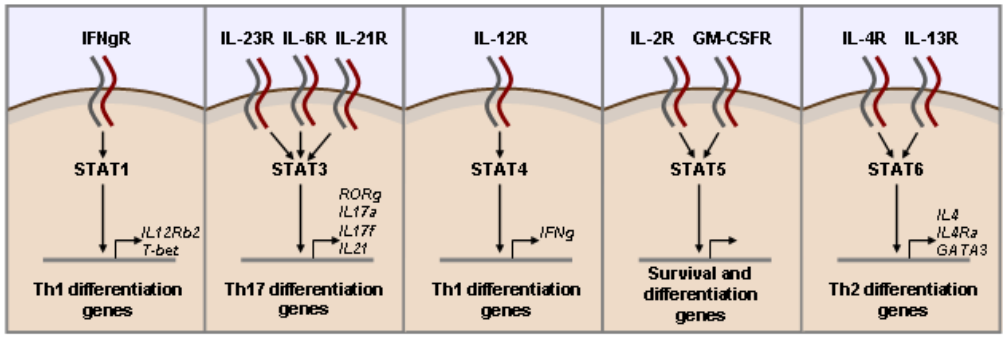


**Figure 2. Cytokine signaling in DC, macrophage and Th cell differentiation and function.** STAT1 and STAT4 signaling starting from IFNγR and IL-12R, respectively, are essential for Th1 cell development. IL-6, IL-23 or IL-21 induced signaling cascade utilizes STAT3 to enhance the differentiation of Th17 cells by increasing directly or indirectly RORγt and RORα expression. STAT5 signaling triggered by cytokine binding to IL-2R or GM-CSFR promotes cell survival or macrophage/DC differentiation, respectively. Th2 cell differentiation is induced by STAT6 that is activated by stimulus through IL-4R or IL-13R. Adapted from Mui *et al.* 1995, Lehtonen *et al.* 2002, O'Shea & Murray 2008.

### 2.1.3 Monocytes, macrophages and DCs in the immune system

In mammals invading pathogens induce an immune response that consists of innate and adaptive parts. Monocytes and macrophages are important mediators of innate immune system, whereas DCs are the major cell type responsible for initiating the adaptive phase of the immune response (Janeway *et al.* 2001).

Monocytes circulate in the blood ready to phagocytose microbes and secrete cytokines when activated. However, upon leaving the blood stream and migrating into the

tissues, monocytes differentiate into macrophages and achieve their full functional maturation (Valledor *et al.* 1998). Macrophages are a heterogenous population of tissue-specific, functionally specialized cells including splenic macrophages, liver Küpffer cells, lung alveolar macrophages, bone osteoclasts and many macrophage subsets in the central nervous system (Stout & Suttles 2004, Gordon & Taylor 2005). Tissue-residing macrophages play an important role in normal tissue homeostasis by phagocytosing senescent cells as well as remodeling and repairing tissues after inflammation (Gordon 1998). Various inflammatory signals, including microbial products (e.g. LPS) and cytokines (e.g. TNFα and IFNγ), induce activation of macrophages and their migration to sites of inflammation (Mosser 2003). Besides recognizing, phagocytosing and destroying infectious agents, activated macrophages also secrete proinflammatory cytokines, oxygen radicals and lipid mediators to maintain the inflammatory responses. Nevertheless, these products can also cause extensive tissue damage and therefore activity of macrophages is carefully regulated by immunosuppressive cytokines, IL-10 and TGFβ (Mosser 2003). Furthermore, macrophages can present microbe-specific peptides to lymphocytes, thus activating adaptive immune responses (Mosser 2003). Development of macrophages from monocytes is regulated by a wide variety of transcription factors, including PU.1, an Ets-family transcription factor, C/EBP proteins and the AML1/CBFβ heterodimer (Valledor *et al.* 1998). The joint expression of PU.1, C/EBPα and AML1/CBFβ synergistically enhances the myeloid-specific expression of the receptors for M-CSF and GM-CSF, which are important lineage-determining cytokines (Valledor *et al.* 1998, Nagamura-Inoue *et al.* 2001). PU.1 also regulates the expression of other genes involved in the acquisition of a functional macrophage phenotype, such as the adhesion molecules CD11b and CD18 and the LPS receptor CD14 (Olson *et al.* 1995, DeKoter *et al.* 1998). In addition, IRF8 is required for the terminal macrophage differentiation as IRF8 knockout mice exhibit markedly reduced monocyte/macrophage population (Holtschke *et al.* 1996, Tamura *et al.* 2000).

DC population comprises several specialized subtypes that differ in their surface marker expression, cytokine production and localization in the body (Ito *et al.* 2005, Villadangos & Schnorrer 2007, Wu & Liu 2007). In humans, there are two main DC subtypes, plasmacytoid (pDCs) and conventional (cDCs) DCs (Shortman & Naik 2007, Villadangos & Schnorrer 2007, Wu & Liu 2007). pDCs are found in the blood and lymphoid organs in a preactivated state (Cella *et al.* 1999, Siegal *et al.* 1999). cDCs can be divided into subsets according to their tissue localization, including skin DCs (Langerhan's cells and dermal DCs), mucosal tissue-associated DCs, lymphoid tissue-associated DCs and interstitial tissue DCs (liver DCs and lung DCs) (Banchereau *et al.* 2003, Wu & Liu 2007). pDCs are potent producers of type I interferons, especially IFNα, and other proinflammatory cytokines in response to viral infections (Cella *et al.* 1999, Siegal *et al.* 1999, Colonna *et al.* 2004, Asselin-Paturel & Trinchieri 2005, Liu 2005), whereas cDC subsets recognize bacterial components and produce proinflammatory cytokines, such as IL-12, IL-6 and TNFα (Banchereau & Steinman 1998, Liu *et al.* 2001, Ito *et al.* 2005). In addition to these primary DC-committed cells, monocytes have been demonstrated to differentiate to immature DCs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Sallusto & Lanzavecchia 1994, Akagawa *et al.* 1996, Kiertscher & Roth 1996, Pickl *et al.* 1996,

Zhou & Tedder 1996, Chapuis *et al.* 1997) or GM-CSF and interferon-α (IFNα) (Santini *et al.* 2000) *in vitro*. However, differentiation of monocytes into functional DCs *in vivo* has remained elusive (Randolph *et al.* 1999, Wu & Liu 2007). Immature DCs reside in peripheral tissues monitoring their surroundings by macropinocytosis, receptor-mediated endocytosis and active phagocytosis for possible invading pathogens (Banchereau *et al.* 2000). Encountering a microbe induces maturation of DC, which leads to increased expression of cell surface adhesion proteins, T cell costimulatory molecules, cytokines and chemokines as well as decreased capacity to endocytose foreign particles (Banchereau *et al.* 2000, Banchereau *et al.* 2003). Mature DCs leave the peripheral tissues and migrate to local lymph nodes to present captured and processed antigens to naïve T cells (Banchereau *et al.* 2000, Villadangos & Schnorrer 2007). Besides bridging innate and adaptive immunity, DCs also participate in maintaining immune tolerance to self-tissues (Banchereau & Steinman 1998, Steinman *et al.* 2003). Several transcriptional regulators and cytokines have been shown to play an essential role in DC development. Nevertheless, distinct DC subpopulations may require different factors (Wu & Liu 2007). Knockout mice studies have demonstrated a defective DC differentiation in the absence of certain transcription factors, including Ikaros, RelB, IRF4, IRF8, STAT3 and PU.1 (Georgopoulos *et al.* 1994, Wu *et al.* 1997, Wu *et al.* 1998, Anderson *et al.* 2000, Guerriero *et al.* 2000, Ardavin *et al.* 2001, Schiavoni *et al.* 2002, Ardavin 2003, Laouar *et al.* 2003, Tsujimura *et al.* 2003, Schiavoni *et al.* 2004, Tamura *et al.* 2005, Zenke & Hieronymus 2006). Furthermore, GM-CSF and Flt3L induce growth and development of different DC subsets, as GM-CSF promotes cDC differentiation and suppresses Flt3L-driven pDC development (Maraskovsky *et al.* 1996, Blom *et al.* 2000, Pulendran *et al.* 2001, Gilliet *et al.* 2002, O'Keeffe *et al.* 2002, D'Amico & Wu 2003, Naik *et al.* 2005, Wu & Liu 2007).

### 2.1.4 T helper cells in the immune system

T lymphocytes are the effector cells of adaptive immune responses. The T cell population is divided into two groups, the CD8+ cytotoxic killer T (Tc) cells and the CD4+ T helper (Th) cells. While Tc cells directly kill the targeted pathogen-infected cells, Th cells regulate the immune defence reactions by secreting cytokines and chemokines that activate and/or recruit target cells (Janeway *et al.* 2001). Th cells can differentiate from the common naïve lymphoid CD4+ Th progenitor (Thp) to functionally distinct effector T cell subsets including Th1, Th2 and Th17 cells, defined by their characteristic profiles of cytokine secretion (Mosmann *et al.* 1986, Parronchi *et al.* 1991, Bettelli *et al.* 2007)) (Figure 3). Th1 cells produce pro-inflammatory cytokines IFNγ, TNFβ (lymphotoxin) and IL-2, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-13 and IL-25 (Abbas *et al.* 1996, Fort *et al.* 2001, Hauber *et al.* 2004, Angkasekwinai *et al.* 2007). The cytokine repertoire produced by Th17 cells includes IL-17 (also known as IL-17A), IL-17F, IL-21, IL-22 and G-CSF (Yao *et al.* 1995, Harrington *et al.* 2005, Park *et al.* 2005). These Th subsets are responsible for specific immune functions. Th1 cells contribute to cell mediated immunity against intracellular pathogens, such as *Mycobacterium tuberculosis, Leishmania major* and *Listeria monocytogenes* (Kaufmann 1993). Cytokines secreted by Th1 cells activate macrophages and Tc cells to kill harmful microbes as well as recruit other Th1 cells to

the site of inflammation to enhance the immune response (Abbas *et al.* 1996). By contrast, Th2 cells are responsible for humoral responses that eventually lead to eradication of extracellular pathogens, such as the parasitic helminths *Nippostrongylus brasiliensis, Trichinella spiralis* and *Schistosoma mansoni* (Scott *et al.* 1989, Sher & Coffman 1992, Maizels & Yazdanbakhsh 2003). Th2 cytokines, IL-4 and IL-13 enhance MHCII expression on B cells, stimulate B cell proliferation and promote the antigen-specific antibody production of B cells (IgE and IgG1) (Finkelman *et al.* 1988, Snapper *et al.* 1988, Defrance *et al.* 1994, Romagnani 1994, Emson *et al.* 1998, Nelms *et al.* 1999). IL-5 mainly controls the growth, differentiation and activation of eosinophils (Campbell *et al.* 1987, Sanderson 1988, Yamaguchi *et al.* 1988), but also stimulates basophils to release histamines and leukotrienes and promotes proliferation and differentiation of murine B lymphocytes (Sanderson *et al.* 1988, Coffman *et al.* 1989, Hirai *et al.* 1990). The task of Th1 and Th2 cells is complemented by the more recently identified Th17 cells that provide immunity against extracellular bacterial and fungal pathogens (Ye *et al.* 2001, Ouyang *et al.* 2008). The Th17 secreted IL-17 family member cytokines play an essential role in host defence by recruiting neutrophils and macrophages to infected tissues (Ye *et al.* 2001, Kolls & Linden 2004, Park *et al.* 2005).

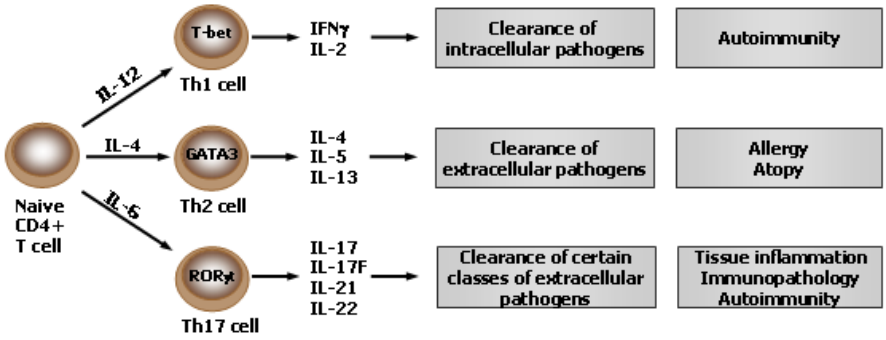


**Figure 3. Overview of the differentiation of Th cells and their implications in immune functions and diseases.** Naïve CD4+ T precursor cells can differentiate towards the Th1, Th2 or Th17 lineages in presence of polarizing cytokines. Subtype specific transcription factors orchestrates the proper development and maintenance of different Th cell types. Cytokines produced by effector Th cells regulate specific immune responses as well as play a role in immunopathogenesis. Modified from Bettelli *et al.* 2008, Dong 2008.

Failures of host defense mechanisms can lead to disorders of the immune system. The balance between Th1/Th2 subsets is crucial, and dysregulated expansion of Th effector cells has been implicated in immunological pathologies (Romagnani 1996, Romagnani 2008). Enhanced Th1 and Th17 responses contribute to various organ-specific autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, Crohn's disease and type 1 diabetes while excessive Th2 responses are associated with the pathogenesis of asthma and allergy (Romagnani 1994, Charlton & Lafferty 1995, Romagnani 2000, Larche *et al.* 2003, Bettelli *et al.* 2007). Thus, dissecting the pathways and regulatory networks controlling the differentiation of Th subsets is of great importance to be able

to develop better ways of diagnosing, providing treatment and even preventing many immunological conditions.

### 2.1.5 T helper cell differentiation

T helper cell differentiation takes place in the peripheral or secondary lymphoid organs, including the lymph nodes, the spleen and lymphoid tissues associated with mucosa, where naïve CD4+ T lymphocytes migrate after their generation in the bone marrow and maturation in the thymus, which are central or primary lymphoid organs (Janeway *et al.* 2001). Many factors influence the Th cell fate, the most potent determinant being the cytokine milieu itself. Furthermore, the differentiation process is affected by type of antigen presenting cell (APC), nature of the antigen (structure and dose), avidity of the T cell receptor (TCR) to the antigen/MHC (strength and duration of TCR signaling), presence of co-stimulatory molecules and number of cell divisions (Abbas *et al.* 1996, Constant & Bottomly 1997, Bird *et al.* 1998, Glimcher & Murphy 2000, Lane 2000, Sperling & Bluestone 2001, Ho & Glimcher 2002, Murphy & Reiner 2002, Szabo *et al.* 2003, Mowen & Glimcher 2004).

#### 2.1.5.1 Th1 cell differentiation

The main cytokine driving Th1 cell differentiation is IL-12 (Hsieh *et al.* 1993, Manetti *et al.* 1993, Seder *et al.* 1993, Manetti *et al.* 1994), which is primarily secreted by activated APCs, including macrophages, DCs and monocytes, but also by neutrophils and B cells in response to various pathogens (D'Andrea *et al.* 1992, Macatonia *et al.* 1993, Flesch *et al.* 1995, Macatonia *et al.* 1995, Ma *et al.* 1996). IL-12 signals through the IL-12 receptor complex composed of two subunits, IL-12Rβ1 and IL-12Rβ2 (Chua *et al.* 1994, Presky *et al.* 1996), which are not expressed by naïve CD4+ T cells but induced by activation through the TCR (Presky *et al.* 1996). Upon IL-12R signaling STAT4 gets phosphorylated and translocated to the nucleus (Manetti *et al.* 1994, Bacon *et al.* 1995, Jacobson *et al.* 1995, Cho *et al.* 1996), where it initiates the transcription of *IFNγ* (Barbulescu *et al.* 1998, Zhang *et al.* 1998, Nakahira *et al.* 2002, Nguyen *et al.* 2002, Park *et al.* 2004). IFNγ promotes Th1 cell differentiation by inducing phosphorylation of STAT1 (Briscoe *et al.* 1996, Leonard & O'Shea 1998) which triggers the transcription of Th1 specific genes, such as *IL12Rβ2* and *T-bet* (Bradley *et al.* 1996, Wenner *et al.* 1996, Szabo *et al.* 1997, Lighvani *et al.* 2001, Zhang *et al.* 2001). The transcription factor T-bet (T-box expressed in T cells), is a master regulator of Th1 development (Szabo *et al.* 2000, Szabo *et al.* 2002) as it creates a positive feedback loop by transactivating the expression of *IFNγ* (Lee *et al.* 2004a) and enhances IL-12 signaling capacity by inducing *IL12Rβ2* chain expression (Afkarian *et al.* 2002, Tong *et al.* 2005). However, a recent study demonstrated STAT4 is required for T-bet to complete IL-12-dependent Th1 cell-fate determination (Thieu *et al.* 2008). The importance of IL-12/STAT4 signaling for the Th1 cell polarization has been demonstrated by knockout (KO) mouse studies. Mice lacking IL-12, IL-12Rβ1, IL-12Rβ2 or STAT4 have profoundly impaired Th1 responses (Kaplan *et al.* 1996b, Magram *et al.* 1996, Thierfelder *et al.* 1996, Cooper *et al.* 1997, Wu *et al.* 1997, Wu *et al.* 2000). Furthermore, T-bet deficiency has been shown to impair Th1

differentiation and be protective of Th1 cell-mediated diseases as well as to increase production of Th2 cytokines and display multiple features of asthma (Finotto *et al.* 2002, Szabo *et al.* 2002, Szabo *et al.* 2003). By contrast, retroviral expression of T-bet promotes Th1-type cytokine profiles in Th2 cells (Szabo *et al.* 2000). Th1 specific transcription factors also play a role in inhibiting Th2 development as STAT4 negatively regulates Th2 cell differentiation (Ouyang *et al.* 1998, Murphy & Reiner 2002) while T-bet represses Th2 development and Th2 cytokine gene expression presumably by interacting with GATA3 (Glimcher & Murphy 2000, Szabo *et al.* 2000, Hwang *et al.* 2005).

#### 2.1.5.2 Th2 cell differentiation

The polarization of Th2 cells is primarily directed by IL-4 (Swain *et al.* 1990, Kuhn *et al.* 1991, Seder *et al.* 1992, Kopf *et al.* 1993, Noben-Trauth *et al.* 2002), which is produced by a number of cell types in the immune system, including Th2 cells, Tc2 cells, basophils, eosinophils, neutrophils, mast cells and natural killer T (NKT) cells (Brown *et al.* 1987, Moqbel *et al.* 1995, Yoshimoto *et al.* 1995, Nelms *et al.* 1999, Weiss & Brown 2001). IL-4 receptor (IL-4R) is composed of IL4Rα chain and the common cytokine receptor γ ($\gamma_c$) chain that is also shared with IL-2, IL-7, IL-9, IL-15 and IL-21 receptors (Nelms *et al.* 1999). In contrast to IL-12 receptor, IL-4R is expressed on naïve CD4+ T cells and further induced by IL-4 making the cells responsive to IL-4 during antigen recognition by TCR (Nelms *et al.* 1999). Nevertheless, the early source of IL-4 *in vivo* as well as its importance as a Th2-initiating factor has remained elusive (von der Weid *et al.* 1996, Noben-Trauth *et al.* 1997, Noben-Trauth *et al.* 2000, Noben-Trauth *et al.* 2002, Mowen & Glimcher 2004, Ansel *et al.* 2006, Sun & Pearce 2007, van Panhuys *et al.* 2008). IL-4 signaling induces phosphorylation of STAT6 (Swain *et al.* 1990, Kaplan *et al.* 1996a, Shimoda *et al.* 1996, Takeda *et al.* 1996, Zhu *et al.* 2001b) which starts the transcription of Th2 promoting genes, including *IL-4*, *IL-4Rα* and *GATA3* (Zheng & Flavell 1997, Ouyang *et al.* 1998, Nelms *et al.* 1999, Zhu *et al.* 2001b). The zinc-finger transcription factor GATA3 further enhances its own expression in a STAT6-independent manner (Ouyang *et al.* 1998). GATA3 plays a key role in the initiation and maintenance of Th2 cell differentiation, especially by orchestrating the remodeling of the Th2 cytokine gene loci (Zhang *et al.* 1997, Zheng & Flavell 1997, Lee *et al.* 1998, Ouyang *et al.* 1998, Ferber *et al.* 1999, Ouyang *et al.* 2000, Avni *et al.* 2002, Yamashita *et al.* 2002, Baguet & Bix 2004, Yamashita *et al.* 2004, Nakayama & Yamashita 2008)). The importance of the IL-4/STAT6 signaling pathway for Th2 cell differentiation and function has been demonstrated by using KO mice. Mice deficient for IL-4, IL-4R or STAT6 have markedly impaired Th2 responses (Kuhn *et al.* 1991, Kopf *et al.* 1993, Noben-Trauth *et al.* 1997, Zhu *et al.* 2001b). GATA3 deficiency is lethal (Pandolfi *et al.* 1995), but using conditional deletion or site-specific recombination the critical role of GATA3 in both Th1 and Th2 responses has been demonstrated (Pai *et al.* 2004, Yamashita *et al.* 2004, Zhu *et al.* 2004). Ectopic expression of STAT6 or GATA3 induces production of Th2 cytokines and decreases IFNγ production in Th1 cells (Zheng & Flavell 1997, Ouyang *et al.* 1998, Kurata *et al.* 1999, Lee & Young 2000). However, the STAT6-independent generation of IL-4 secreting Th2 cells has been suggested by several studies (Kaplan *et al.* 1999, Jankovic *et al.* 2000, van Panhuys *et al.* 2008). Introducing

GATA3 into STAT6 depleted T cells not only restores Th2 transcription factor expression and cytokine production but also completely reconstitutes Th2 cell development (Ouyang *et al.* 2000). Furthermore, Notch signaling was shown to directly transactivate GATA3 expression and drive Th2 cell differentiation possibly by substituting STAT6 (Amsen *et al.* 2007, Fang *et al.* 2007, Nakayama & Yamashita 2008). Very recently, WNT signaling was also linked to Th2 differentiation as T cell factor 1 (TCF1) and β-catenin were demonstrated to induce GATA3 expression upon TCR stimulation, independent of IL-4R signaling (Yu *et al.* 2009). IL-4/STAT6 signaling also plays a role in inhibiting Th1 development and Th1 cytokine production (Kurata *et al.* 1999, Nelms *et al.* 1999, Szabo *et al.* 2003).

#### 2.1.5.3 Th17 cell differentiation

In mouse, the differentiation of Th17 lineage is induced by TGFβ and IL-6 (Bettelli *et al.* 2006, Mangan *et al.* 2006, Veldhoen *et al.* 2006) and, in the absence of IL-6, by TGFβ in combination with IL-21 (Korn *et al.* 2007, Nurieva *et al.* 2007, Zhou *et al.* 2007). However, early Th17 cell differentiation also requires IL-1 signaling (Chung *et al.* 2009). The cytokines initiating human Th17 cell development are less clear as several groups have reported that TGFβ plus IL-6 do not drive the polarization at least *in vitro* (Acosta-Rodriguez *et al.* 2007, Chen *et al.* 2007, van Beelen *et al.* 2007, Wilson *et al.* 2007). Initially, TGFβ was observed to inhibit differentiation of human Th17 cells (Acosta-Rodriguez *et al.* 2007, Chen *et al.* 2007) but recent studies have shown that this cytokine is required for this differentiation process in human (Manel *et al.* 2008, Volpe *et al.* 2008, Yang *et al.* 2008a). However, Th17 polarization can be induced with IL-1β alone or in combination with IL-6 (Acosta-Rodriguez *et al.* 2007) or IL-23 (Wilson *et al.* 2007). IL-6, IL-1β and TGFβ are secreted by the cells of the innate immune system (Diehl & Rincon 2002, Li *et al.* 2006a, Acosta-Rodriguez *et al.* 2007) while TGFβ is also produced by regulatory T cells (Treg) (Faria & Weiner 2006, Li *et al.* 2007). IL-21 is expressed mainly by CD4+ T cells and NKT cells (Parrish-Novak *et al.* 2000, Wurster *et al.* 2002, Coquet *et al.* 2007). IL-6 and IL-21 mediated signaling activates STAT3 which induces the expression of Th17 promoting lineage specific transcription factors, RORγt and RORα (Laurence *et al.* 2007, Yang *et al.* 2007, Yang *et al.* 2008b). These two members of the retinoic-acid-receptor-related orphan nuclear hormone receptor family (Jetten 2004) trigger the production of key Th17 cytokines, IL-17, IL-17F and IL-22 (Ivanov *et al.* 2006, Yang *et al.* 2008b). IL-6 also induces STAT3-dependent production of IL-21 that reinforces the Th17 fate in an autocrine manner (Korn *et al.* 2007, Nurieva *et al.* 2007, Zhou *et al.* 2007). IL-23, secreted by APCs, was the first cytokine suggested to selectively regulate IL-17 expression (Cua *et al.* 2003, Murphy *et al.* 2003). However, IL-23 receptor (IL-23R) is not expressed by the naïve CD4+ T cells but induced by IL-6 in developing Th17 cells (Yang *et al.* 2007, Zhou *et al.* 2007). In fact, instead of initiating Th17 differentiation, IL-23 has been demonstrated to sustain the expression of Th17 selective transcription factors and promote Th17 cell survival and cytokine secretion (Ivanov *et al.* 2006, Veldhoen *et al.* 2006, Dong 2008, McGeachy & Cua 2008). The essential role of Th17 specific transcription factors in Th17 cell function has been demonstrated by using mice deficient for STAT3, RORγt or RORα. Deletion of STAT3 results in defective Th17 cell differentiation concordant with decreased expression of IL-17, IL-17F, IL-

22, IL-23R, RORγt and RORα and protection against experimental autoimmune encephalomyelitis (EAE) (Harris *et al.* 2007, Mathur *et al.* 2007, Yang *et al.* 2007, Yang *et al.* 2008b). Similarly, Th17 development is impaired in mice lacking both RORγt and RORα (Yang *et al.* 2007). Interestingly, IL-2, an important growth factor for most T cell subsets, has inhibitory effects on the expansion of Th17 cells by suppressing the expression of RORγt (Laurence *et al.* 2007). ETS1 transcription factor was recently shown to inhibit polarization of Th17 cells in a IL-2-dependent manner as $ETS1^{-/-}$ T cells exhibited increased Th17 development in response to TGFβ and IL-6 only in the presence of IL-2 (Moisan *et al.* 2007). However, differentiating ETS-1 deficient Th cells were also resistant to the suppressive effect of IL-2 (Moisan *et al.* 2007). IRF4 transcription factor, essential for Th2 differentiation (Hu *et al.* 2002, Rengarajan *et al.* 2002a), was also demonstrated to be important for Th17 differentiation as T cells from IRF4-deficient mice failed to differentiate to Th17 cells (Brustle *et al.* 2007). In addition, IRF4 was suggested to function upstream of the nuclear receptors RORγt and RORα and play a key role in IL-21-mediated steps of Th17 development (Huber *et al.* 2008). Furthermore, generation of Th17 cells is antagonized by Th1-type cytokines IFNγ and IL-27 as well as cytokines produced by Th2 cells, IL-4 and IL-25 (Harrington *et al.* 2005, Park *et al.* 2005, Batten *et al.* 2006, Stumhofer *et al.* 2006, Kleinschek *et al.* 2007).

## 2.2 Regulation of gene expression

### 2.2.1 *Cis*-regulatory elements

Eukaryotic promoter, located directly upstream of the transcribed region, provides a site for assembly of the basal transcription machinery, including general transcription factors and RNA polymerase II, and is sufficient for subsequent initiation of transcription of any given gene. However, the promoter-driven transcription produces only minimal amounts of transcripts and thus, *cis*-regulatory elements, located on the same chromosome, are required to modify transcriptional rates (Lee & Young 2000). Developmental and cell lineage-specific regulation of gene expression is largely affected by both gene-proximal elements as well as long-range interactions of various *cis*-regulatory elements. Gene expression modulating *cis*-regulatory sequences include: (1) enhancers, (2) silencers, (3) insulator/boundary elements, (4) locus control regions (LCRs) and (5) matrix attachment regions (MARs) which are found even hundreds of kilobytes upstream or downstream of the transcription start site (Ogbourne & Antalis 1998, Rowell *et al.* 2008) (Figure 4). Furthermore, co-regulated genes are frequently located in linear proximity on the same chromosome indicating that gene organization in the genome is non-random and evolutionarily conserved gene clusters are likely to be advantageous (Singer *et al.* 2005, Sproul *et al.* 2005, Kosak *et al.* 2007).

Enhancer is a regulatory sequence that greatly increases expression of a neighboring gene regardless of its orientation and location (upstream, downstream or even within introns) by providing a site for assembling enhancer complexes or enhanceosomes which induce chromatin remodeling and recruit the RNA polymerase II machinery to the promoter (Szutorisz *et al.* 2005, Li *et al.* 2006b). In contrast, transcriptional repression is achieved either through an active mechanism by position-independent

silencer elements or a passive mechanism by position-dependent negative regulatory elements (NREs) (Ogbourne & Antalis 1998). Insulators or boundary DNA sequence elements demarcate different chromatin environments (euchromatin and heterochromatin) as well as genomic loci, and prevent inappropriate interactions between adjacent chromatin domains (Kellum & Schedl 1991, Gaszner & Felsenfeld 2006). There are two types of insulators: an enhancer-blocking insulator that prevents genes from inappropriate activation by neighboring enhancers by blocking the interaction between enhancers and promoters when placed between these two elements, and a barrier insulator that protects against silencing by surrounding heterochromatin when placed at the junction between the two chromatin environments (Sun & Elgin 1999). Interestingly, a ubiquitously expressed vertebrate zincfinger protein, CCCTCbinding factor (CTCF) plays a crucial role especially in the enhancer-blocking type insulation (Chung *et al.* 1997, Bell *et al.* 1999). Insulating elements can interact with each other through CTCF molecules to form clusters or tether the chromatin fiber to structural elements within the nucleus and thereby establish chromatin loop domains which separate enhancers and promoters (Corces 1995, Yusufzai *et al.* 2004, Gaszner & Felsenfeld 2006, Wallace & Felsenfeld 2007). Furthermore, recent studies have shown that CTCF recruits cohesin and together insulate promoters from distant enhancers and create chromatin domain boundaries between accessible and repressive chromatin (Parelho *et al.* 2008, Wendt *et al.* 2008). In addition, high-throughput studies using ChIP-Chip and ChIP-Seq have shown that CTCF often binds between genes that are in close genomic proximity, but have distinct expression patterns and opposing chromatin modifications (Barski *et al.* 2007, Kim *et al.* 2007b, Xie *et al.* 2007). CTCF may also contribute to tissue-specific promoter usage as CTCF binding sites are found between alternative promoters of a single gene (Kim *et al.* 2007b).

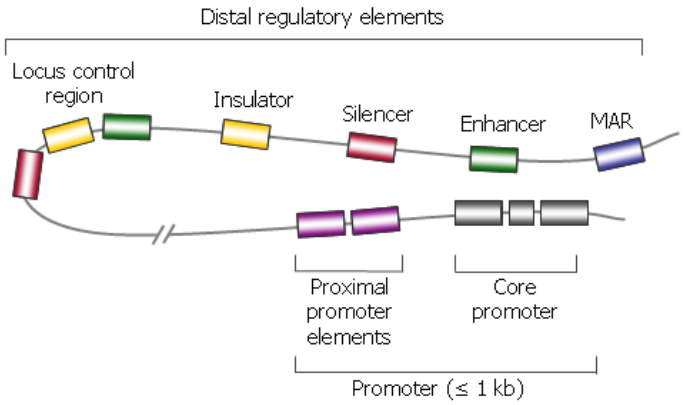


**Figure 4. Schematic presentation of a typical gene regulatory region.** A core promoter and proximal promoter elements form the promoter that typically span less than 1 kb pairs. Distal *cis* regulatory elements, including enhancers, silencers, insulators, locus control regions and MARs, can be located up to 1 Mb pairs, depending on the type, up or downstream of the promoter. These distal elements may contact the promoter elements by looping out the intervening DNA. Modified from Maston *et al.* 2006.

Coordinated and synchronized gene expression is required especially in complex loci that contain multiple genes. The LCR is an important player in this type of regulation. The best-studied LCR, found in the human β-globin locus, is involved in the regulation of the five globin genes that are activated at different stages of development (Choi & Engel 1988, Engel & Tanimoto 2000, Li *et al.* 2002). LCRs are defined as *cis*-acting regulatory elements exhibiting both enhancer and insulator activity that confer physiological, tissue-specific and copy number-dependent expression of linked genes regardless of any flanking suppressive sequences (Grosveld *et al.* 1987, Milot *et al.* 1996, Kioussis & Festenstein 1997, Festenstein & Kioussis 2000, Li *et al.* 2002). LCRs mediate gene regulation through the recruitment of coactivators, the formation of intra- and inter-chromosomal loops and the positioning of the genes to nuclear regions that favor transcription (Dean 2006). Interestingly, MARs also play important role in organizing chromatin loop domains and thereby regulating gene expression by providing sequences through which chromatin loops are anchored to the nuclear matrix (Galande *et al.* 2007). MAR-binding proteins, such as SATB1, recognize and bind AT-rich sequences possessing strong nucleotide-unpairing properties and subsequently may function as critical transcriptional control proteins (Bode *et al.* 1992, Dickinson *et al.* 1992).

### 2.2.2 Chromatin accessibility

The regulatory elements described above orchestrate gene expression through the binding of tissue-specific transcription factors and other regulatory proteins. However, the binding is largely determined by factor abundance and ability to recognize target regulatory sequences in chromatin (Lee *et al.* 2006, Rowell *et al.* 2008). The chromatin structure is generally considered to be an impediment to transcriptional activity and needs to be remodeled via epigenetic mechanisms without altering the base sequence of DNA in order to allow local access to transcriptional machinery (Kornberg & Lorch 1999a). The critical epigenetic changes are: (1) post-translational histone modifications, (2) nucleosome composition, position and interactions with DNA, and (3) methylation of cytosines within CpG dinucleotides (Bird & Wolffe 1999, Kornberg & Lorch 1999a, Lee *et al.* 2006, Rowell *et al.* 2008).

#### 2.2.2.1 Histone modifications

Two basic chromatin states based on cytological staining are decondensed transcriptionally permissive euchromatin and condensed transcriptionally repressive heterochromatin. Heterochromatin can be further divided into two subtypes; constitutive heterochromatin, which is obligately silenced, and constitutive heterochromatin, which is silenced only in certain contexts (Trojer & Reinberg 2007). The basic unit of chromatin is the nucleosome that is composed of 146 base pairs of DNA wrapped nearly twice around an octamer of core histones (two copies of histones H2A, H2B, H3 and H4) (Kornberg & Lorch 1992, Kornberg & Lorch 1999b). The core histones are globular except for their N-terminal tails that protrude from the core (Luger *et al.* 1997). Nucleosomes are dynamic structures: both histone tails and globular domains are subject to a number of posttranslational modifications (Strahl &

Allis 2000, Zhang *et al.* 2003, Xu *et al.* 2005) whereas the histone-DNA contacts are altered by the protein complexes that utilize ATP hydrolysis (Flaus & Owen-Hughes 2004, Smith & Peterson 2005). The post-translational modifications of histones influence gene expression either by facilitating or impeding transcription factor binding and transit of RNA polymerases. Modification of over 60 different histone residues has been detected using mass spectrometry or specific antibodies and there are at least eight distinct types of histone modifications (reviewed in Kouzarides 2007). These modifications include methylation and deimination of arginines (R); methylation, acetylation, ubiquitylation, ADP-ribosylation and sumoylation of lysines (K); phosphorylation of serines (S) and threonines (T); ADP-ribosylation of glutamic acids (E); and isomerization of proline (P) residues. Furthermore, extra complexity is provided by the fact that lysine side chains can be mono-, di- or tri-methylated, while arginine side chain can be mono-methylated or (symmetrically or asymmetrically) di-methylated (Bannister *et al.* 2002) (Table I). Most histone modifications have been found to be dynamic and rapidly changing and enzymes adding and removing different types of modifications have been identified. Furthermore, histone modifications can also be evicted by histone replacement that is involved in nucleosome positioning (Berger 2007).

**Table I Chromatin modifications involved in transcription.** PTM: post translational modification. Adapted from Cuthbert *et al.* 2004, Hassa *et al.* 2006, Berger 2007, Kouzarides 2007, Li *et al.* 2007.

| Chromatin Modifications | Residues Modified | Transcriptionally relevant sites | Transcriptional role |
|---|---|---|---|
| **Histone PTMs** | | | |
| Acetylation | **K**-ac | H3 (9,14,18,56), H4 (5,8,13,16), H2A, H2B, H2A.Z | Activation |
| Methylation (lysines) | **K**-me1 **K**-me2 **K**-me3 | H3 (4,79) | Activation |
| | | H3 (27), H4 (20) | Repression |
| | | H3 (9,36) | Activation/ repression |
| Methylation (arginines) | **R**-me1 **R**-me2a **R**-me2s | H3 (2,17,26), H4 (3) | Activation |
| Phosphorylation | **S**-ph **T**-ph | H3 (3,10,28), H2A, H2B | Activation |
| Ubiquitylation | **K**-ub | H2B (120) | Activation |
| | | H2A (119) | Repression |
| Sumoylation | **K**-su | H2B (6/7), H2A (126) | Repression |
| Proline isomerization | **P-cis > P-trans** | H3 (30,38) | Activation/ repression |
| ADP ribosylation | **E**-ar | | Activation |
| Deimination | **R > Cit** | H3 (2,8,17,26) | Repression |
| **DNA methylation** | | | |
| Methylation (cytosines) | me**C** | CpG islands | Repression |

Acetylation is one of the most studied histone modification (Grunstein 1997). Histone acetyltransferases (HATs) transfer an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of certain lysine side chains within a histone whereas histone deacetylases (HDACs) rapidly remove these acetyl groups (Loidl 1994, Brownell *et al.* 1996, Hassig & Schreiber 1997, Pazin & Kadonaga 1997, Sterner & Berger 2000). Acetylation has strong potential to unfold chromatin and activate transcription as acetyl groups neutralize the basic charge of histones, thereby reducing strength of binding to negatively charged DNA (Taverna *et al.* 2007). In contrast,

methylation of histones does not reduce their charge, but can create binding sites for regulatory proteins that influence chromatin accessibility (Bannister & Kouzarides 2005). Moreover, in combination with other modifications methylation may induce alterations in chromatin architecture. Interestingly, histone methylation was believed to be irreversible until the recent discovery of enzymes that demethylate lysine (LSD1) (Shi *et al.* 2004) and arginine (PADI4) (Cuthbert *et al.* 2004, Wang *et al.* 2004). LSD1 (lysine-specific demethylase 1) uses an amine oxidase reaction to demethylate a specific lysine (K4) within histone H3 (Shi *et al.* 2004) whereas PADI4 (peptidyl arginine deiminase) converts methyl-arginine to citrulline in a process termed deimination to antagonize methylation on the arginine residues (Cuthbert *et al.* 2004, Wang *et al.* 2004). Furthermore, enzymes involved in phosphorylation (Nowak & Corces 2004), ubiquitylation (Shilatifard 2006), sumoylation (Nathan *et al.* 2006), ADP-ribosylation (Hassa *et al.* 2006) and proline isomerization (Nelson *et al.* 2006) have also been identified.

The histone modifications have been shown to functionally impact transcription, replication and repair (Groth *et al.* 2007, Li *et al.* 2007). In case of transcription, modifications including acetylation and phosphorylation correlate with transcriptional activation whereas sumoylation, deimination and proline isomerization correlate with repression (Kouzarides 2007). Furthermore, methylation and ubiquitylation are correlated with both activation and repression of transcription. Important regulatory elements such as promoters and enhancers are enriched with specific histone modifications correlating with transcriptional activity (Roh *et al.* 2005, Barski *et al.* 2007, Heintzman *et al.* 2007, Roh *et al.* 2007). Promoter regions of active genes have usually high levels of histone acetylation (H3K9Ac and H3K14Ac) and methylation (H3K4me3, H3K36me3 and H3K79me1) (Bernstein *et al.* 2005, Kim *et al.* 2005, Roh *et al.* 2005, Roh *et al.* 2006, Li *et al.* 2007) while repressed genes have elevated levels of methylation on different lysine residues (H3K9me3, H3K27me3, H3K79me3 and H4K20me) (Bannister & Kouzarides 2005, Boyer *et al.* 2006, Lee *et al.* 2006, Roh *et al.* 2006). Furthermore, some of these modifications, including the H3 acetylation and mono- and di-methylated H3K4, are also detected in intergenic regions and correlate with functional enhancers (Roh *et al.* 2005, Heintzman *et al.* 2007, Roh *et al.* 2007). Interestingly, promoters and other regulatory regions may also posses both permissive H3K4me3 and repressive H3K27me3 modifications (Azuara *et al.* 2006, Bernstein *et al.* 2006, Lee *et al.* 2006, Roh *et al.* 2006, Barski *et al.* 2007, Chang & Aune 2007, Mikkelsen *et al.* 2007, Taverna *et al.* 2007). Originally, this type of bivalent mark was found in embryonic stem cells (Azuara *et al.* 2006, Bernstein *et al.* 2006) but recently similar modification pattern was observed also in resting T cells (Barski *et al.* 2007). CpG rich promoters of developmentally regulated genes that are inactive but poised for induction or silencing depending on differentiation-driving signals are generally enriched with bivalent histone modifications, suggesting that in this context an active regulation is needed for silencing gene expression (Rowell *et al.* 2008). Thus, plasticity needed for precursor cells to respond to their environment and adopt new fates is provided by the dynamic nature of the chromatin landscape. Moreover, according to the histone code hypothesis, each individual histone modification acts in a combinatorial manner and thereby each pattern of modifications leads to a biological consequence (Strahl & Allis 2000, Jenuwein & Allis 2001, Margueron *et al.* 2005).

This hypothesis predicts that a pre-existing modification affects subsequent modifications on the same or different histones in the same or adjacent nucleosomes, and that these modifications serve as marks for the recruitment of different proteins or protein complexes which function to ensure the acquisition and maintenance of developmental and cell type–specific expression and silencing of genes (Kouzarides 2007, Li *et al.* 2007).

#### 2.2.2.2 Nucleosome positioning

The positioning of nucleosomes relative to the underlying DNA plays a critical role in regulating transcription as it determines the accessibility of regulatory elements to transcription factors (Henikoff *et al.* 2004, Li *et al.* 2007, Henikoff 2008). Micrococcal nuclease (MNase) digestion followed either by ligation-mediated PCR combined with DNA microarrays containing tiling oligonucleotide probes or massively parallel sequencing by Solexa high-throughput sequencing technique have been used to study the nucleosome positioning on whole genome level (Yuan *et al.* 2005, Schones *et al.* 2008). Interestingly, majority of nucleosomal DNA appears as ~140 bp peaks in a microarray survey (Yuan *et al.* 2005) and the same phasing is displayed in the H3K4me3 profiles of promoter regions suggested to mark nucleosome positions (Barski *et al.* 2007, Schones *et al.* 2008), indicating that nucleosomes are relatively well-positioned. *In vitro* transcription experiments have shown that the nucleosome forms a strong barrier to RNA polymerase II (Kireeva *et al.* 2005, Bondarenko *et al.* 2006). Interestingly, active genes have reduced nucleosome occupancy in their promoter regions (Bernstein *et al.* 2004, Lee *et al.* 2004, Sekinger *et al.* 2005, Yuan *et al.* 2005, Ozsolak *et al.* 2007), which is not observed in enhancer elements (Yuan *et al.* 2005)). In addition, active and silent genes exhibit differential positioning of the first nucleosome downstream of the transcription start site (Schones *et al.* 2008). Moreover, RNA polymerase II binding directly correlates with nucleosome phasing relative to the start site in active genes (Schones *et al.* 2008). Chromatin-remodeling complexes, of which the two best-studied families are SWI/SNF and ISWI, utilize ATP hydrolysis to alter the histone-DNA contacts (Saha *et al.* 2006). Several strategies are used for accessing the nucleosomal DNA: (1) DNA can be transiently unwrapped from the histone octamer surface (Langst & Becker 2001, Li & Widom 2004), (2) conformational change in the octamer can lift the DNA off the internal surface of the nucleosome (Narlikar *et al.* 2001), (3) the entire nucleosome can be exchanged in and out of chromatin (replacement) (Lorch *et al.* 1999, Phelan *et al.* 2000, Reinke & Horz 2003, Boeger *et al.* 2004) or (4) the histone octamer can move translationally along the DNA (sliding) (Hamiche *et al.* 1999, Langst *et al.* 1999, Whitehouse *et al.* 1999, Flaus & Owen-Hughes 2003). In order to move a nucleosome, DNA-bound histones must be transferred to another acidic component, such as neighboring DNA (sliding) or a histone chaperone (replacement) (Rando & Ahmad 2007).

#### 2.2.2.3 DNA methylation

Cytosine methylation of CpG dinucleotides is a crucial mechanism through which transcriptional activity is controlled (Jones & Takai 2001, Bird 2002, Jaenisch & Bird 2003). Increased DNA methylation strongly correlates with gene silencing whereas

transcriptionally active regions are relatively demethylated. DNA methylation represses gene expression by recruiting methyl-CpG binding proteins, such us MeCP2, MBD1 and MBD2, corepressors and histone deacetylases, which can block the binding of transcription factors and generate inaccessible chromatin structure (Bird & Wolffe 1999). CpG methylation is mediated by DNA methyltransferases (Dnmts), including Dnmt1, which maintain patterns of DNA methylation from one generation to the next by copying CpG methylation patterns from the parental DNA strand to the daughter strand during S phase (Jones & Takai 2001). DNA can be demethylated through a passive or an active mechanism (Bird 2002). In the passive model of demethylation, the DNA methylation is gradually lost upon consecutive DNA replications by inhibiting the recruitment of Dnmt1 that normally targets hemimethylated sites and thereby preserves the pattern of methylation. In contrast, in the active model of demethylation the methyl groups are catalytically removed by demethylating enzymes. This latter mechanism of demethylation is yet not fully established in mammalian cells.

### 2.2.3 Regulation of cytokine expression in CD4+ T cells

The key effector function of activated CD4+ T helper cells is to produce cytokines which direct the recruitment, proliferation and function of many cell types involved in immune and inflammatory responses (Abbas *et al.* 1996). A number of different types of *cis*-regulatory elements, including enhancers, silencers and LCRs, have been identified in the *Ifng* and Th2 cytokine loci using (1) bioinformatics to identify evolutionarily conserved non-coding sequences (CNSs) between species, (2) biochemical methods to identify sites susceptible to digestion by DNase I and (3) functional analysis using reporter assays, transgenic mice and knockout mice to characterize *in vivo* role of identified regulatory elements (Stalder *et al.* 1980, Keene *et al.* 1981, Gross & Garrard 1988, Nardone *et al.* 2004). In parallel, the distribution of epigenetic marks on the histones has been unraveled using modification-specific antibodies in chromatin immunoprecipitations (ChIP) (Kouzarides 2007). More recently, DNase I hypersensitivity assay and ChIP have been coupled to high-throughput methods including genome tiling arrays (DNase-chip and ChIP-chip) and sequencing (DNase-seq and ChIP-seq) to detect modifications on whole genome level (Barski *et al.* 2007, Boyle *et al.* 2008, Schones *et al.* 2008). Furthermore, correlating these high-resolution genome wide modification maps to gene expression profiles in a particular cell type provides important insights to the function of specific elements (Rowell *et al.* 2008).

#### 2.2.3.1 Regulation of Ifng expression

***cis-regulatory elements in Ifng locus***

IFN-γ is the hallmark cytokine of Th1 effector cells produced in response to viral or intracellular bacterial infection (Szabo *et al.* 2003). The *Ifng* proximal promoter contains binding sites for an array of transcription factors including ATF-2, c-Jun and other Jun proteins, CREB, ATF-1, Oct-1, YY1, NFAT and T-bet (Penix *et al.* 1996, Agarwal & Rao 1998, Sweetser *et al.* 1998, Soutto *et al.* 2002a). Recently, Djuretic *et al.* showed that Runx3 and T-bet, both of which are upregulated during Th1 polarization, bind to the *Ifng* promoter interdependently and cooperate to ensure the

maximal IFN-γ production (Djuretic *et al.* 2007). However, the proximal regulatory elements are not sufficient to confer lineage-specific expression of *Ifng* indicating the crucial role for distal regions in the regulation of *Ifng* locus (Young *et al.* 1989, Zhu *et al.* 2001a, Soutto *et al.* 2002b). Multiple *cis*-regulatory elements have been identified within ~110 kb surrounding *Ifng* including enhancers and insulators (Young *et al.* 1989, Agarwal & Rao 1998, Lee *et al.* 2004a, Shnyreva *et al.* 2004, Hatton *et al.* 2006, Schoenborn *et al.* 2007). IFNgCNS1 located 5 kb upstream and IFNgCNS2 located 18 kb downstream of the transcription start site of the murine *Ifng* gene demonstrated enhancer activity in transient transactivation assays and Th1-specific DNase I hypersensitivity (Lee *et al.* 2004a, Shnyreva *et al.* 2004). Furthermore, binding of T-bet to CNS-2 induces transcriptionally favorable chromatin structure (Shnyreva *et al.* 2004) while binding of both T-bet and NFAT to CNS-1 in stimulated Th1 cell lines augments enhancer activity (Lee *et al.* 2004a) indicating that these sites play a crucial role in promoting *Ifng* expression. Recently, Schoenborn *et al.* performed comprehensive high-resolution mapping of *Ifng* locus and identified several novel distal regulatory elements and characterized their role in Th cell lineage-specific regulation of *Ifng* expression (Schoenborn *et al.* 2007). They mapped eight CNSs surrounding *Ifng* of which the most distal ones were observed to function as boundary elements. The more proximal CNSs were shown to exhibit enhancer activity. IfngCNS-22 is an enhancer possessing permissive histone marks in Th1 and Th2 cells as well as in naïve CD4+ cells poising the *Ifng* gene for induction during the initiation of Th1 differentiation (Hatton *et al.* 2006, Schoenborn *et al.* 2007) whereas IfngCNS-46 is an insulator which may orchestrate spreading of repressive histone modifications in Th2 cells and block silencing effects from downstream regions in Th1 cells (Schoenborn *et al.* 2007, Rowell *et al.* 2008). Interestingly, all strong DNase I hypersensitivity sites detected in Th1 cells locate in a CNS whilst Th2 cell or naïve CD4+ cell specific hypersensitivity sites are adjacent to but not in a CNS suggesting a Th cell lineage specific role of the multiple distal regulatory elements in the *Ifng* locus (Schoenborn *et al.* 2007).

***Epigenetic regulation of Ifng***

Dynamic regulation of histone modifications across *Ifng* locus reflecting its transcriptional activity has been observed both in Th1 and Th2 polarized cells. Interestingly, TCR stimulation induces initial histone hyperacetylation as well as histone methylation of *Ifng* locus irrespective of polarizing cytokines (Avni *et al.* 2002, Fields *et al.* 2002, Chang & Aune 2005, Chang & Aune 2007, Schoenborn *et al.* 2007). However, during the differentiation "bivalent" histone marks disappear and the histone modifications are regulated in a lineage- and transcription factor-dependent fashion. In Th1 cells *Ifng* transcription is upregulated and sustained by increased histone hyperacetylation mediated by T-bet and STAT4 (Avni *et al.* 2002, Fields *et al.* 2002, Chang & Aune 2005). Nevertheless, a very recent study showed that in the absence of Jak3-dependent signals, binding of T-bet to the *Ifng* promoter is impaired possibly due to reduced accessibility of the *Ifng* locus caused by decreased histone acetylation indicating that Jak3 plays a crucial role in regulation of epigenetic modification of the *Ifng* locus during Th1 cell differentiation (Shi *et al.* 2008). Furthermore, permissive H3K4 methylation is acquired and repressive H3K27 methylation is nearly completely removed in the *Ifng* locus in Th1 differentiated cells (Schoenborn *et al.* 2007). In

contrast, during Th2 differentiation methylation of H3K27 is increased in a GATA3- and STAT6-dependent mechanism accompanied by recruitment of the Polycomb family histone methyltransferase EZH2 to the *Ifng* locus (Chang & Aune 2007, Schoenborn *et al.* 2007). Moreover, progressive demethylation of DNA in the proximal and distal regulatory elements of the *Ifng* gene has been observed during Th1 differentiation (Young *et al.* 1994, Mullen *et al.* 2002, Schoenborn *et al.* 2007) while in Th2 cells and other cells that do not express IFN-γ the CpG nucleotides are increasingly methylated reducing transcription factor binding (Penix *et al.* 1996, Winders *et al.* 2004, Schoenborn *et al.* 2007). The essential role of DNA methylation in *Ifng* regulation is further confirmed by following knockout mice studies. Dnmt1 deficient T cells have markedly reduced levels of DNA methylation and *in vitro* activated naïve Dnmt1$^{-/-}$ CD4+ T cells produce ~5-10-fold more IFN-γ. However, in response to polarizing culture conditions the IFN-γ production is appropriately induced or silenced in Th1 and Th2 cells, respectively (Makar & Wilson 2004). Methyl CpG-binding domain protein-2 (MBD2) recruits the multiprotein nucleosome remodeling and deacetylase (NuRD) repressive complex, together comprising the methyl CpG binding protein 1 (MeCP1) complex, to methylated DNA thereby linking CpG methylation to repressive chromatin structure (Ng *et al.* 1999, Feng & Zhang 2001, Hendrich *et al.* 2001). IFN-γ production of MBD2-deficient T helper cells is increased under all polarizing conditions indicating that DNA methylation and MBD2 are required for repressing *Ifng in vivo* (Hutchins *et al.* 2005).

#### 2.2.3.2 Regulation of Th2 cytokine locus in CD4+ T cells

Th2 cells produce cytokines IL-4, IL-5 and IL-13 to protect against extracellular pathogens (Murphy & Reiner 2002). The Th2 cytokine gene cluster provides an important model system in which to study transcriptional regulation and lineage-dependent chromatin changes. The evolutionarily conserved locus comprises three cytokine genes *IL-4*, *IL-5* and *IL-13* as well as two housekeeping genes *Rad50* and *Kif3a* (Frazer *et al.* 1997, Loots *et al.* 2000). As *Rad50* is constitutively expressed whereas expression of the adjacent cytokine genes needs to be highly regulated in both lineage-restricted and temporal fashion, coordinated long-range regulatory mechanisms are required (Smale & Fisher 2002).

***cis-regulatory elements in Th2 cytokine locus***

Several DNase I-hypersensitivity sites, including HSS1, HSS2, HSII, HSV, HSVa, RHS4 and RHS7/RAD50-C, spread across the *IL4-IL13-Rad50-IL5* locus are Th2 specific (Agarwal & Rao 1998, Takemoto *et al.* 1998, Agarwal *et al.* 2000, Fields *et al.* 2004, Lee & Rao 2004b). HSS1 and HSS2, located in the intergenic region between *IL-13* and *IL-4* genes, are included in CNS-1 (Loots *et al.* 2000), which is an important coordinate regulator of Th2 cytokine genes. CNS-1 exhibits strong enhancer activity shown by studies in transgenic reporter mice (Lee *et al.* 2001) and furthermore, deletion of CNS-1 results in a clear reduction of cells producing IL-4, IL-5 and IL-13 (Loots *et al.* 2000, Mohrs *et al.* 2001). IL-4 promoter activity is also enhanced by HSII (also termed as the intronic enhancer (IE)) in the second intron of the *IL-4* gene and this enhancement is GATA3-dependent when HSII acts in combination with CNS-1 (Lee *et al.* 2001). HSV that overlaps CNS-2, and HSVa are located 3' of *IL-4* in close

proximity to each other (Agarwal & Rao 1998, Agarwal *et al.* 2000). HSVa is an activation-dependent HS that binds GATA3 and NFAT1 (Agarwal *et al.* 2000) and functions as an enhancer (Agarwal *et al.* 2000, Lee *et al.* 2001). Interestingly, HSVa is specifically induced in IL-4-producing Th2 cells compared to IL-4-nonproducers suggesting that HSVa controls probabilistic expression of IL-4 (Guo *et al.* 2004). Moreover, the expression of IL-4, IL-5 and IL-13 is clearly reduced in HSV+HSVa knockout mice Th2 cells (Solymar *et al.* 2002). RHS4 and RHS7 are located in 3' region of the *rad50*, and RHS7 has been shown to exhibit enhancer activity, but more importantly, they are part of the Th2 cytokine LCR which includes two additional HS sites: RHS5/RAD50-O and RHS6/RAD50-(A+B) (Lee *et al.* 2003, Fields *et al.* 2004, Lee & Rao 2004b, Lee *et al.* 2005). The presence of an LCR within the Th2 cytokine locus was indicated using bacterial artificial chromosome (BAC) transgenic mice which showed strong, position independent, Th2 specific and copy number dependent luciferase reporter activity in effector CD4+ T cells (Lee *et al.* 2003). Using the chromosome conformation capture assay (3C) the Th2 LCR was shown to participate in a chromatin core configuration where the promoters for the genes encoding Th2 cytokines are brought in close spatial proximity in CD4+ T cells (Spilianakis & Flavell 2004). GATA3 and STAT6 are essential factors for the establishment and/or maintenance of these intrachromosomal interactions (Spilianakis & Flavell 2004). Furthermore, GATA3 binds RHS7 *in vivo* and deletion of RHS7 clearly reduce formation of these long-range interactions of various *cis*-regulatory elements in Th2 cytokine locus, suggesting a central role of RHS7 in the proper regulation of Th2 cytokine expression (Lee *et al.* 2005).

An exception to the HS sites described above is provided by HSIV, the *IL-4* silencer, located in a conserved noncoding sequence toward the 3' end of the *IL-4* locus (Ansel *et al.* 2004). HSIV is constitutively hypersensitive in naïve CD4+ T cells, Th1 and Th2 cells (Agarwal & Rao 1998, Ansel *et al.* 2004). Deletion of HSIV by gene targeting led to increased *IL-4* and *IL-13* transcription by naïve T cells. Notably, the IFN-γ expression of HSIV-deficient Th1 cells was normal, but part of the cells also produced aberrantly large amounts of IL-4, indicating that the HSIV site is a crucial *cis*-regulatory region responsible for *IL-4* silencing during differentiation of Th1 cells (Ansel *et al.* 2004). Recently, the transcription factor Runx3 was shown to mediate HSIV-dependent *IL-4* silencing (Djuretic *et al.* 2007, Naoe *et al.* 2007). The Runx complexes are composed of two subunits, including Cbfβ protein and one of the Runx family proteins possessing a conserved DNA-binding Runt domain (Speck 2001). The repression of IL-4 in Th1 cells is impaired in Cbfβ - or Runx3-deficient T cells (Djuretic *et al.* 2007, Naoe *et al.* 2007). Furthermore, asthma-related symptoms develop in these mice, including infiltration of eosinophils into the lung in Runx3-deficient mice (Fainaru *et al.* 2004, Fainaru *et al.* 2005) and elevated serum IgE and airway infiltration in mice lacking Cbfβ in their T cells (Naoe *et al.* 2007). Runx3 is induced in Th1 cells in a T-bet-dependent manner and cooperates with T-bet to silence *IL-4* in Th1 cells (Djuretic *et al.* 2007). Runx3 and T-bet bind to the *IL-4* silencer, and binding of Runx complexes is inhibited by GATA3, indicating that lineage-restricted transcription factors are involved in Runx mediated Th subtype-specific regulation of *IL-4* expression (Djuretic *et al.* 2007, Naoe *et al.* 2007).

***Epigenetic regulation of Th2 cytokines***

Besides *cis*-regulatory elements, the epigenetic changes play an important role in the regulation of the Th2 cytokines. Similarly to *Ifng* locus, also *IL-4* locus acquires initial histone hyperacetylation as well as histone methylation modifications upon TCR stimulation in a lineage-independent fashion (Avni *et al.* 2002, Fields *et al.* 2002). Nevertheless, later during Th2 differentiation, selective histone hyperacetylation occurs at regulatory regions of *IL-4*, *IL-5* and *IL-13* and this process is strongly dependent on the continued presence of IL-4, STAT6 and GATA3 (Avni *et al.* 2002, Fields *et al.* 2002, Yamashita *et al.* 2002, Baguet & Bix 2004). GATA3-mediated targeting and downstream spreading of histone hyperacetylation within *IL-4* and *IL-13* loci may be orchestrated by a conserved GATA3 response element (CGRE), located upstream of the *IL-13* gene, which binds GATA3, histone acetyltransferase complexes including CBP/p300 and RNA polymerase II (Yamashita *et al.* 2002). Furthermore, in Th1 cells permissive histone modifications, H3K9/K14ac, H3K4me2 and H3S10ph, are almost completely removed from the *IL4-IL13* locus whereas H3K27 methylation, a repressive histone modification, is acquired correlating with silenced Th2 cytokine expression (Grogan *et al.* 2003, Baguet & Bix 2004, Koyanagi *et al.* 2005). Interestingly, methylated H3K27 occurs selectively at HSIV and HSS3, the only two lineage-nonspecific hypersensitivity sites at the *IL4-IL13* locus in naïve CD4+ cells and Th1-polarized but not Th2-polarized cells (Takemoto *et al.* 1998, Ansel *et al.* 2004), and the enzyme responsible for the methylation is EZH2 which associates with HSIV and HSS3 (Koyanagi *et al.* 2005). Moreover, the *IL-4* silencer, HSIV, exhibits an unusual pattern of both activating and silencing histone modifications of which the repressive marks are selectively lost under Th2 culture conditions suggesting a critical role for this element in silencing the inappropriate expression of IL-4 and IL-13 in naïve and Th1 T cells (Baguet & Bix 2004, Koyanagi *et al.* 2005).

In naïve cells, the *IL4-IL13* locus is predominantly methylated (Lee *et al.* 2002, Santangelo *et al.* 2002). Dnmt1, a DNA methyltransferase, maintains DNA methylation from one generation to the next by copying patterns of CpG methylation from the parental DNA strand to the daughter strand during S phase (Bird & Wolffe 1999). During development of Th2 cells recruitment of Dnmt1 is prevented, accompanied by CpG demethylation of the Th2 cytokine locus (Agarwal & Rao 1998, Bird *et al.* 1998, Guo *et al.* 2002) strikingly correlating with the degree of expression of IL-4 (Guo *et al.* 2002, Lee *et al.* 2002). Using transgenic mice, it was shown that the loss of GATA3 expression results in decreased Th2 cytokine production and increased DNA methylation at the *IL-4* gene locus (Yamashita *et al.* 2004). Interestingly, even if activated Dnmt1-deficient CD4+ T cells produce substantially more IL-4 without affecting GATA3 expression, the Th2 cytokine expression is appropriately up- and downregulated in the presence of polarizing cytokines suggesting that DNA methylation and lineage-specific transcription factors account for complementary and nonredundant mechanisms by which the Th2 effector program is regulated (Makar *et al.* 2003, Makar & Wilson 2004). Moreover, helper T cells lacking MBD2, a methyl CpG binding domain protein, (Hendrich *et al.* 2001) express IL-4 ectopically (Hutchins *et al.* 2002). According to the quantitative competition model, *Mbd2* gene dosage is inversely correlated to the increasing efficiency of GATA3 to promote IL-4 production. High levels of GATA3 can displace MBD2 from the *IL-4* locus prior to the

onset of CpG demethylation and thereby overcome the effects of MBD2-mediated silencing (Hutchins *et al.* 2002). Furthermore, DNA can be demethylated via either a passive mechanism, where methylation is depleted upon replication of DNA and cell division, or an active process that involves catalytic removal of the methyl groups by enzymatic activity. Interestingly, a slow DNA demethylation process is observed upon exclusion of Dnmt1 from the *IL-4* locus and this may play an important role in stabilizing high-level Th2 cytokine expression (Wilson & Merkenschlager 2006). Nevertheless, upon initiation of Th2 cell differentiation, the RHS7 located to Th2 LCR undergoes the most efficient demethylation in the entire *IL-4* locus in an active, STAT6-dependent manner, and may thereby facilitate early Th2 cytokine production (Fields *et al.* 2004, Kim *et al.* 2007a).

#### 2.2.3.3 Regulation of IL-5 expression

IL-5 predominantly produced by activated Th2 cells but also by mast cells and eosinophils (Mosmann & Coffman 1989, Plaut *et al.* 1989, Dubucquoi *et al.* 1994), is involved uniquely in control of the growth, differentiation and activation of eosinophils (Campbell *et al.* 1987, Sanderson 1988, Yamaguchi *et al.* 1988). Dysregulated expression of IL-5 causing an abnormally large number of eosinophils (eosinophilia) accumulating in blood and tissues has been strongly linked with the pathogenesis of a number of allergic and inflammatory diseases, most notably asthma, rhinitis and dermatitis (De Monchy *et al.* 1985, Leung 1998). Besides its role in eosinophil function, IL-5 activates basophils and promotes proliferation and differentiation of murine B lymphocytes (Sanderson *et al.* 1988, Hirai *et al.* 1990).

Even if Th2 cytokines are generally considered to be co-regulated, several observations indicate that the expression of *IL-5* is regulated independently of other Th2 cytokines, in particular *IL-4*. Using murine T helper type 2 cell clones, it was shown that T-cell receptor-mediated and cytokine receptor-mediated signals induce different pattern of cytokine gene expression suggesting a differential regulation especially for the *IL-4* and *IL-5* genes (Bohjanen *et al.* 1990). Furthermore, Jung *et al.* observed that human T-cells rarely co-express IL-4 and IL-5 (Jung *et al.* 1995). Interestingly, histone hyperacetylation of the *IL-5* gene during differentiation of Th2 cells displays a significantly delayed kinetics compared with that of the *IL-4* and *IL-13* genes suggesting a distinct remodeling mechanism for the *IL-5* gene locus (Yamashita *et al.* 2002). Finally, the biological specificity of eosinophilia and its control by IL-5 implies a unique and independent regulation of *IL-5* expression (Sanderson 1992).

Efficient production of IL-5 requires activation of both TCR and a second signaling pathway (Tominaga *et al.* 1988). *In vitro* this can be achieved by using PMA in combination with anti-CD28, cyclic adenosine 3',5'-monophosphate (cAMP) or the lectin concanavalin A (ConA) (Lee *et al.* 1993, Schandene *et al.* 1994, Van Straaten *et al.* 1994, Karlen *et al.* 1996). Interestingly, IL-5 expression induced with PMA and cAMP is inhibited by the immunosuppressant drug cyclosporine A (CsA) whereas it cannot block the production stimulated by PMA and anti-CD28 (Karlen *et al.* 1996, Umland *et al.* 1999) or PMA and Con A (Van Straaten *et al.* 1994, Naora & Young 1995), suggesting that the induction of IL-5 expression can occur via at least two

distinct pathways. The production of IL-5 is regulated mainly at the level of transcription as protein synthesis inhibitors cyclohexamide (CHX) and anisomycin completely inhibit *IL-5* mRNA synthesis (Van Straaten *et al.* 1994, Naora & Young 1995, Stranick *et al.* 1995). In addition, Umland *et al.* showed that regulation of mRNA stability also plays a role in the control of IL-5 production as *IL-5* mRNA is significantly more stable than other cytokine transcripts (Umland *et al.* 1998). However, *de novo* protein synthesis is an absolute requirement for the initiation of *IL-5* transcription (Umland *et al.* 1998).

In the *IL-5* promoter, the conserved lymphokine element 0 (CLE0; -42 bp to -56 bp) acts as an on/off switch for *IL-5* transcription (Yamagata *et al.* 1995). This crucial element binds the transcription factor activator protein-1 (AP-1) members JunD and Fra-2 as well as Oct-1 and Oct-2 factors (Mori *et al.* 1997, Thomas *et al.* 1999). Schwenger *et al*. (2002) observed that the amount of IL-5 produced correlates with the formation of the AP-1 complex and that the *de novo* synthesis of JunD and Fra-2 induced by PMA-stimulation is the rate-limiting step of the production of the AP-1 complex. Furthermore, at least 3 positive (-80 bp to -45 bp (also known as IL-5-REI), -123 bp to -92 bp (IL-5-REII), -312 bp to -227 bp) and 4 negative (-90 bp to -79 bp, -170 bp to -130 bp (IL-5-REIII), -312 bp to -404 bp and -448 bp to -460 bp) regulatory elements of the human *IL-5* promoter have been identified (Gruart-Gouilleux *et al.* 1995, Stranick *et al.* 1997, Mordvinov *et al.* 1999, Schwenger *et al.* 1999).

The 5' flanking region of the human *IL-5* gene has been reported to interact with a variety of transcription factors that play important roles on *IL-5* expression. GATA3 binds to at least three sites in the *IL-5* promoter. Positive regulation is mediated by -70 and -152 sites whereas binding to -400 site results in strong inhibition of *IL-5* expression (Yamagata *et al.* 1995, Blumenthal *et al.* 1999, Schwenger *et al.* 2001). In addition, NFAT, ETS1, ETS2, YY1, C/EBPβ and glucocorticoid receptor (GR) are involved in the complex regulation of *IL-5* transcription (Prieschl *et al.* 1995, Blumenthal *et al.* 1999, De Boer *et al.* 1999, Mordvinov *et al.* 1999, Schwenger *et al.* 1999, Cousins *et al.* 2000, Li-Weber *et al.* 2001, Wang *et al.* 2006). Some of these factors have been shown to cooperate on *IL-5* promoter and synergistically regulate *IL-5* expression (Blumenthal *et al.* 1999, Mordvinov *et al.* 1999, Schwenger *et al.* 1999, Liu *et al.* 2004). Interestingly, a recent study reported that GR represses *IL-5* expression by binding to an NFAT-AP-1 site in the *IL-5* proximal promoter. This repression is mediated by recruiting histone deacetylases as the HDAC inhibitor Trichostatin A (TSA) caused a partial relief of repression whereas co-transfection of cells with HDAC1 augmented the repression. Furthermore, the NFAT-AP-1 site juxtaposes a crucial GATA3 site in the proximal promoter of *IL-5* and the recruitment of GR strongly inhibits the GATA3-dependent transcriptional activation of *IL-5* whilst NFAT/AP-1 protein complex can compete with GR for DNA binding and impair this repression (Jee *et al.* 2005). Recent studies have further elucidated the role of histone modifications in the dynamic regulation of *IL-5* expression. Histone hyperacetylation of the *IL-5* gene in CD4+ cells is Th2-specific and occurs in a STAT6- and GATA3-dependent manner (Yamashita *et al.* 2002). The acetylation status of histones at the *IL-5* promoter and thereby the activity of the *IL-5* promoter is reciprocally controlled by HDAC4 and p300, a HAT. HDAC4 forms protein complexes with GATA3 and YY1

and this mediates the recruitment of HDAC4 to the *IL-5* promoter with concurrent change in the expression of IL-5 (Han *et al.* 2006b). CBP/p300 activate the *IL-5* promoter synergistically with the transcription factors C/EBP, NFAT and c-Fos, and the HAT activity of CBP/p300 is required to activate IL-5 expression (Liu *et al.* 2004).

**2.2.3.4 Interchromosomal regulation of Ifng and Th2 cytokine loci in CD4+ T cells**

Currently it is well accepted that coordinate regulation of gene expression involves higher-order organization of chromatin where large intervening chromosomal regions between two regulatory sites are looped out bringing crucial distant regulatory elements in a close spatial proximity through intrachromosomal interactions (de Laat 2007, Dekker 2008). Th2 cytokine locus undergoes excessive folding in Th2 cells as well as in Th1 and naïve CD4+ T cells forming an initial chromatin core configuration containing the promoters for the genes encoding *IL-4*, *IL-5* and *IL-13*, which may facilitate their coordinate expression (Spilianakis & Flavell 2004). In activated Th2 cells the looping is orchestrated by SATB1 which binds to several sites within Th2 cytokine locus extending from *IL-5* past *Kif3a* driving the formation of additional, smaller loops (Cai *et al.* 2006).

Intriguingly, a new dimension to our understanding of the higher-order regulation of gene expression was provided by genome-wide 4C analyses (3C-on chip or circular 3C) showing that chromosomal interactions also occur between regions on different chromosomes (Simonis *et al.* 2006, Zhao *et al.* 2006, Simonis *et al.* 2007). In theory, interaction of separate chromosomes in the nucleus could permit regulatory elements from one chromosome to control the transcription of a gene located on another chromosome (Rowell *et al.* 2008). Using 3C technique and 2D-FISH, Spilianakis *et al.* (2005) demonstrated that in mouse the *Ifng* promoter on chromosome 10 physically interacts with the regulatory regions in the Th2 cytokine locus, including the *IL-5* promoter, *Rad50* promoter and RHS6 DNase I hypersensitive site of the Th2 LCR, on chromosome 11 (Spilianakis *et al.* 2005). During the differentiation of naïve T cells to effector Th1 or Th2 cells interchromosomal interactions between these mutually exclusive loci are abrogated and intrachromosomal contacts within the *Ifng* and Th2 cytokine loci increase upon gene activation. The Th2 LCR is required for the T-cell-specific dynamic hub formation as mutation of RHS7 within the LCR not only abolished these long-range interchromosomal associations but also impaired transcription on both chromosomes: *Ifng* expression was delayed and *IL-5* expression was decreased when naïve T cells were differentiated under Th1 and Th2 conditions, respectively. Proximity of these cytokine loci in naïve T cells may help initiating or enforcing opposite epigenetic states while the switch from inter- to intrachromosomal interactions may contribute to lineage-specific transcriptional initiation or silencing of these loci (Spilianakis *et al.* 2005).

**2.2.3.5 miRNA regulated cytokine expression in CD4+ T cells**

The microRNAs (miRNAs) have distinct temporal and spatial expression patterns and play critical regulatory roles in various physiological processes, including hematopoiesis, cell differentiation, proliferation and survival, as well as organ development (Chen *et al.* 2004, Mendell 2005). Furthermore, besides affecting the

outcome of immune responses to infection and development of diseases of immunological origin (reviewed in Garzon & Croce 2008), miRNA expression can distinguish normal cells from cancer cells (Lu *et al.* 2005). Using microarray technology and high-throughput sequencing the miRNA expression profiles in the hematopoietic system have been investigated globally (Monticelli *et al.* 2005, Landgraf *et al.* 2007). Interestingly, several studies using hematopoietic stem cells, progenitor cells and/or fully differentiated cells have shown that miRNA expression pattern and abundance, yet partially overlapping, varies strikingly according to the developmental stage, cell lineage and activation (Monticelli *et al.* 2005, Cobb *et al.* 2006, Garzon *et al.* 2006, Taganov *et al.* 2006, Georgantas *et al.* 2007, Neilson *et al.* 2007, O'Connell *et al.* 2007, Wu & Liu 2007, Zhan *et al.* 2007). Moreover, the diversity of affected protein-coding transcripts is combinatorially increased by the fact that each mRNA can be targeted by a number of miRNAs and each miRNA can target several transcripts (Lewis *et al.* 2003, Krek *et al.* 2005, Lewis *et al.* 2005, Lim *et al.* 2005).

miRNAs are endogenous ~22 nt non-coding RNAs transcribed mainly by RNA polymerase II from a specialized set of miRNA genes (Bartel 2004, Lee *et al.* 2004). The primary transcripts (pri-miRNAs) are 5' 7-methyl guanosine capped and 3' poly(A) tailed sequences (Cai *et al.* 2004) of even several kilobases (Lee *et al.* 2002) and contain a mature miRNA sequence and a varying amount of flanking region (Cai *et al.* 2004). Pri-miRNA transcript forms a stem loop structure (hairpin) due to sequence complementarity and is cleaved by the nuclear RNase III enzyme Drosha into a precursor ~70 nt miRNA (pre-miRNA) (Lee *et al.* 2002, Lee *et al.* 2003) which is exported from the nucleus to the cytoplasm by nuclear export factor Exportin 5 (Exp5) and its RAS-related nuclear protein-guanosine triphosphate (RAN-GTP) cofactor (Yi *et al.* 2003, Bohnsack *et al.* 2004, Lund *et al.* 2004, Han *et al.* 2006a). In the cytoplasm, the pre-miRNA is further processed by the cytoplasmic RNase III enzyme Dicer to generate ~22 nt miRNA duplexes (Grishok *et al.* 2001, Hutvagner *et al.* 2001, Ketting *et al.* 2001, Lee *et al.* 2003). One strand of the Dicer product forms the mature miRNA, which is finally incorporated into a large protein complex, termed the RNA-induced silencing complex (RISC), containing Argonaute and other proteins (Khvorova *et al.* 2003, Schwarz *et al.* 2003, Liu *et al.* 2004, Chendrimada *et al.* 2005, Chendrimada *et al.* 2007). miRNA guides RISC to complementary mRNA targets where it binds to sequences in the 3' untranslated regions (UTRs) of target and act predominantly by regulating mRNA stability or inhibiting the initiation of translation (Jing *et al.* 2005, Kiriakidou *et al.* 2007, Mathonnet *et al.* 2007).

The significance of miRNAs and RNAi pathway during hematopoiesis has been examinated by disrupting miRNA biogenesis at specific steps using genetic deletion and transgenic approaches. As loss of Dicer is lethal early in development (Bernstein *et al.* 2003), the functional role of Dicer in T lymphocytes has been studied using conditional deletion (Cobb *et al.* 2005, Muljo *et al.* 2005). Dicer deficiency in T lymphocytes blocks peripheral CD8+ T cell development whereas Dicer-deficient CD4+ T cells, although reduced in numbers in thymus and periphery, are viable and express appropriate lineage-specific markers (Cobb *et al.* 2005, Muljo *et al.* 2005). Interestingly, Dicer-null helper T cells proliferate poorly and undergo increased apoptosis upon TCR stimulation. Furthermore, under Th2 culture conditions these cells

fail to repress *Ifng*, which may be due to reduced GATA3 expression in Dicer-deficient Th2 cells (Muljo et al 2005). Further insights in the role of miRNAs in T helper cell differentiation have been provided by targeted disruption or ectopic expression of specific miRNAs. Probably the best-studied miRNA in this context is miR-155. The evolutionarily conserved miR-155 is produced from the non-protein-coding transcript of the *bic* gene that was discovered as a common retroviral integration site in avian leukosis virus-induced lymphomas (Tam *et al.* 1997). In T lymphocytes the expression of the *bic* transcript and of mature miR-155 is absent in naïve CD4+ T cells but is rapidly induced after cross-linking of the antigen receptor (Tam 2001, Haasch *et al.* 2002, Thai *et al.* 2007). Mice lacking miR-155 are immunodeficient and display increased lung airway remodeling (Rodriguez *et al.* 2007). Using transcriptome analysis on miRNA-155-null CD4+ T cells, a wide spectrum of genes, including cytokines, chemokines, and transcription factors was shown to be regulated by miRNA-155 (Rodriguez *et al.* 2007). Furthermore, when miR-155 deficient CD4+ T cells were activated without any polarizing cytokines or they were cultured in suboptimal Th2 conditions, the cells produced more IL-4 and less IFN-γ than control cells (Rodriguez *et al.* 2007, Thai *et al.* 2007). These results strongly suggest that miRNA-155 plays a key role in the function of the immune system (Rodriguez *et al.* 2007, Thai *et al.* 2007). The miRNA miR-146a is suggested to play a role in the immune system as it is induced in response to different microbial components and proinflammatory cytokines in an NF-κB-dependent manner (Taganov *et al.* 2006). miR-146a is substantially increased in T helper 1 cells whereas its expression is decreased in T helper 2 cells compared to naïve CD4+ T cells (Monticelli *et al.* 2005). Interestingly, induction of miR-146a in Th1 cells may be explained by the action of cytokine IL-18 that synergistically with IL-12 controls Th1 differentiation and secretion of IFN-γ (Murphy & Reiner 2002). IL-18 receptor signaling triggers NF-κB signaling among other pathways and employs TRAF6 and IRAK1 to upregulate miR-146a (Taganov *et al.* 2006). Mice ectopically expressing miR-17-92 in lymphocytes develop lymphoproliferative disease and autoimmunity and die prematurely (Xiao *et al.* 2008). In addition, CD4+ T cells from these mice show substantial increase in proliferation in parallel with decreased activation-induced cell death. Moreover, miR-17-92 transgenic mice CD4+ T cells produce increased amounts of IFN-γ and IL-10 under Th1 condition whereas expression of IL-4 is not altered in Th2 condition (Xiao *et al.* 2008).

# 3 AIMS OF THE STUDY

The main goal of this PhD thesis was to investigate the pathways involved in the IL-4 signaling induced differentiation of two important cell types of the immune system, Th2 cells and DCs.

The specific aims of this thesis study were to:

(1) identify novel GM-CSF/IL-4 regulated genes in human monocyte-derived macrophages and DCs and find out key regulators of IL-4 induced DC differentiation,

(2) elucidate the early target genes of IL-12 or IL-4 in polarizing human Th1 and Th2 cells,

(3) define the functional role of SATB1 in Th cell commitment,

(4) study the regulation of *IL-5* by SATB1 and GATA3.

# 4 MATERIALS AND METHODS

## 4.1 Plasmids and oligonucleotides

### 4.1.1 Plasmid constructs and siRNA oligonucleotides (III, unpublished)

The SATB1-shRNA and STAT6-shRNA plasmid constructs were generated by cloning the SATB1 shRNA oligonucleotides (DNA Technology, Aarhus, Denmark) into the *Eco*RI (or *Bgl*II in case of STAT6-shRNA) and *Xho*I sites of the previously modified pSUPER-H-2K$^k$ plasmid, which contains a truncated H-2K$^k$ cell surface selection marker (Tahvanainen *et al.* 2006). Scramble control plasmids, pSUPER-H-2K$^k$-Scramble-shRNA and pSUPER-H-2K$^k$-Scramble2-shRNA, were cloned similarly. Furthermore, synthetic small interfering RNA (siRNA) oligonucleotides were used to knockdown SATB1, STAT6 or GATA3 (Sigma/Proligo, Evry Cedex, France). In parallel experiments, two different shRNA/siRNA sequences targeting a different part of the gene to be knocked down were used to minimize the amount of unspecific targets (Jackson *et al.* 2003). See Table S6 (*Report III*) for sequences.

The *IL-5* promoter sequence (-581 bp to +34 bp) was amplified from gDNA isolated from human umbilical cord blood CD4+ T cells with PCR using IL5p-F and IL-5p-R primers (*Report III, Table S6*) and cloned into *Kpn*I and *Hind*III sites of pGL3-basic vector (Promega, Madison, WI, USA) to create pGL3-IL-5 vector. Predicted SBSs were removed from the *IL-5* promoter sequence using various combinations of primers (*Report III, Table S7*).

Cloning of GST:CD+HD has been described elsewhere (Purbey *et al.* 2006). GATA3 was cloned into pBluescript II SK (+/-) phagemid vector (Stratagene, La Jolla, CA, USA) using PCR primers GATA3- *Bgl*II-F and GATA3-*Eco*RI-R. GATA3 was subcloned into pIRES2-H-2K$^k$ vector (Tahvanainen *et al.* 2006) using PCR primers pIRES2-GATA3-F and pIRES2-GATA3-R and further subcloned into *Eco*RI and *Xho*I sites of pC6-2 vector (Purbey *et al.* 2006) using PCR primers GATA3-F and GATA3-R (*Report III, Table S6*).

### 4.1.2 Preparation of probes for EMSA (III)

For EMSA probe preparation, the ~600 bp *IL-5* promoter and its truncations were amplified with PCR (*Report III, Table S6*) using wt or mutated pGL3-IL-5 reporter constructs in presence of α-$^{32}$P dATP and α-$^{32}$P dCTP (both from Perkin Elmer, Boston, MA) as well as unlabeled dATP, dCTP, dGTP and dTTP to efficiently label the probes.

## 4.2 Cell culture

### 4.2.1 Differentiation of monocytes to DCs and macrophages (I)

Monocytes were purified from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki,

Finland). Human PBMC were isolated by a density gradient centrifugation over a Ficoll-Paque gradient (Amersham-Pharmacia Biotech, Uppsala, Sweden), as described previously. Mononuclear cells were collected, and monocytes were purified further as described previously (Veckman *et al.* 2004). Briefly, mononuclear cells were centrifuged over a Percoll gradient (Amersham-Pharmacia Biotech). Next, T and B cells were depleted by using anti-CD3 and anti-CD19 magnetic beads (Dynal, Oslo, Norway). Monocytes were adhered onto plastic six-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) for 1 h at 37°C in RPMI-1640 medium without FCS ($2.5\times10^6$ cells/well). After incubation, nonadherent cells were removed, and the wells were washed with PBS. Monocytes were allowed to differentiate into macrophages in macrophage serum-free medium supplemented with antibiotics and recombinant human (rh) GM-CSF (10 ng/ml, Nordic Biosite, Täby, Sweden). Immature, monocyte-derived DC were generated using RPMI-1640 medium plus 10% FCS, rhGM-CSF (10 ng/ml), and rhIL-4 (20 ng/ml, R&D Biosystems, Minneapolis, MN, USA). In vitro-differentiated cell populations were devoid of CD3+ and CD19+ cells. pDC were isolated from PBMC by positive selection using blood DC antigen-4 (BDCA-4)-conjugated, paramagnetic beads (Miltenyi Biotec, Gladbach, Germany). To enhance cell purity, the labeled pDC were separated twice through MACS LS columns. Otherwise, the protocol recommended by the manufacturer was followed. The purity of isolated CD123/BDCA-2, doublepositive cells was over 95% constantly, and no CD11c expression was detected. mDC were isolated from PBMC by using the BDCA-1 DC isolation kit, according to the manufacturer's instructions (Miltenyi Biotec). Briefly, CD19+ cells were removed, first by positive selection, after which BDCA-1+ DC were positively selected. Again, the BDCA-1+ cells were run twice through an LS column to enhance purity. Isolated BDCA-1+ cells were over 95% CD11c+.

### 4.2.2 Polarization of primary human T helper cells (II, III, unpublished)

CD4+ T cells were purified 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) using Ficoll-Paque gradient centrifugation (Amersham-Pharmacia Biotech) and anti-CD4 conjugated magnetic beads (Dynal). Cells were cultured in Yssel's medium (Yssel *et al.* 1984) supplemented with 1% human AB-serum (Finnish Red Cross) in the density of $1.5 - 4 \times 10^6$ cells/ml. Cells were activated with plate-bound anti-CD3 (2.5 μg/ml for coating) and 0.5 μg/ml soluble anti-CD28 (Immunotech, Marseille, France). Alternatively, (*Report II; validation of oligonucleotide array results with quantitative RT-PCR*) cord blood CD4+ T cells were activated with irradiated (6000 rad) CD32-B7 transfected mouse L fibroblasts and 100 ng/ml phytohemagglutinin (PHA; Murex Diagnostics, Chatillon, France) (Yang *et al.* 1995). Th1 polarization was induced with IL-12 (2.5 ng/ml) and Th2 differentiation with IL-4 (10 ng/ml; both from R&D Systems). Alternatively activated cells cultured in Th2 conditions were supplemented with 10 μg/ml anti-IL-12 (R&D Systems). Part of the cells was cultured without any polarizing cytokines, in "Th0" conditions. Where indicated, the cells were also supplemented with 3 ng/ml TGFβ or 17 ng/ml IL-10 (both from R&D Systems) (*Report II*). Amount of IL-10 used in the experiments was selected based on the efficiency to suppress IFNγ production

(~50 % decrease) in PBMCs cultured in Th1 conditions for 18 hours (data not shown). TGFβ-mediated suppression of IFNγ production by Th1 cells in these conditions has been previously described (Lund *et al.* 2003). After 48h of priming, IL-2 (17 ng/ml; R&D Systems) was added into the cultures. If cultured more than one week, the cells were reactivated every 7 days with plate-bound anti-CD3 and soluble anti-CD28 as on day 0 and cultured in the presence of concordant cytokines. The cord blood cell cultures were generated from four to nine individuals and buffy coat cell cultures from one to four individuals. Cells were harvested at indicated time points. In case of nucleofected cells, the cultures were started 24 hours post-nucleofection for siRNA oligonucleotide transfected cells or 48 hours post-nucleofection for plasmid transfected cells to ensure the efficient gene overexpression or knockdown at the priming of the cells.

## 4.3 Transfections

### 4.3.1 Human primary T cell nucleofection (III, unpublished)

Human cord blood or buffy coat primary CD4+ T cells were transfected as described elsewhere (Tahvanainen *et al.* 2006). Briefly, $4\times10^6$ cord blood cells were nucleofected with 1.5 μg siRNA oligonucleotides targeting STAT6, GATA3 or SATB1 or with scramble control siRNAs. Alternatively, 5×106 buffy coat cells were nucleofected with 10 μg of shRNA plasmid DNA or, for transactivation assay, with 2 μg reporter vector and 8 μg shRNA plasmid DNA. In the experiment measuring the effect of silencing the expression of two genes simultaneously, the total amount of oligonucleotides used per transfection was 3 μg (1.5 μg of both siRNAs) to ensure the efficient knockdown of the target genes. Nucleofections were carried out in 100 μl Opti-MEM I (Invitrogen, Carlsbad, CA, USA) using Nucleofector™ device (program U-14) (Amaxa, Cologne, Germany). Several parallel nucleofections were performed. After the pulse, the cells were kept in RPMI-1640 medium containing 10 % FBS, L-glutamine and antibiotics (pen/strep) in the density of ~$2\text{-}2.5\times10^6$ cells/ml at 37 °C, in 5 % $CO_2$ until activated or proceeded for enrichment.

### 4.3.2 Enrichment of transfected cells (III, unpublished)

Cells nucleofected with shRNA plasmids used for Illumina beadarray gene expression analysis were enriched for transfected cells. Removal of dead and apoptotic cells as well as enrichment of H-$2K^k$ positive cells was carried out as described elsewhere (Tahvanainen *et al.* 2006). Dead Cell Removal Kit and MACSelect $K^k$ MicroBeads coated with the H-$2K^k$ antibody for positive selection were used according to manufacturer's instructions (Miltenyi Biotech). Buffy coat CD4+ T cells nucleofected with vectors containing H-$2K^k$ marker gene were harvested 16 h after transfection and dead cells were magnetically labeled. Viable cells were separated from labeled cells using MACS LS columns (Miltenyi, Biotech), after which transfected cells were labeled with MACSelect $K^k$ MicroBeads and magnetically purified. The purified H-$2K^k$ positive cells were cultured in Th0, Th1 or Th2 conditions as described above.

After enrichment of cells and before starting the culture, the cells were kept in Yssel's medium at 37 °C, in 5 % $CO_2$.

### 4.3.3 Transactivation assay (III)

For transactivation assay, buffy coat CD4+ T cells were cotransfected with wt or mutated pGL3-IL-5 luciferase reporter constructs and SATB1-shRNA or scramble control plasmids. Cells were harvested and analyzed for the luciferase activity using BriteLite reagent and Victor™ plate reader (both from Perkin Elmer). Relative luciferase activity was calculated by normalizing the measurements of wt or mutated *IL-5* reporter to the empty pGL3-basic control measurement.

## 4.4 Gene expression analyses

### 4.4.1 Oligonucleotide array studies

#### 4.4.1.1 Affymetrix gene expression analyses (I, II)

For the Affymetrix sample preparations (Affymetrix, Santa Clara, CA, USA), 20 μg (*Report I*) or 5 μg (*Report II*) total cellular RNA, pooled from several individuals, was used as starting material. The sample preparation was performed according to the instructions and recommendations provided by the manufacturer. The samples were hybridized by the Finnish DNA Microarray Centre to human genome (HG) U133A containing approximately 22 000 probes covering approximately 13 000 human genes (*Report I*) or HG-U95Av2 arrays containing probes for approximately 9 300 genes (*Report II*). Two biological repeats for each microarray experiment were performed except that kinetic data for 3, 6 and 24 h differentiated DCs and macrophages was studied from one culture (a pool of four individuals). The data were analyzed and filtered with GeneChip Microarray Suite software version 5 (MAS5, Affymetrix) according to recommendations of the manufacturer. Briefly, the probe set was excluded if the detection call for both target and reference was "absent", if the change call gave "no change" in comparison analysis or if the signal log ratio between target and reference was between -1 and 1. Gene expression was considered upregulated if the signal log ratio between the reference and the target samples was higher than one (greater than two-fold increase) and the target sample was "present". Similarly, a gene was defined as downregulated if the signal log ratio was less than minus one (two-fold decrease) and the reference sample was "present". Genes were considered as differentially expressed when they exhibited a consistent change in two separate biological repeats (*Report I and II*) or in the case of the immediate differentiation response of early DCs and macrophages, consistent regulation at all the three time points (*Report I*). All the genes fulfilling these criteria in at least one of the comparisons and one of the time points were selected for further analysis, where the expression of the genes was explored in parallel in different conditions without fold-change threshold. Microsoft Access and Excel for Windows softwares (*Report I and II*) and GeneSpring 6.0 software (Silicon Genetics, Redwood, CA, USA) (*Report II*) were used for data analysis and processing. Visualization was performed using Eisen's Treeview (Eisen *et al.* 1998). The gene annotations were obtained from the NetAffx

database (Liu *et al.* 2003). The DC and macrophage differentiation data (*Report I*) was also analyzed using Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA, USA). The significance is expressed as a P value, which is calculated by comparing the number of user-specified genes of interest participating in a given pathway, relative to the total number of occurrences of these genes in all pathway annotations stored in the Ingenuity Pathways Knowledge Base (right-tailed Fisher's Exact test). The early Th differentiation data (*Report II*) has been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE2770.

#### 4.4.1.2 Illumina gene expression analysis (III, unpublished)

The starting material used for the Illumina sample preparations (Illumina, San Diego, CA) was 50-200 ng total RNA, pooled from several individuals. The sample preparation was performed according to the instructions and recommendations provided by the manufacturer. The samples were hybridized by the Finnish DNA Microarray Centre to Illumina Sentrix® Human-6 Expression BeadChip arrays containing over 46 000 probes covering approximately 20 000 human genes. Total of five biological repeats for each microarray experiment were performed including two cultures of buffy coat cells nucleofected with SATB1-shRNA or scramble control plasmids and three cultures of cord blood cells nucleofected with SATB1 targeting or control oligonucleotides (*Report III, Table S8*). The data were quantile normalized (Bolstad *et al.* 2003) and log-transformed using the R package limma (Wettenhall & Smyth 2004) that is a part of the Bioconductor project (http://www.bioconductor.org). The different versions (V1 and V2) of Illumina BeadChIP array were combined using annotations (Ensembl BioMart database; hsapiens dataset) from the R package biomaRt (Durinck *et al.* 2005). The R package RankProd (Hong *et al.* 2006) was used to identify SATB1 regulated genes across the five biological repeats. For each biological replicate, the maximum or the minimum signal log ratios (SLR) over all time points were used as an input for the RP function. Genes having the estimated percentage of false positive predictions pfp<0.05 were considered to be differentially expressed. Fold changes were calculated as $2^{|SLR|}$ if SLR>0 and $-2^{|SLR|}$ if SLR<0, where SLR is the average maximum (for upregulated genes) or minimum (for downregulated genes) signal log ratio over the biological replicates. The gene annotations were obtained from Illumina. The microarray data were also analyzed using Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The SATB1 RNAi gene expression data can be found at the NCBI Gene Expression Omnibus with accession number GSE17241.

### 4.4.2 Quantitative RT-PCR analyses (I-III)

For macrophage and DC study qRT-PCR analyses (*Report I*), total RNA was isolated from macrophages or DCs derived from two to four donors using RNeasy Midi Kit (Qiagen, Valencia, CA, USA). RNA from different donors was pooled before purification. The primers used in TaqMan analyses, corresponding to *C3* (Hs00163811_m1), *TCF7L2* (Hs00181036_m1), *FcγR1A* (Hs02340030_m1), *FcεR1A* (Hs00758599_m1), *TGFα* (Hs00177401_m1), *SOCS1* (Hs00705164_s1), *FZD2*

(Hs00361432_s1), and *WNT5A* (Hs0018013_m1) genes, were obtained from Applied Biosystems (Foster City, CA, USA).

For Th differentiation studies (*Report II, III*), total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. DNase I (Qiagen) treatment was included in the RNA isolation procedure to eliminate genomic DNA from the samples. cDNA was prepared using either Superscript II Kit (Gibco BRL, Life Technologies, Paisley, Scotland) or Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) and subsequently used as a template for gene expression analyses. qRT-PCR was performed using TaqMan ABI Prism 7700 and 7900HT (Applied Biosystems, Foster City, CA, USA) as described elsewhere (Hamalainen *et al.* 2000, Lund *et al.* 2003). The housekeeping gene *EF1α* (Elongation factor 1 alpha) was used as a reference transcript (Hamalainen *et al.* 2001b). Primers and probes (*Report II, Table 4; Report III, Table S9*) (MedProbe, Oslo, Norway; Oligomer Oy, Helsinki, Finland or Roche Applied Science) used for the quantification of gene expression were designed using Primer Express (Applied Biosystems) and Universal ProbeLibrary Assay Design Center (Roche Applied Science) softwares. The quantitative value obtained from TaqMan real-time RT-PCR is a threshold cycle (Ct). The fold differences between different conditions can be calculated from the normalized Ct values ($\Delta Ct = Ct_{geneX} - Ct_{housekeeping\ gene}$) with the formula: fold difference $= 2^{|\Delta Ct1 - \Delta Ct2)|}$ (Hamalainen *et al.* 2001a, Lund *et al.* 2003). Quantitative ChIP-PCRs were performed using iCycler (BioRad) and iQ SYBR Green mix (BioRad). Change in threshold (ΔCt) values were calculated using the formula: $\Delta Ct = (Ct_{Target} - Ct_{Input})$ and $\Delta(\Delta Ct) = (\Delta Ct_{Target} - \Delta Ct_{IgG})$. Fold differences in occupancy were calculated as follows: Fold difference $= 2^{-\Delta(\Delta Ct)}$.

## 4.5 Protein expression analysis

### 4.5.1 Western blotting (I-III)

Monocyte-derived macrophages and DCs (*Report I*) were harvested and samples treated as previously described (Veckman *et al.* 2004). For direct Western blot analyses, cells were lysed and 30 µg protein aliquots were separated on 10 % SDS-PAGE (8 % for detection of FN) using Laemmli buffer system. Proteins separated on gels were transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). Binding of primary and secondary antibodies was performed in PBS (pH 7.4) containing 5 % nonfat milk for 1 h at room temperature. Primary antibodies used in immunoblotting were anti-WNT5A (sc-23698), anti-FN (sc-9068) and anti-NFATc1 (sc-7294; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). HRP-conjugated, anti-guinea pig (P0141, Dako A/S, Glostrup, Denmark), anti-goat (P0449, Dako A/S) and anti-rabbit (P0448; Dako A/S) Igs were used as secondary antibodies. The protein bands were visualized on Hyper-Max film using the ECL system (Amersham Biosciences, Piscataway, NJ, USA).

Expression of T-bet (TBX21), GATA3, SATB1, BCL6, TCF7, SOCS1 and ID3 was studied in the intact cord blood CD4+ T cells (*Report II*) and expression of STAT6, GATA3 and SATB1 in the nucleofected cord blood CD4+ cells (*Report III*) cultured in

Th0, Th1 or Th2 conditions. The cells were lysed in SDS buffer (62.5 mM Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 50 mM DTT) and equal protein amounts of each whole cell lysate sample were loaded on 5-15 % SDS-PAGE gels after having added bromophenol blue (0.1 %). Proteins were transferred into either nitrocellulose membranes (Hybond™-ECL™, Amersham Biosciences) or polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were blocked in 0.01 % TBS-T or 0.03 % PBS-T containing 5 % nonfat milk either overnight at +4 °C or 30 min at room temperature. For detection of the proteins, the membranes were incubated with the primary antibodies including mouse anti-BCL6 (M7211, Dako A/S), mouse anti-TBX21 (sc-21749), mouse anti-GATA3 (sc-268), goat anti-SATB1 (sc-5989), goat anti-TCF7 (sc-8589), rabbit anti-SOCS1 (sc-9021), rabbit anti-ID3 (sc-490) or mouse anti-STAT6 (sc-981) (all from Santa Cruz Biotechnology) in 0.01 % TBS-T (or 0.03 % PBS-T) with 5 % milk for either 1 h at room temperature or overnight at +4°C. Subsequently, membrane was probed with HRP-conjugated anti-mouse (M15345, BD Transduction Laboratories or sc-2005, Santa Cruz Biotechnology Inc), anti-goat (705–035–003, Jackson ImmunoResearch Laboratories) or anti-rabbit (554021, BD Pharmingen) secondary antibody was performed at 1:10 000 dilution in 0.01 % TBS-T (or 0.03 % PBS-T) with 5% milk for 30-60 min at room temperature. The bound antibodies were visualized with ECL reaction (Amersham Biosciences).

### 4.5.2 Flow cytometry

#### 4.5.2.1 Cell surface proteins (I-III)

The expression of DC and macrophage specific cell surface proteins was analyzed by flow cytometry (*Report I*). One-, 3- and 7-day cells were collected and washed with PBS and unspecific binding of antibodies was blocked by incubating cells in PBS containing 10 % FCS. Cells were stained with fluorescence label-conjugated anti-CD1b (BD Biosciences), anti-CD14 (Dako A/S) or anti-DC-SIGN (specific ICAM-grabbing nonintegrin; BD Biosciences) antibodies and their respective isotype control antibodies (Caltag Laboratories, San Francisco, CA, USA). The expression of cell surface proteins was analyzed with FACScan flow cytometer and CellQuest software (BD Biosciences).

Cord blood CD4+ T cells cultured in Th0, Th1 or Th2 conditions for 0, 2, 6, 24 or 48 h were studied for IL-10R expression (*Report II*). After harvesting the cells were washed with PBS containing 2 % FCS and 0.01 % azide and subsequently stained with anti-human IL-10R-PE (BD Pharmingen) for 15 min. The cells were washed with PBS containing 2 % FCS and 0.01 % azide and then with PBS containing 0.01 % azide. The IL-10R expression on the fixed cells (1% formalin in PBS) was studied with FACScan and CellQuest Software (BD Biosciences).

The cells nucleofected with plasmids containing H-2K$^k$ marker were stained with H-2K$^k$-FITC (Miltenyi Biotec) antibody approximately 20 hours after nucleofection to measure the transfection efficiency (*Report III*). MACSelect Control-FITC (Miltenyi Biotec) antibody staining was used to assess the positivity of the purified cells after

enrichment of nucleofected cells. Samples were analyzed with FACScan and CellQuest Software (BD Biosciences).

#### 4.5.2.2 Intracellular cytokine staining (unpublished)

After 7-8 days of polarization, the intracellular cytokine production (IFNγ and IL-4 cytokines) of SATB1-siRNA or scramble control oligonucleotide nucleofected cells or non-nucleofected cells was measured. Cells were activated with PMA (5 ng/ml; Calbiochem) and ionomycin (500 mg/ml; Sigma-Aldrich) in the density of $1\times10^6$ cells/ml for 5 h. Half of the cells were left as untreated control cells. Brefeldin A (10 μg/ml; Alexis Biochemicals, Lausen, Switzerland) was added to all samples 2 h after activation. The cells were harvested and washed with ICS buffer (PBS containing 0.5 % BSA and 0.01 % azide). Cells were fixed with 4 % paraformaldehyde for 15 min and permeabilized with 0.5 % saponin for 10 min after which they were stained with anti-IFNγ-FITC (Caltag Laboratories, Burlingame, CA, USA) and anti-IL-4-PE (BD Pharmingen) antibodies for 20 min. The cytokine expression profiles were measured with FACScan and CellQuest Software (BD Biosciences).

### 4.5.3 Cytokine secretion assay (III)

Secreted cytokines were measured from the culture supernatants of siRNA or scrambled control transfected cells using Luminex assay and multiplex bead kits from Bio-Rad Laboratories, Inc. (Hercules, CA). The assays were conducted in duplicate according to the manufacturer's instructions. Measurements and data analysis were performed with the Bio-Plex system in combination with the Bio-Plex Manager software (Bio-Rad Laboratories). Fold induction was calculated by dividing the measurement of siRNA treated sample to corresponding scrambled control sample.

## 4.6 DNA binding assays

### 4.6.1 DNA affinity binding assay (I)

Monocyte-derived macrophages and DCs (*Report I*) were harvested at 3 and 7 days and samples were treated as previously described (Veckman *et al.* 2004). Both strands of the DNA elements containing a NFAT/AP-1-binding site of the human *IL-2* gene promoter (proximal -135 and distal -280 (Rooney *et al.* 1995, Serfling *et al.* 1995)) were synthesized with BamHI overhangs as spacers, with 5'-biotinylation of the upper strand oligonucleotide (DNA Technology). The oligonucleotides were annealed in 0.5 M NaCl and kept with streptavidin-agarose beads (Neutravidin, Pierce, Rockford, IL, USA) at +4 °C for 2 h, in a ratio to yield maximum saturation of the beads with the biotinylated oligonucleotide. Samples were incubated with agarose beads, which were saturated with the oligonucleotide, at +4 °C for 2 h. After washing, the bound proteins were released in SDS sample buffer and equal aliquots were subjected to SDS-PAGE and Western blotting.

### 4.6.2 Electrophoretic mobility shift assay (III)

For EMSA (electrophoretic mobility shift assay) reactions (*Report III*), recombinant proteins, GST:CD+HD (the Cut and homeo domains) containing the DNA binding domain of SATB1 (Purbey *et al.* 2008) and GST:GATA3, were expressed in DH5α strain of *Escherichia coli* and purified according to standard procedures. Nuclear extracts from Th1 or Th2 cells were prepared essentially as described (Kumar *et al.* 2007). Briefly, newly harvested cells were washed with phosphate buffer and the pellet was frozen at -80 °C for 30 min. The cell pellet was thawed rapidly, 5 volumes of rapid extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 450 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT and 1 × protease inhibitor cocktail) were added and the cells were resuspended by pipetting. Cells were kept on ice for 10 min followed by three cycles of freeze/thawing. The lysate was centrifuged and the supernatant was collected. The protein content of the lysate was estimated using Bio-Rad DC Protein Assay. Binding reactions were performed in a 10 µl total volume containing 10 mM HEPES pH 7.9, 1 mM DTT, 50 mM KCl, 2.5 mM MgCl2, 10 % glycerol, 0.5-1 µg of double-stranded poly (dI-dC), 10 µg BSA and the recombinant protein or nuclear lysate. Samples were incubated at room temperature for 5 min before adding $^{32}$P-labeled IL-5p probe. After 10 min incubation at room temperature, the reactions were loaded on 6 % native polyacrylamide gels to resolve the binding products. For antibody-mediated supershifts, reaction mixtures were supplemented with anti-SATB1 (Kumar *et al.* 2006) or normal rabbit IgG (Santa Cruz Biotechnology) 5 min after adding the probe. Incubation was continued for 10 min at room temperature followed by electrophoresis. The gels were dried under vacuum and exposed to X-ray film. The purified GST proteins were used for the EMSA with various probes to determine the dissociation constants (Kd), which is the concentration (Molar) of protein required to bind 50 % of the substrate DNA.

### 4.6.3 Chromatin immunoprecipitation and ChIP-on-chip (III)

Chromatin immunoprecipitations were performed as previously described (Kumar *et al.* 2005). Briefly, cord blood CD4+ T cells were crosslinked for 10 min at room temperature by adding formaldehyde to a final concentration of 1% directly to the culture medium. Cells were washed with wash buffer 1 (0.25 % Triton-X-100, 10 mM EDTA pH 8.0, 0.5 mM EGTA, 10 mM HEPES pH 7.5, 10 mM sodium butyrate and 1 × protease inhibitor cocktail) and wash buffer 2 (0.2 M NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA, 10 mM HEPES pH 7.5, 10 mM sodium butyrate and 1 × protease inhibitor cocktail) followed by lysing the cells by resuspending the cell pellet in lysis buffer (150 mM NaCl, 25 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 1 % Triton-X-100, 0.1 % SDS, 0.5 % sodium deoxycholate, 10 mM sodium butyrate and 1 × protease inhibitor cocktail). After cell lysis, chromatin was sonicated such that genomic DNA was sheared into 300–1000 bp fragments using Bioruptor XL (Diagenode, Belgium). Sheared chromatin was immunoprecipitated with anti-SATB1 (Kumar *et al.* 2006), GATA3 or HDAC1 antibodies (Santa Cruz Biotechnology) or control normal rabbit IgG (Upstate Biotechnology) and processed further as described (Kumar *et al.* 2005). Part of the sheared chromatin was saved as Input/Whole Cell Extract (WCE) sample that was treated similarly excluding the immunoprecipitation step. DNA was PCR

amplified using primers IL5p-F1 and IL5p-R (*Report III, Table S7*). PCR products were resolved by agarose gel electrophoresis, stained with EtBr and visualized under UV light.

For the ChIP-on-chip sample preparations, two hundred nanograms of WCE and anti-SATB1 immunoprecipitated DNA pooled from several individuals were used as the starting material. The sample preparation was performed according to the instructions and recommendations provided by the manufacturer. The samples were processed and hybridized by Genotypic Technology Ltd (Bangalore, India) with Human genome promoter microarrays (Agilent Technologies Inc). The arrays were custom designed at Genotypic Technologies to contain over 244 000 probes covering -5.5kb upstream to +2.5kb downstream of approximately 15 000 known transcripts start site and known ENCODE regions. The text output file generated from the images using Agilent feature extraction software was used for the analysis. The normalization including Median Blanks substraction, Inter-array median normalization and Dye-bias median normalization was done using Agilent DNA Analytics software. Genes having the normalized log ratio >2 and enriched with at least 3 probes were considered as specifically enriched. Visualization was performed using Eisen's Treeview (Eisen *et al.* 1998). The SATB1 ChIP-on-chip data can be found at the NCBI Gene Expression Omnibus with accession number GSE17380.

### 4.6.4 Matrix-loop partitioning assay (unpublished)

Nuclear matrix and loop-associated fractions of genomic DNA were prepared as previously described (Kumar *et al.* 2007). Briefly, cord blood isolated $CD4^+$ T cells were washed with phosphate buffer followed by sequential lysis with CSK-1 (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100 and 1 × protease inhibitor cocktail) and CSK-2 buffers (10 mM EDTA pH 8.0, 2 M NaCl, 1 mM DTT and 1 × protease inhibitor cocktail). Genomic DNA was digested with restriction enzymes (*Eco*RI, *Eco*RV, *Bam*HI, *Bgl*II, and *Not*I or *Sal*I) for 16 h, at 37 °C. Isolated fractions of matrix and loop-associated DNAs were prepared by separating digested loop DNA from undigested material (nuclear matrix and MARs) by centrifugation. The restriction enzymes were heat inactivated at 85 °C for 10 min. Proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation was used to purify DNA from both fractions, which were subsequently used as templates for qRT-PCR amplification with different sets of primers designed for *IL-2* and *IL-5* promoters using Universal ProbeLibrary Assay Design Center (Roche Applied Science, Germany) software. Primer and probe sequences used in analysis were as follows: IL-5p proximal-fwd: 5'-tgagacctcaatgggaaagg-3', IL-5p-proximal-rev: 5'-gatggcttcagtgactcttcct-3', IL-5p proximal-probe: Universal Probe Library #58, IL-5p distal-fwd: 5'-aaaggcaaacgcagaacg-3', IL-5p distal-rev: 5'-cagctccaagagctagcaaac-3', IL-5p distal-probe: Universal Probe Library #21, IL-2p-fwd: 5'-ggtttaaagaaattccaaagagtca-3', IL-2p-rev: 5'-ttcaaagactttacctgtctgaaaaa-3' and IL-2p-probe: Universal Probe Library #35.

## 4.7 Statistical methods

### 4.7.1 Statistical analysis (III, unpublished)

The statistical significance between means in the quantification of mRNA and secreted cytokines was calculated with paired two-tailed Student's t test. $p < 0.05$ was considered statistically significant.

### 4.7.2 Linear modeling (III)

The association between the SBSs and the luciferase activity was investigated in terms of a multivariable linear model, assuming that the SBSs have an additive effect on the activity. More specifically, we considered the linear regression model $y = \beta_0 + \sum_{j=1}^{4} \beta_j x_j + \varepsilon$, where y is the relative activity of a reporter construct, $x_j$ is the binary indicator for the presence of the individual SBS j in the construct, and $\varepsilon$ is an error term. The model was fitted with the least squares method using the function lm in the statistical software R. For each coefficient $\beta_j, j = 0,...,4$, the null hypothesis $H_0, \beta_j = 0$ was tested under the assumption of normally distributed errors.

# 5 RESULTS AND DISCUSSION

## 5.1 Identification of genes involved in cytokine-driven macrophage and DC differentiation (I)

### 5.1.1 Differentially regulated genes in macrophages and DCs

Affymetrix HG-U133A oligonucleotide arrays were used to obtain genome-wide view of the early and late stages of the differentiation of macrophages and DCs from human monocytes. Previously, the differences in gene expression profiles of these cells have been studied mainly using various infection models in mouse and human (Granucci *et al.* 2001, Ahn *et al.* 2002, Richards *et al.* 2002, Chaussabel *et al.* 2003, Grolleau *et al.* 2003, Hacker *et al.* 2003, Wells *et al.* 2003, Jenner & Young 2005, Martinez *et al.* 2006). These studies have shown macrophages and DCs to respond in cell type-specific manner to different pathogens or their components, reflecting their roles as primary instigators of immune responses and effectors in local infections and inflammation. However, our study provided the first direct comparison of transcriptomes of differentiating human macrophages and DCs on whole genome level. In humans, peripheral blood monocytes treated with GM-CSF or GM-CSF+IL-4 generate macrophages and DCs *in vitro*, respectively. We studied the differentiation of macrophages and DCs at two stages: the early stage samples revealed immediate response genes which were regulated by differentiating cytokines after 3, 6 or 24 hours of treatment whereas the late stage samples displayed stable cell-type specific genes which showed differential expression after 3 or 7 days of treatment with cytokines. Interestingly, a large number of genes was up- or downregulated only after 3 hours of differentiation in both cell types, indicating that monocytes responded rapidly and robustly to differentiation-driving cytokines. In macrophages 526 and 972 genes were upregulated and 380 and 453 genes were downregulated at the 3+6h and 6+24h time points, respectively (*Report I, Table 1*). In DCs the number of regulated genes was even higher as 529 and 1051 genes were upregulated and 489 and 441 genes were downregulated at these time points, respectively. Comparison of the expression profiles of genes which were constantly regulated in all three time points between macrophages and DCs showed that 17 genes, including *FGFR1*, *TKTL1* and *ADFP*, were expressed at a higher level in cells differentiating to macrophages whereas 22 genes, including *FN1*, *WNT5A*, *MAOA*, *TGM2*, *KIAA0992*, *CTNS* and *CCL22*, were expressed at a higher level in cells differentiating to DCs (*Report I, Table 1 and Figure 1*). Intriguingly, the monocyte/macrophage marker CD14 was downregulated both in GM-CSF- and GM-CSF+IL-4-stimulated cells as compared with untreated monocytes (*Report I, Figure 1*).

Even though hundreds of genes responded to the differentiation promoting cytokines in monocytes, cell-type specific gene expression patterns of macrophages and DCs only started to emerge during the first day of differentiation. The complete differentiation of macrophages and DCs *in vitro* takes roughly 6-7 days and therefore we also carried out gene expression profiling of these cells after 3 and 7 days of differentiation. In contrast to the experiment described above exploring the short kinetics of macrophage and DC differentiation, we observed differential expression of fewer genes at 3 and 7 days but

the cell-type specific differences were evident and the gene expression patterns stable (*Report I, Figure 2*). Altogether, 342 and 354 genes were upregulated and 194 and 236 genes were downregulated both at 3 and 7 day-time points in macrophages and DCs, respectively (*Report I, Figure 2*). The majority of these genes was regulated similarly in both of these cell types, which is not surprising as GM-CSF is involved in the differentiation process of macrophages as well as DCs. In total, 76 genes were expressed at a higher level in macrophages whilst 120 genes were more expressed in DCs (*Report I, Figure 2*). These differentially regulated genes included several known cell-surface molecules, such as *CD14*, *FcγR1A* and *CD163* in macrophages and *CD1a*, *CD1e*, *FcεR1A/R2*, *CD1c*, *CD1b*, *LY75* (*DEC-205*) and *CD209* (*DC-SIGN*) in DCs (*Report I, Figure 3*). Moreover, the genes of HLA Class II were also expressed more in DCs than in macrophages, reflecting the high antigen-presenting capacity of DCs. Similarly, a DC-specific expression was exhibited by *TNFRSF11A* (receptor activator of NF-κB) that encodes for an important costimulatory molecule regulating DC-T cell interactions. Furthermore, differentially expressed receptors for cytokines and other ligands included *CSF3R* in macrophages and *FZD2*, *IL21R*, and *IL10RA* in DCs (*Report I, Figure 3*). Cell-type specific expression profile was detected also for several cytokines, chemokines and extracellular ligands: *GDF15/MIC-1* was upregulated almost exclusively in macrophages whereas *WNT5A*, *CCL17*, *CCL22* and *TGFA* were greatly induced during DC differentiation. Most importantly, genes involved in transcription, such as *BHLHB3*, *MCM2*, *MCM6* and *NR1H3* in macrophages and *IRF4*, *R4A3*, *NFIL3*, *SMAD1*, *EHF* and *ETV5* in DCs, also showed preferential expression pattern in these cell types (*Report I, Figure 3*).

The proportion of differentially expressed transcriptional regulators was relatively high as 13 % of the cell type specific genes encoded members of various transcription factor families, like Ets, leucine zipper, forkhead and bHLH families. However, this is in agreement with the fact that differentiation of hematopoietic cells, including that of macrophages and DCs, is known to be regulated by transcription factors directing the sequential expression of more cell type specific genes (Valledor *et al.* 1998, Nagamura-Inoue *et al.* 2001). Furthermore, some of the differentially expressed transcription factors identified in our study have previously shown to be linked to macrophage or DC differentiation and function. *IRF4* is specifically induced during DC differentiation in humans (Lehtonen *et al.* 2005) and *IRF4* knockout mice show impaired DC differentiation and functional deficiencies (Suzuki *et al.* 2004, Honma *et al.* 2005, Tamura *et al.* 2005), possibly due to the importance of IRF4 in TLR signaling. *C/EBPβ* was expressed less in DCs than in macrophages, which is in concordance with its well-established role in macrophage biology. Surprisingly, *MafB*, which is considered as a macrophage-specific transcription factor, exhibited higher expression in DCs (*Report I, Figure 3*). It has been suggested that MafB and PU.1 reciprocally regulate macrophage and DC differentiation, respectively (Bakri *et al.* 2005). However, *PU.1* expression does not vary significantly between macrophages and DCs (Lehtonen *et al.* 2005) that would be needed to inhibit MafB expression and activity, suggesting that other mechanisms, apart from the mere expression of these transcription factors, may be involved. In addition, the function of some of the differentially regulated transcription factors is established in other hematopoietic lineages, including *BCL6* and *ABF-1* in B cells (Massari *et al.* 1998, Niu 2002) and

*SOX-4* in T cells (Schilham & Clevers 1998), but there are no previous reports of their role in macrophages or DCs. This suggests that depending on the cell type and other proteins available many transcription factors may have broader functions than anticipated previously.

Several transcription factors identified in this study have been reported to function as repressors of gene expression, indicating that in parallel with positive regulation of cell type specific factors, fine-tuning of gene expression during terminal differentiation and selective downregulation of factors of other lineages play important roles. Cytokines IL-4 and GM-CSF activate Jak-STAT signaling pathway that is negatively regulated by specific cytoplasmic tyrosine phosphatases of the SOCS family. In our study, expression of *SOCS-1* was detected almost exclusively in DCs but not in macrophages. In mouse, the SOCS-1 expression in DCs has been reported to be regulated by IL-4 (Jackson *et al.* 2004) and SOCS-1-deficient DCs are hyperactivated in response to cytokines and show enhanced antigen presentation (Hanada *et al.* 2003, Shen *et al.* 2004). Furthermore, SOCS-1 knockout mice develop systemic autoimmune diseases (Hanada *et al.* 2003).

To get a better insight of our oligonucleotide array results, we processed the data with Ingenuity Pathway Analysis software, which can be used to build networks and detect pathways from a set of regulated genes. Macrophage and DC specific genes from 24h and 7d timepoints were analyzed for the presence of genes belonging to canonical signaling pathways (*Report I, Figure 4*). Genes linked to antigen presentation, NF-κB signaling and IL-4 signaling were specifically regulated in DCs whereas genes related to death receptor signaling pathway were regulated in macrophages and not in DCs. Furthermore, genes involved in chemokine, p38 MAPK and VEGF signaling showed regulation in both cell types especially at 24h timepoint, and IL-6 signaling and apoptosis related genes exhibited regulation after 7 days of differentiation. Thus, differentiation of macrophages and DCs results in the acquisition of cell type specific signaling capacities reflected by the transcriptomes of these cells.

### 5.1.2 Validation of the oligonucleotide array results by flow cytometry and qRT-PCR

To confirm the phenotype of the differentiated cells and the gene expression profiles detected with oligonucleotide arrays, a panel of genes was analyzed using flow cytometry and qRT-PCR. The expression of the cell lineage specific marker molecules CD14, CD1b and DC-SIGN was studied by staining nonfixed monocytes and 3- and 7-day macrophages and DCs with specific antibodies and detecting the cell surface expression by flow cytometry (*Report I, Figure 5A*). CD14 expression was high in monocytes and 3- and 7-day macrophages while 3-day DCs showed weak and 7-day DCs negligible CD14 expression. CD1b was expressed in all cell types but 3- and 7-day DCs displayed higher expression levels. Similarly, DC specific induction was also observed for DC-SIGN that was absent in monocytes and 7-day macrophages. Moreover, qRT-PCR analysis confirmed the preferential expression of *FcγR1A*, *C3* and *TCF7L2* in macrophages and DC specific expression of *WNT5A*, *FZD2*, *SOCS-1*, *TGFA* and *FcεR1A* (*Report I, Figure 5B*). These data show that the gene expression

patterns detected with microarray were in full concordance with the qRT-PCR results and the protein level expression of CD14, CD1b and DC-SIGN.

### 5.1.3 WNT5A, FN and FZD2 in DCs

As described above, *WNT5A* was found to be greatly induced in DCs differentiated from monocytes. Besides monocyte-derived DCs, in humans there are at least two other DC subtypes, namely plasmacytoid (pDCs) and myeloid (mDCs) dendritic cells, which are found in blood in a differentiated but immature form (Ito *et al.* 2005). We studied the basal expression and the effects of DC-differentiation driving cytokines GM-CSF and IL-4 on the expression of *WNT5A* in pDCs and mDCs. Freshly isolated pDCs and mDCs were stimulated with GM-CSF or IL-4 or with their combination for 4 hours and the *WNT5A* mRNA expression was analyzed by qRT-PCR. Part of the cells was left unstimulated. *WNT5A* expression was low in unstimulated pDCs whereas it was readily detectable in unstimulated mDCs and 7-day-differentiated, monocyte-derived DCs obtained from the same blood donors (*Report I, Figure 6*). IL-4 stimulation induced a four-fold increase in *WNT5A* expression in pDCs and mDCs, resulting in greatly higher expression levels than in monocyte-derived DCs, indicating that *WNT5A* is a target of IL-4/STAT6 signaling. The cell type specific expression pattern of *WNT5A* in DC subtypes suggests that WNT proteins in general and WNT5A in particular may play an important role in DC development and function. WNT5A has been shown to inhibit macrophage colony formation and the differentiation of monocytes to macrophages (Brandon *et al.* 2000). Mice infected with *Mycobacterium tuberculosis* develop a transient protective Th1 response (Orme 1987, Orme *et al.* 1993, Rhodes & Graham 2002) and recently it has been shown that WNT5A expression is induced via TLR2 in *Mycobacterium tuberculosis*-infected human macrophages and other professional antigen-presenting cells (Blumenthal *et al.* 2006). This enhanced WNT5A expression was shown to be required for IL-12 production in macrophages, which subsequently induces Th1 differentiation, and IFN-γ production in T cells, which plays an essential role in resistance to this infection (Flynn *et al.* 1993). WNT5A is an important mediator in the control of T helper cell differentiation during *Mycobacterium tuberculosis* infection and furthermore, this protein provides the first implication of the involvement of WNT signaling in bridging innate and adaptive immunity to infections (George 2008).

Similarly to *WNT5A*, also *fibronectin* (*FN*) was found to be preferentially expressed in monocyte-derived DCs. FN is an important component of extracellular matrix (ECM) whilst WNT proteins are known to associate with proteins of the ECM. We studied the expression of FN and WNT5A on protein level using western blotting and observed that both of these proteins were expressed in monocytes (*Report I, Figure 7*). FN expression was greatly upregulated during DC differentiation whereas macrophages were completely devoid of FN. WNT5A expression was induced in 3-day-differentiated macrophages and DCs. However, at 7-day-timepoint, WNT5A was barely detectable in macrophages but in DCs the expression was maintained at a higher level. Even though the expression of ECM linked proteins WNT5A and FN was high in 7-day DCs, attempts to detect a direct association of these proteins by coimmunoprecipitation were not successful. Furthermore, FN has been shown to be

expressed by IL-4 activated macrophages (Gratchev *et al.* 2001), suggesting that macrophages may induce FN in inflammatory conditions.

In the canonical WNT signaling pathway, WNT5A binds to FZD4 or FZD5 and stabilizes β-catenin to induce the expression of TCF/LEF-regulated target genes (Clevers 2006, Staal *et al.* 2008) whereas in the non-canonical WNT signaling WNT5A can use the FZD2 receptor which activates the intracellular $Ca^{2+}$ pathway (Slusarski *et al.* 1997, Kuhl *et al.* 2000). As shown above, *FZD2* expression exhibited a similar DC-specificity than *WNT5A*, and thus, a functional ligand-receptor pair is expressed during DC differentiation and may exert a direct functional activity in an autocrine or paracrine manner. Intracellular $Ca^{2+}$ influx results in the activation and DNA binding of NFAT and therefore, we studied whether the basal activation of NFAT was different in 7-day-differentiated macrophages and DCs. We carried out oligonucleotide-binding experiments using proximal (-135) and distal (-280) NFAT-binding sites of the human *IL-2* gene promoter (Rooney *et al.* 1995, Serfling *et al.* 1995). The constitutive binding of NFATc1 to the *IL-2* promoter NFAT sites was high in DC extracts, and the corresponding binding of NFATc1 in macrophage cell extracts was clearly weaker (*Report I, Figure 8A*). To study whether the constitutively high NFAT DNA-binding activity in DCs was mediated by G protein-coupled receptors, such as FZD2, we used pertussis toxin (PT) to block the activity of such receptors. PT treatment reduced the basal NFAT DNA-binding activity in DCs but not in macrophages (*Report I, Figure 8B*), suggesting that FZD2, which is a PT-sensitive receptor, may be involved in constitutive NFAT activation in human monocyte-derived DCs. Similarly, CsA treatment also reduced the constitutive NFAT-binding activity in DCs but not in macrophages, suggesting that the calcineurin-dependent signaling is involved in NFAT activation in monocyte-derived DCs. Hence, constitutive activation of the NFAT was observed specifically in DCs and this may be induced by an autocrine/paracrine loop of WNT5A/FZD2 signaling. Interestingly, *Streptococcus pyogenes*-stimulated monocyte-derived DCs are capable of producing IL-2 (Veckman *et al.* 2004), a cytokine considered as T cell-specific whereas NFAT activity is required for the expression of IL-2. Thus, the constitutively active WNT signaling pathway could provide sufficient NFAT activity for IL-2 production in microbe-stimulated DC.

In conclusion, we observed that cytokine induced differentiation of human monocytes to macrophages or DCs is associated with a number of changes in their gene expression. We found that over 200 genes were differentially expressed in monocyte-derived macrophages and DCs. Many of these genes encode for cell surface molecules and receptors regulating DC or macrophage interactions with other cells, microbes or their components, as well as soluble mediators such as cytokines and immunoglobulins. Furthermore, an essential group of differentially expressed genes was transcription factors which may regulate crucial cell type-specific gene expression patterns in macrophages and DCs. Our global gene expression profiling of human monocyte-derived macrophages and DCs forms a solid basis for further functional analyses, which ultimately will give us a complete picture of the molecular determinants regulating the functional differences of these important cell types and provide practical tools for modulating macrophage activity in chronic inflammatory conditions and *in vitro* production of DC for immunotherapeutic purposes.

## 5.2 Early target genes of IL-12 and IL-4 during T helper cell polarization (II)

### 5.2.1 Differentially expressed genes during the early polarization of Th1 and Th2 cells

Large scale gene expression analysis was performed using Affymetrix U95Av2 arrays to identify the early targets of IL-12 and IL-4 during Th cell differentiation. Previously, similar transcriptome profiling has been utilized to discover genes involved in the differentiation process of human Th cells after 2 days or later (Rogge *et al.* 2000, Hamalainen *et al.* 2001a, Lund *et al.* 2003). However, the initiation of the polarization process has remained poorly characterized even though defining the upstream factors involved in the very earliest phase of Th cell differentiation is essential for understanding the molecular mechanisms leading to the commitment of Th1 and Th2 subsets. We compared the gene expression profiles of cells induced to polarize to Th1 and Th2 directions after 2 and 6 h of differentiation. Totally, 63 genes were differentially expressed by Th1 and Th2 polarizing cells. To identify genes regulated by IL-12 or IL-4, the expression profiles of Th1 or Th2 polarizing cells were compared to the profiles of the cells cultured without any polarizing cytokines (Th0 cells). This comparison demonstrated that the effects of IL-4 induced gene regulation emerged earlier than those of IL-12 signaling. In addition, at 2 h timepoint most of the IL-4 regulated genes were induced whereas at 6 h timepoint several genes were repressed by IL-4. The delay in IL-12 signaling is in concordance with the previous reports showing that IL-12R is not expressed by the naïve Thp cells but is induced in response to TCR activation (Rogge *et al.* 1997, Szabo *et al.* 1997, Rogge *et al.* 1999, Hibbert *et al.* 2003). The only IL-12 regulated genes were *IFNG* (1.87-fold) and the interferon regulated *GBP1* (1.62-fold) at the 6-h time point, indicating that minute amounts of IL-12R is, however, present in the Thp cells. Our previous study with cells cultured in Th1 or Th2 conditions for 2 days demonstrated that the effects of IL-12 are clearly seen after 2 days of differentiation (Lund *et al.* 2003). These observations are further supported by the results of our study on mouse Th1 cell differentiation showing that *IFNG* is the earliest gene regulated in an IL-12- and STAT4-dependent manner whereas other genes are regulated by STAT4 predominantly after 48 h of Th1 polarization (Lund *et al.* 2004). Interestingly, 26 genes of the 63 genes differentially expressed in Th1 and Th2 conditions were constantly regulated by IL-4 or/and exhibited Th1 vs. Th2 difference both at 2 and 6-h timepoints (*Report II, Table 1*). In addition, a subset of genes was differentially regulated in Th1 and Th2 conditions after either 2 h (17 genes) or 6 h (20 genes) of polarization (*Report II, Table 2*). Furthermore, the newly identified genes showing differential regulation in Th1 and Th2 culture conditions were grouped into the functional categories based on Gene Ontology annotations (Ashburner *et al.* 2000) to demonstrate their putative functional roles in T helper cell differentiation. The dominating functional groups consisted of transcription factors, cell adhesion molecules and receptors and enzymes and other intracellular signaling molecules (*Report II, Tables 1, 2*), indicating that the majority of the genes regulated during the early stage of Th1 and Th2 cell differentiation code for factors involved in signaling events from the cell surface to nucleus. Most of the genes in these groups were induced by IL-4 already after 2 hours of differentiation.

Nevertheless, a large number of the genes coding for extracellular molecules and ligands were regulated only after 6 hours of polarization. Interestingly, several members of the group of enzymes and other intracellular signaling molecules participate in RAS and MAPK signaling. Such genes included *NFIL3* (*Report II, Table 1A*); *DUSP6* and *MAP3K14/NIK* (*Report II, Table 1C*); *RASGRP1*, *RASA2*, *SOS1* and *PPM1A/ PP2Calpha*, (*Report II, Table 2C*); *LRRN3* (*Report II, Table 2F*) and previously identified *ERK3* (Corbalan-Garcia *et al.* 1996, Li *et al.* 1996, Malinin *et al.* 1997, Camps *et al.* 1998, Ebinu *et al.* 1998, Hanada *et al.* 1998, Kawasaki *et al.* 1998, Takekawa *et al.* 1998, Kuribara *et al.* 1999, Fukamachi *et al.* 2002). This is consistent with the earlier reports that RAS/MAPK pathway favors Th2 polarization by enhancing the IL-4R signaling (Yamashita *et al.* 1999).

As described above, only a few genes were differentially regulated across the studied time scale. Among the genes displaying constant changes throughout the early polarization were the well-known mediators of Th1 and Th2 differentiation, including *GATA3*, *MAF* (*Report II, Tables 1, 2A*) and *IFNG* (Glimcher & Murphy 2000). In addition, several novel genes with similar kinetics were differentially regulated by IL-4 and/or IL-12. Such genes included the transcriptional regulators *NFIL3*, *SATB1*, *STK17B* and *BCL6* (*Report II, Tables 1A, 2A*); cell adhesion molecules and receptors *IL-10RA*, *CXCR4* and *EDG1* (*Report II, Table 1B*); enzymes and intracellular signaling molecules *DUSP6*, *SPINT2*, *SOCS1*, *EBI2*, *GNAI1* and *GBP1* (*Report II, Tables 1C, 2C*); transporters *ABCD3* and *SLC39A8* (*Report II, Table 1D*); chemokine *CCL20* (*Report II, Table 2D*) and other genes with miscellaneous functions *XCL1*, *LRRN3*, *NKG7* and *HPCAL1* (*Report II, Tables 1E, 2G*). As differential regulation of these genes was maintained throughout the early polarization of human Th1 and Th2 cells (Rogge *et al.* 2000, Nagai *et al.* 2001, Lund *et al.* 2003, Lund *et al.* 2003), these genes are likely to play an essential role in the regulation of Th cell differentiation.

### 5.2.2 Confirming the gene expression profiles by TaqMan and western blotting

To validate the gene expression profiles detected with oligonucleotide arrays, a subset of genes was analyzed using TaqMan (qRT-PCR) and western blotting. The expression kinetics of *CHST10*, *NKG7*, *TCF7* and *ID3* was analyzed on mRNA level during the first week of Th1 and Th2 differentiation (*Report II, Figure 1*). *NKG7* and *CSTH10* were induced by IL-4 and preferentially expressed in the cells polarizing to Th2 direction during the early stage of differentiation as observed with oligonucleotide arrays (*Report II, Tables 2B, 2F and Figure 1*). However, after 2 days *NKG7* was also induced by IL-12 (4-fold, $p=0.017$) and became preferentially expressed by the polarizing Th1 cells (at 7 days), as described previously (Nagai *et al.* 2001) whereas *CHST10* maintained Th2 specific during the first week of differentiation. Similarly, oligonucleotide array results for *ID3* and *TCF7* were confirmed, as both these genes were downregulated by IL-4 and preferentially expressed in the Th1 conditions at 6 h (*Report II, Tables 1A, 2A and Figure 1*). Thus, the TaqMan results were in concordance with the oligonucleotide array results.

As shown above, oligonucleotide array and TaqMan results clearly demonstrated that *ID3*, *SATB1*, *TCF7*, *BCL6* and *SOCS1* were differentially regulated already at the

initiation of Th1 and Th2 differentiation. Interestingly, similar expression kinetics has been previously shown for the known key regulators of Th1 and Th2 differentiation, *TBX21* and *GATA3*, respectively, in mouse (Zhang *et al.* 1997, Szabo *et al.* 2000) and human (Ylikoski *et al.* 2005). To validate the expression pattern of these factors on protein level, western blot analysis was carried out using cells cultured in Th1 or Th2 conditions for 0, 2, 6, 24 and 48 h. TBX21 and GATA3 were used as control proteins to monitor the differentiation of the cells, and as expected, TBX21 was preferentially expressed in Th1 and GATA3 in Th2 polarized cells (*Report II, Figure 2*). The expression of BCL6 was barely detectable in Thp cells on protein level (*Report II, Figure 2*). As anticipated based on the results on mRNA level, BCL6 showed Th1-specific expression after 6 h and the difference was maintained at least until 48 h. A low level of BCL6 expression was observed also in the cells cultured in Th2 conditions. The importance of BCL6 in suppression of Th2 differentiation has been previously demonstrated with BCL6 knockout mice that display lethal Th2 hyperresponse (Dent *et al.* 1997, Ye *et al.* 1997). BCL6 is suggested to promote Th1 differentiation by inhibiting expression of GATA3 protein and *IL-5* gene (Arima *et al.* 2002, Kusam *et al.* 2003). In contrast to BCL6, SATB1 was already expressed in the Thp cells and its expression was enhanced at 6 h in Th2 conditions reflecting the oligonucleotide array results (*Report II, Figure 2*). SATB1 was also expressed by Th1 polarizing cells but the preferential expression was seen, however, in Th2 conditions at 24 and 48 h. Similarly to SATB1, also TCF7 was already expressed in the naïve Thp cells. Interestingly, at the 24-h time point the Th1/Th2 difference in the expression of TCF7 vanished temporarily (*Report II, Figure 2*), in agreement with the changes observed in the expression profile on mRNA level (*Report II, Figure 1*). After 48 h of culturing in Th2 conditions, TCF7 was downregulated by IL-4 and exhibited preferential expression in Th1 polarizing cells, which is in accordance with the oligonucleotide array and TaqMan results. Intriguingly, the role of SATB1 and TCF7 in the regulation of Th cell differentiation has not yet been fully determined, although they have functional characteristics similar to GATA3. All these three proteins play important roles in early thymocyte development (Ting *et al.* 1996, Alvarez *et al.* 2000). SATB1 regulates gene expression over long distances and, as GATA3, is involved in chromatin remodeling (Farrar *et al.* 2002, Yasui *et al.* 2002, Cai *et al.* 2003). Furthermore, SATB1 and GATA3 have been suggested to mutually control the transcription of the *CD8B* gene (Kieffer *et al.* 2002). Hence, it would be interesting to determine whether these factors together regulate the expression of genes involved in Th cell differentiation. TCF7 is localized in the Th2 cytokine cluster in chromosomal region 5q31 in human and, similarly to GATA3, it can bind to the enhancer element of the *TCRα* gene (Kuo & Leiden 1999). Most importantly, both SATB1 and TCF7 have been associated with the diseases or phenotypes mediated by Th1 or Th2 responses. SATB1 was demonstrated to be preferentially expressed on both mRNA and protein level in the peripheral blood T cells of patients with atopic dermatitis (Matsumoto *et al.* 2002) whereas a recent study reported a polymorphism in the *TCF7* gene to be associated with type-1 diabetes, which is a disease with dominating Th1 phenotype (Noble *et al.* 2003). As the transcription factors GATA3, SATB1 and TCF7 have similarly important roles in the regulation of thymocyte maturation, it is possible that they also regulate later differentiation of Th cells. Contrary to the results on mRNA

level, the SOCS1 protein did not show any preferential expression in Th1 or Th2 cells during their early polarization (*Report II, Figure 2*). In addition, we were not successful in detecting the ID3 protein in Th cells. Based on these observations, we concluded that the transcriptional regulators BCL6, TCF7 and SATB1 are differentially regulated during the early Th1 and Th2 cell polarization both on mRNA and protein level.

### 5.2.3 Effects of immunosuppressive cytokines TGFβ and IL-10 on human Th differentiation

TGFβ is an immunosuppressive mediator that is able to inhibit the differentiation of Th1 and Th2 cells (Gorelik & Flavell 2002). Previously, we demonstrated that after 48 h of polarization, TGFβ antagonizes effects of IL-12 or IL-4 on certain genes including NFIL3 and SATB1 (Lund *et al.* 2003). To study the effects of TGFβ on the initiation of Th1 and Th2 polarization, we compared the gene expression profiles of cells induced to polarize to Th1 or Th2 directions in presence and absence of TGFβ after 2 and 6 h of polarization. Totally, 20 genes were regulated by TGFβ in Th1 and/or Th2 conditions (*Report II, Table 3*). However, the effects of TGFβ on the genes regulated by IL-4 were modest, as only five of the TGFβ regulated genes, including *ID3*, *LAMA3*, *CCL20*, *DDIT4* and *C19orf6*, were also clearly regulated by IL-4 (over 2-fold change). Genes *CCL20*, *ID3* and *LAMA3* were repressed by IL-4 in the absence of TGFβ while their expression, especially that of *CCL20*, was induced in the presence of TGFβ in both Th1 and Th2 conditions. The positive regulation of *ID3* and *LAMA3* by TGFβ has been previously described in fibroblasts and epidermal keratinocytes, respectively (Korang *et al.* 1995, Chambers *et al.* 2003). The chemokine CCL20 is a ligand for CCR6 and is involved in enhancing chemotaxis of T helper cells. Its production by rheumatoid arthritis-derived activated synoviocytes has been shown to be inhibited by Th2 cytokines IL-4 and IL-13 (Chabaud *et al.* 2001, Ambrosini *et al.* 2003, Veckman *et al.* 2004). Moreover, preferential expression of CCL20 by Th1 cells, which are activated and polarized for 12–14 days, has also been previously reported (Nagai *et al.* 2001). The *DDIT4* gene, which also was repressed by IL-4, showed similar induction than *CCL20*, however, only in cells cultured in Th1 conditions. The *C19orf6* gene was upregulated by IL-4 and this induction was strongly antagonized by TGFβ. This gene codes for membralin protein that is highly expressed in central nervous system (Andersson & vol Euler 2002). However, the function of the C19orf6 protein is poorly characterized. In conclusion, although TGFβ regulated several genes during the initiation of both Th1 and Th2 differentiation processes, few genes were clearly also regulated by IL-4. Opposite regulation of the genes *ID3*, *LAMA3*, *CCL20*, *DDIT4* and *C19orf6* by IL-4 and TGFβ may play a role in the TGFβ mediated suppression of Th1 and Th2 differentiation.

Besides TGFβ, the cytokine IL-10 is involved in the suppression of Th1 or Th2 differentiation. IL-10 is produced by a subset of immunosuppressive CD4+ T cells, Treg cells, which have been shown to inhibit the function of Th cells (Levings *et al.* 2002). Interestingly, the receptor for IL-10 (IL-10RA) was induced by IL-4 on mRNA level after both 2 and 6 h (*Report II, Table 1B*). IL-10R has previously been reported to be preferentially expressed in Th2 conditions also after 2 and 3 days of polarization

(Rogge *et al.* 2000, Lund *et al.* 2003). The expression of IL-10R in cells cultured in Th1 or Th2 conditions or without any polarizing cytokines for 0, 2, 6, 24 and 48 h was studied on protein level using flow cytometry. Surprisingly, 40-60% of the naïve Thp cells expressed the receptor (*Report I, Figure 3*). The expression was further increased in response to CD3+CD28 activation alone and after 24 h of activation nearly all of the cells (73–95%) expressed the IL-10R. Even though the expression of IL-10R was preferentially induced by Th2 conditions on mRNA level, on protein level there was no apparent reproducible difference between the cells polarized to Th1 and Th2 directions. Thus, our study demonstrated that the expression of the IL-10R protein is highly and rapidly induced by TCR activation alone and is expressed at similar levels by the differentiating Th1 and Th2 cells, indicating the responsiveness of all these cell types (Th0, Th1, Th2) to IL-10 cytokine.

To examine whether the IL-10 signaling was functional in these developing Th1 and Th2 subtypes, we studied the effects of IL-10 on the expression of *GATA3* and *TBX21*. In addition, we analyzed the expression of *SATB1* and *DUSP6*, two genes differentially regulated by Th1- and Th2-inducing cytokines, in response to IL-10. Concordant with our previous studies on human cells, three out of five individuals showed increased expression (3.2-fold, p=0.05) of *TBX21* at 6 h in the cells polarizing to Th1 direction compared to the cells in Th2 conditions (*Report II, Figure 4*) (Ylikoski *et al.* 2005). Interestingly, *TBX21* expression was reduced in the presence of IL-10 in all of these individuals. At 48 h, *TBX21* was preferentially expressed (14.9-fold, p=0.005) in the cells polarizing to the Th1 direction in each of the five individuals studied whereas IL-10 decreased the *TBX21* expression (2.0-fold, p=0.05) in four of five individuals. The IL-10 caused suppression in *TBX21* expression is consistent with previous reports describing inhibition of IFN-γ production and STAT1 signaling by IL-10 (Ito *et al.* 1999, Conti *et al.* 2003). Similarly in agreement with our previous observations in human cells, *GATA3* was preferentially expressed (-14.3-fold, p=0.01) at 6 h in the Th2 polarizing cells when compared to the cells cultured in Th1 conditions (*Report II, Figure 4*) (Ylikoski *et al.* 2005). Differences in *GATA3* expression between Th1 and Th2 polarizing cells were measured at 24 h (-17.9-fold, p=0.0005) and 48 h (-9.8-fold, p=0.05). A suppressive effect of IL-10 on *GATA3* expression (1.65-4.79-fold change) was observed in two of five individuals at all the timepoints studied (6, 24 and 48 h). However, in three other individuals *GATA3* expression was not suppressed by IL-10 during Th2 differentiation. Moreover, the expression of SATB1 and DUSP6 was not altered upon IL-10 treatment (*Report II, Figure 4*). Our results demonstrated that the IL-10 signaling was functional during development of both Th1 and Th2 cells, as in four of five individuals IL-10 rapidly decreased the expression of the two key regulators of Th1 and Th2 differentiation *TBX21* and/or *GATA3*, respectively. Nevertheless, IL-10 did not inhibit the expression of *SATB1* and *DUSP6*, which are similarly to GATA3 immediately induced by IL-4 in the Th2 polarizing conditions. Intriguingly, the immunosuppressive effects of IL-10 were not observed in all individuals. Furthermore, in some of the individuals IL-10 changed gene expression in either Th1 or Th2 conditions by affecting *TBX21* or *GATA3* expression, respectively. This intrinsic variation in the responsiveness of different individuals to the immunosuppressive cytokine IL-10 may be critical and merits further investigation. Moreover, the early sensitivity of developing Th cell subtypes to the

immunomodulatory effects of IL-10 is likely to be a crucial mechanism to control the magnitude of the Th1- and Th2-mediated responses in different individuals by the IL-10 producing cells.

Taken together, the early differentiation of human Th1 and Th2 cells is first orchestrated by IL-4. The regulation of gene expression is dynamic at the initiation of Th development and only a subset of genes is differentially expressed by the polarizing Th1 and Th2 cells throughout the early differentiation. The immediate response to IL-4 induces expression of genes that code mainly for mediators and components of the intracellular signaling pathways, such as receptors, cell adhesion molecules, signal transducers, enzymes and their inhibitors and transcription factors. Later, additional factors in these groups as well as extracellular molecules and ligands become regulated. The presence of TGFβ in Th1- and Th2-inducing conditions results in changes in the expression pattern of several genes, few of which are regulated also by IL-4, which may play a role in the TGFβ mediated inhibition of Th cell differentiation. In addition to TGFβ, these early developing Th cells are responsive to the immunosuppressive cytokine IL-10, which in most of the individuals studied downregulated the expression of the key regulators of Th1 and Th2 differentiation.

## 5.3 Regulation of Th cell differentiation by SATB1 (III, unpublished)

Transcription factors play a key role in driving cell differentiation, whereas the regulation of chromatin structure is required to maintain these changes. SATB1 encompasses both these functions: SATB1 is a T-cell-enriched transcription factor and chromatin organizer essential for controlling a large number of genes participating in T cell development and activation (Alvarez *et al.* 2000). SATB1 regulates gene expression by periodically anchoring matrix attachment regions (MARs) to the nuclear matrix (Cai *et al.* 2003) and by directly recruiting chromatin modifying factors (Yasui *et al.* 2002, Kumar *et al.* 2006). Depending on its post-translational modifications, SATB1 activates or represses multiple genes (Kumar *et al.* 2006). Upon activation of mouse Th2 cells, SATB1 has been shown to orchestrate the expression of Th2 cytokine genes (Cai *et al.* 2006). In our investigations we have studied the role of SATB1 in T helper cell differentiation.

### 5.3.1 STAT6-dependent SATB1 expression in Th2 cells

Our laboratory has previously shown that SATB1 expression is induced by TCR stimulation in human CD4+ T cells cultured in Th1 (anti-CD3 + anti-CD28 + IL-12), Th2 (anti-CD3 + anti-CD28 + IL-4) and Th0 (anti-CD3 + anti-CD28) conditions, as compared to naïve Thp cells and further increased by IL-4 in cells polarizing to Th2 direction (Lund *et al.* 2003, Lund *et al.* 2005). We investigated whether STAT6, which is activated by stimulation through IL-4R and is crucial for a number of IL-4-mediated effects, including Th2 cell differentiation and IgE response (Swain *et al.* 1990, Kaplan *et al.* 1996a, Shimoda *et al.* 1996, Takeda *et al.* 1996), regulates SATB1 expression in polarizing Th2 cells. Interestingly, the inhibition of the expression of STAT6 using RNAi in CD4+ T cells isolated from cord blood or buffy coat and cultured in Th2

conditions led to a markedly diminished inductions of SATB1 at mRNA and protein level analyzed by quantitative RT-PCR and western blotting, respectively (*Report III, Figures 1A and 1B*). The regulation of SATB1 expression by STAT6 is not mediated via GATA3, a key transcription factor of Th2 cells regulated by STAT6, as knockdown of GATA3 using specific siRNA oligonucleotides in differentiating cord blood CD4+ T cells did not affect SATB1 expression (*Report III, Figures 1B and 1C*). Thus, STAT6, activated by IL-4 signaling positively regulates SATB1 expression during Th2 differentiation.

### 5.3.2 Over three hundred genes are regulated by SATB1 in polarizing T helper cells

To investigate the role of SATB1 in T helper cell polarization, SATB1 target genes were studied using Illumina beadarray analysis of gene expression on total RNA from differentiating CD4+ T cells in which SATB1 had been downregulated using RNAi. SATB1 regulated genes have previously been identified using SATB1 null mouse, human cancer cell lines as well as normal and immortalized human mammary epithelial cells (Alvarez *et al.* 2000, Kumar *et al.* 2006, Han *et al.* 2008). Our study is the first genome wide screen of SATB1 target genes in primary human CD4+ T cells. To identify the SATB1 regulated genes in cells polarizing to Th1 or Th2 direction, the gene expression profiles of SATB1-siRNA or SATB1-shRNA treated cells were compared with corresponding scrambled siRNA/shRNA nucleofected control cells. We found that a total of 319 genes were directly or indirectly regulated by SATB1 in CD4+ T cells (*Report III, Tables S1 and S2*). In Th1 differentiating cells, SATB1 knockdown selectively affected 43 genes, whereas the expression of 70 genes was altered specifically in Th2 polarizing cells (*Report III, Figure 2A*). Furthermore, 14 genes were regulated by SATB1 in both Th1 and Th2 driving conditions but not in Th0 conditions, and thus altogether the expression of 40 % (43+70+14=127 genes) of the SATB1 regulated genes was altered only in the presence of a polarizing cytokine (IL-12 or IL-4). Moreover, approximately 30 % (99 genes) of SATB1 regulated genes were dependent on SATB1 expression only in Th0 subtype, i.e. cells cultured without any polarizing cytokines. These results indicate that SATB1 target genes are partly Th subtype specific. Intriguingly, in naïve CD4+ Thp cells and in cells cultured in Th0 and Th2 conditions, the majority of target genes were upregulated by SATB1 knockdown, indicating that SATB1 is a repressor of gene expression in these cells (*Figure 5A*). In contrast, 60 % of SATB1 target genes in Th1 polarizing cells were downregulated upon SATB1 knockdown, suggesting that SATB1 is mainly an activator of gene expression in these cells. The differential impact of SATB1 on gene expression in various Th cell subtypes may be due to post-translational modifications of SATB1, which are probably induced by T cell activation and cytokine signaling, that affect the transcriptional role of SATB1 (Kumar *et al.* 2006). In addition, the post-translational status of SATB1 may change in the different Th subtypes according to the differential availability of various kinases, phosphatases, HATs and HDACs. This may explain our data showing that *IL-2*, a key cytokine involved in T cell activation was downregulated in Th0 cells where SATB1 expression had been knocked down (*Report III, Table S2*), which is contrary to earlier reports demonstrating that SATB1 represses *IL-2* and its

receptor, *IL-2R* (Alvarez et al. 2000, Kumar et al. 2005) depending on the phosphorylation status of SATB1 (Kumar et al. 2006). Nevertheless, our findings are in agreement with the observations of Kumar *et al.* (2006) elucidating that SATB1 can both activate and repress gene expression. The regulation of SATB1 by TCR and Th1/Th2 polarizing cytokines requires further investigation.

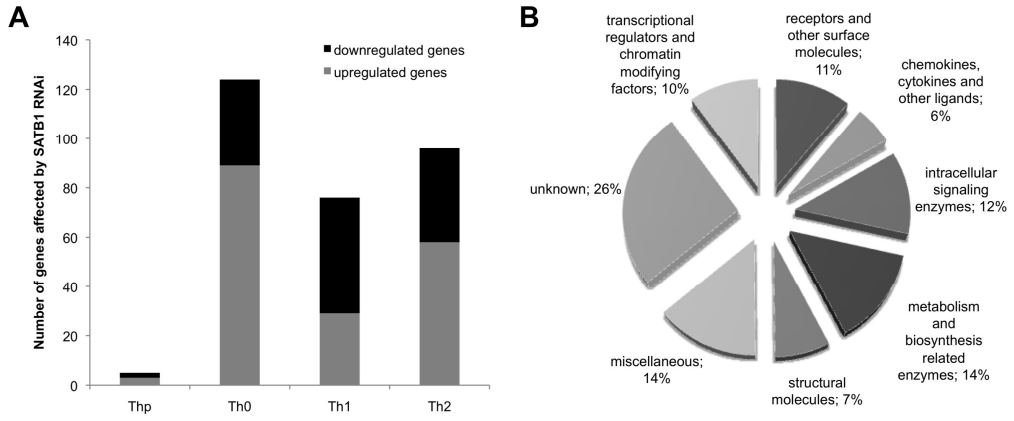


**Figure 5. Genes regulated by SATB1 during Th1 and Th2 cell differentiation.** Expression profiles of SATB1-siRNA/shRNA and scramble control siRNA/shRNA treated cells were studied using Illumina beadarrays. (A) Black and grey parts of the bar indicate the number of genes downregulated and upregulated, respectively, upon SATB1 knockdown in different Th subtypes. (B) SATB1 target genes were grouped according to their GO annotations.

To interpret the putative functional roles of SATB1 in CD4+ T cells, SATB1 target genes were categorized according to their Gene Ontology annotations (Ashburner *et al.* 2000). The grouping of these genes revealed that one third of the SATB1 target genes coded for factors participating in signal transduction, including receptors and other cell surface molecules, intracellular signaling enzymes as well as transcriptional regulators and chromatin modifying factors (*Figure 5B*). Intriguingly, several cytokines and chemokines, such as *IL-1B*, *IL-2, IL-3, IL-6, CCL3, CCL5, CSF2* and *EBI3* (*Report III, Table S1: IL-4 target genes*); *CCL4L2 and CXCL10* (*Report III, Table S1: IL-12 and IL-4 target genes*); *IL-5, IL-18* and *CCL3L1* (*Report III, Table S2*), and their receptors, including *IL7R* and *CXCR4* (*Report III, Table S1: IL-4 target genes*); *IL12Rβ2, IL18R1* and *IL18RAP* (*Report III, Table S1: IL-12 and IL-4 target genes*) were regulated by SATB1 in differentiating Th cells. Moreover, a substantial group of SATB1 regulated genes coded for enzymes related to metabolism and biosynthesis (*Figure 5B*).

SATB1 knockdown upregulated the expression of an IL-4 induced chemokine receptor *CXCR4* (*Report III, Table S1: IL-4 target genes*) (Jourdan *et al.* 1998). Interestingly, CXCR4, besides other cell surface molecules CD4 and CCR5, has been shown to be used by HIV-1 as a coreceptor to enter into CD4+ T cells, which is associated with clinical progression to AIDS (Connor *et al.* 1997, Copeland 2006). Furthermore, HIV-1 infection involves upregulation of CXCR4 (Wang *et al.* 1998) and a shift from Th1-

to Th2-type cytokine production by CD4+ T cells (Meyaard *et al.* 1996, Klein *et al.* 1997), which favors replication of HIV-1 (Maggi *et al.* 1994). Moreover, HIV-1 Tat protein is able to displace HDAC1 that is bound to SATB1, resulting in derepression of SATB1-regulated gene expression in T cells (Kumar *et al.* 2005). These findings suggest that SATB1 interferes with HIV infection and thus, downregulation of SATB1 might play a role in the course of infection.

### 5.3.3 SATB1 target genes include several factors involved in Th2 differentiation

Following our observation of the impact of SATB1 on the regulation of a large set of genes in CD4+ T cells, we next studied whether SATB1 regulated genes are involved in T helper cell differentiation. The gene expression profiles of scramble treated Thp, Th0, Th1 and Th2 cells were compared with each other to determine genes regulated by T cell activation (Th0 vs. Thp), IL-12 (Th1 vs. Th0) or IL-4 (Th2 vs. Th0). In addition, IL-12, IL-4 or TCR regulated genes were determined by detailed gene expression kinetics study carried out in our laboratory on differentiating human cord blood Th1 and Th2 cells at the whole genome level (Elo, L.L., Järvenpää, H., Tuomela, S. *et al.*, *manuscript in preparation*). This information of cytokine or TCR specific regulation of SATB1 target genes was added to their annotation. Notably, 35 % (111 genes) of the SATB1 target genes were regulated by IL-4 and additional 13 % (41 genes) were regulated by both IL-12 and IL-4, revealing that altogether 48 % (152 of 319 genes) of SATB1 targets are IL-12 and/or IL-4 regulated genes (*Report III, Figure 2B*). Furthermore, TCR stimulation alone regulated one third (108 genes) of SATB1 target genes and only 18 % (=100-35-13-34 %) of SATB1 regulated genes were not regulated by TCR or Th1/Th2 polarizing cytokines.

SATB1 knockdown affected the expression profiles of a number of Th1/Th2 differentiation involving transcription factors (*IRF8, STAT1 and SMAD3)*, receptors (*IL12Rβ2, CXCR4, IL18R1* and *IL18RAP*) and cytokines (*IFNγ, IL-6* and *IL-18*). SATB1 regulated two members of IRF family of transcription factors, namely *IRF7* and *IRF8*. When SATB1 expression was knocked down, *IRF8* was 1.6-fold decreased in Th0 cells and 6.5-fold decreased in cells cultured in Th1 or Th2 conditions (*Report III, Table S1: IL-4 regulated genes*). In contrast, SATB1 knockdown induced the expression of *IRF7* 2.7-fold in Th0 and Th1 differentiating cells (*Report III, Table S1: IL-4 regulated genes*). Intriguingly, IRF family members are involved in Th cell differentiation, as IRF1- or IRF8-deficient mice show defective Th1 responses due to impaired production of IL-12 (Giese *et al.* 1997, Lohoff *et al.* 1997, Scharton-Kersten *et al.* 1997, Taki *et al.* 1997, Salkowski *et al.* 1999, Masumi *et al.* 2002), whereas IRF4 is required for Th2 differentiation and plays a role in inhibiting aberrant Th1 development (Lohoff *et al.* 2002, Tominaga *et al.* 2003). Furthermore, *STAT1* was 1.6-fold downregulated in Th1 polarizing cells and *SMAD3* was 1.5-fold induced in Th0 cells upon SATB1 silencing (*Report III, Table S1: IL-4 regulated genes*). SMAD3, a key signal transducer in TGFβ signaling (Attisano & Wrana2002), has been shown to promote Th2 differentiation, as SMAD3 knockout mice exhibit increased GATA3 and IL-4 levels (Anthoni *et al.* 2007). In addition, mice lacking STAT1 are unresponsive to IFNγ and have defective Th1 responses (Meraz *et al.* 1996). As SATB1 knockdown upregulated the expression of *SMAD3* and downregulated *IRF8* and *STAT1*, our

findings indicate a shift in the gene expression profile of SATB1 silenced polarizing CD4+ T cells towards Th2 subtype.

Using the ChIP-on-chip approach, we investigated whether the promoters of SATB1 regulated genes are bound *in vivo* by SATB1 in CD4+ T cells. SATB1 enriched chromatin from cord blood CD4+ T cells cultured in Th1 and Th2 polarizing conditions for 24 h was hybridized on Agilent Human Promoter CoC 244k arrays together with corresponding input controls. SATB1 was bound to promoters of 3279 and 2729 genes in Th1 and Th2 cells, respectively (data not shown). Twenty-seven % (86 genes) of SATB1 target genes identified using RNAi approach were also bound *in vivo* by SATB1 (*Report III, Figure 2C*). In addition, direct SATB1 target genes were further enriched with IL-4 regulated genes as 60 % (40+12=52 genes) of SATB1 CoC and siRNA targets were regulated by IL-4. These results, together with the finding that IL-4 activated transcription factor STAT6 positively regulates SATB1 expression, suggest that SATB1 might have a role in IL-4 mediated signaling and thereby in control of Th2 differentiation or function.

### 5.3.4 SATB1 regulates cytokine expression

#### 5.3.4.1 IFNγ centered signaling network is affected by SATB1 knockdown

To investigate the protein and biomolecule interaction networks associated with SATB1 target genes, analysis was made with the Ingenuity Pathways Analysis (IPA) software. These results indicated that an interferon-gamma centered gene regulatory network was greatly affected by SATB1 knockdown across the different Th subtypes suggesting that SATB1 plays a role in the regulation of IFNγ expression which mediates Th1 cell differentiation (Figure 6). Even if IFNγ transcription was not affected by SATB1 knockdown during the early differentiation of Th1 cells, IFNγ target genes, including *IL12Rβ2, IFI44, IFI44L, IFI6, IFIT3 and MX1* (*Report III, Table S1: IL-12 and IL-4 regulated genes*) as well as *IFIT2* and *STAT2* (*Report III, Table S1: IL-4 target genes*) were induced in Th subtypes.

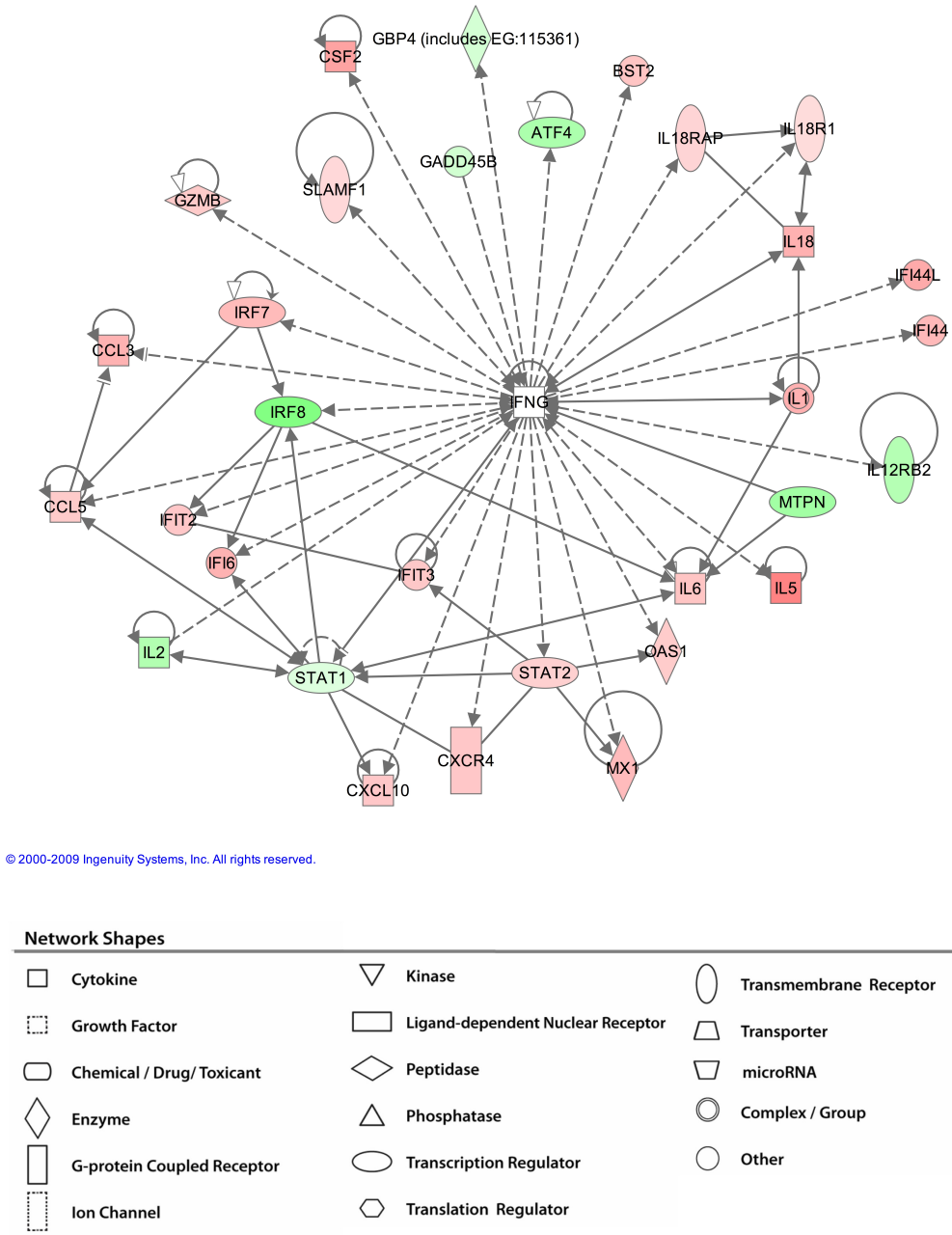


**Figure 6. IFNγ centered pathway in greatly regulated by SATB1 knockdown.** The SATB1 target genes identified using microarray were analyzed and the IFNγ network was generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). In the network the molecular relationships between genes/gene products are represented as nodes and their biological relationship is represented as an edge. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product. Several SATB1 target genes are either directly or indirectly connected to IFNγ.

We studied the effect of SATB1 knockdown on the IFNγ production of Th1 polarized cells (7-8 days). Intracellular cytokine staining analyzed with flow cytometry showed that the downregulation of SATB1 led to an increased IFNγ production by Th1 cells (Figure 7), but not by Th2 cells (data not shown). These results were in agreement with the Illumina beadarray analysis results obtained from the early differentiating Th cells, showing that IFNγ regulated genes are upregulated by SATB1 knockdown. Thus, our results suggest that SATB1 is involved in the regulation of genes of IFNγ centered signalling network during Th cell differentiation.

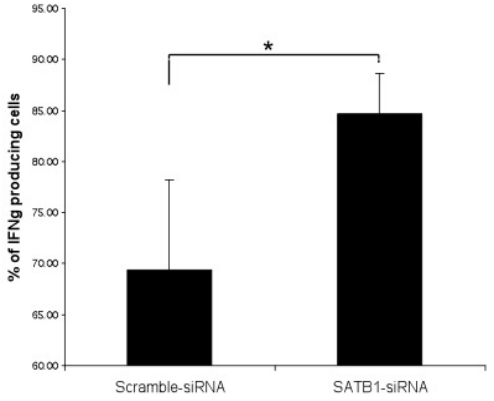


**Figure 7. IFNγ production is increased in SATB1 silenced Th1 polarized cells at day 8.** Naïve CD4+ T cells isolated from cord blood were nucleofected with SATB1 siRNA or scramble control oligonucleotide. 24 hours post transfection, the cells were activated with plate-bound anti-CD3 and soluble anti-CD28 and Th1 polarization was induced with IL-12. After 7 days of polarization, the cells were restimulated using PMA and IM as described in experimental procedures, cells were stained for intracellular cytokines and the IFNγ cytokine production was measured with flow cytometry. The figure shows average percentage and standard deviation from four independent experiments. The statistical difference in the IFNγ production was determined using paired *t* test. *$p<0.05$.

Intriguingly, downregulation of SATB1 changed the expression profiles of several factors that regulate IFNγ expression. SATB1 knockdown induced the expression of the cytokine *IL-18* (3.3-fold across the Th subtypes; *Report III, Table S2*) and both chains of its receptor: a ligand binding chain *IL18R1* (1.7-fold in Th0 cells; *Report III, Table S1: IL-12 and IL-4 regulated genes*) and a signal transducing chain *IL18RAP* (on average 1.8-fold in Th0, Th1 and Th2 polarizing cells; *Report III, Table S1: IL-12 and IL-4 regulated genes*). These results are in agreement with the observations by Han *et al.* (2008) showing that *IL-18* and *IL18R1* are repressed by SATB1. IL-18 is a proinflammatory cytokine produced by a wide variety of immune cells, including monocytes, macrophages and DCs (Okamura *et al.* 1995, Ushio *et al.* 1996, Stoll *et al.* 1998). However, IL-18 has not been reported to be expressed by CD4+ cells. Interestingly, IL-18 alone cannot promote Th1 differentiation but in combination with IL-12 or IFNα it has been shown to induce IFNγ production and Th1 cell proliferation (Okamura *et al.* 1995, Micallef *et al.* 1996, Robinson *et al.* 1997, Sareneva *et al.* 1998,

Takeda *et al.* 1998). However, in combination with IL-2, IL-18 promotes Th2 polarization and the expression of Th2 cytokines (Hoshino *et al.* 2000, Xu *et al.* 2000, Yoshimoto *et al.* 2000), indicating that the role of IL-18 in T helper cell differentiation is dependent on the cytokine milieu. Moreover, *IL18R1* gene encoding IL-18Rα is induced via the IL-12/STAT4 pathway in Th1 cells (Kunikata *et al.* 1998, Xu *et al.* 1998, Yoshimoto *et al.* 1998, Lawless *et al.* 2000, Sareneva *et al.* 2000, Nakahira *et al.* 2001, Yu *et al.* 2007) and inhibited via the IL-4/STAT6 pathway in Th2 cells (Smeltz *et al.* 2001). Recently, GATA3 was shown to bind *IL18R1* locus and mediate repression of *IL18R1* expression (Yu *et al.* 2008). SATB1 knockdown induced expression of *IL-18* and both chains of its receptor could lead to an aberrant autocrine IL-18 signaling in Th1 polarizing cells, which, in the presence of IL-12 in the culture conditions, might explain the increased IFNγ production by the Th1 cells. Alternatively, the source of IL-18 could be any other immune cell types likely to be present in trace amounts in the culture conditions. Collectively, these findings suggest that SATB1 regulates IFNγ network, but do not necessarily control *IFNγ* transcription directly by itself. However, SATB1 might also regulate the *IFNγ* transcription in Th1 polarizing cells by affecting the loopscape of the *IFNγ* locus, as IFNγ treatment was shown to alter the SATB1 and PML governed chromatin structure and expression of a subset of MHC class I genes (Kumar *et al.* 2007).

#### 5.3.4.2 IL-5 expression is greatly induced by SATB1 knockdown during Th2 differentiation

As described above, SATB1 regulates a large variety of cytokines, chemokines and their receptors in differentiating T helper cells. One of the most highly upregulated genes in Th2 differentiating cells upon SATB1 knockdown is the Th2 hallmark cytokine, *IL-5* (*Report III, Table S2*). Quantitative RT-PCR was performed to further analyze the SATB1 knockdown induced transcription of *IL-5*. Additional cultures were generated from cord blood isolated CD4+ T cells that were nucleofected with SATB1-siRNA or scrambled control oligonucleotides. SATB1 expression was markedly downregulated by SATB1-siRNA at mRNA and protein level (*Report III, Figures 3A and 3B*). Suppression of SATB1 expression greatly induced IL-5 expression at 48 hours of culture in Th2-driving conditions when SATB1-siRNA cells were compared to scrambled control treated cells (*Report III, Figure 3C*). Induction of *IL-5* expression was not detected in Th1 polarizing cells where SATB1 was knocked down (data not shown). Furthermore, Bio-Plex assay was applied to measure secreted IL-5 from the culture media of SATB1-siRNA or control siRNA treated cells. At 24 and 48 h time points the production of IL-5 was significantly increased upon SATB1 knockdown (*Report III, Figure 3D*). Collectively, these results indicate that SATB1 participates in a negative feedback loop in which it is induced during Th2 differentiation and represses *IL-5* expression in polarizing Th2 cells.

The control of IL-5 by SATB1 has been reported previously, as Cai *et al.* (2006) demonstrated the positive regulation of *IL-5* by SATB1 upon activation in the mouse D10 Th2 cell clone. The contrasting results might be explained by the difference in the polarization status of the cells (naïve/early differentiating vs. effector/memory CD4+ cells) and by the origin of the cells (human vs. mouse). Notably, similar dual function

in cytokine gene control has recently been shown for IRF4, which differentially regulated Th2 cytokine production, especially IL-4, in naïve CD4+ T cells compared to effector/memory CD4+ T cells (Honma *et al.* 2008). Even if Th2 cytokines are generally considered to be co-regulated as they are located in a close proximity in human chromosome 5 and mouse chromosome 11, several observations indicate that the expression of *IL-5* is regulated independently of other Th2 cytokines, in particular *IL-4* (Bohjanen *et al.* 1990, Sanderson 1992, Jung *et al.* 1995, Yamashita *et al.* 2002). This is also supported by our results showing that downregulation of SATB1 dramatically upregulated the expression of *IL-5,* but not *IL-4* or *IL-13* (data not shown). Thus, these results indicate that SATB1 specifically represses *IL-5* expression during early polarization of Th2 cells.

### 5.3.5 Regulation of IL-5 expression by SATB1

#### 5.3.5.1 SATB1 binds to the *IL-5* promoter *in vitro* at multiple sites

To understand the molecular mechanism of how SATB1 inhibits *IL-5* transcription and to test whether SATB1 acts by directly binding to the *IL-5* promoter as in case of other SATB1 regulated genes (Kumar *et al.* 2005), we performed electrophoretic mobility shift assay (EMSA) to monitor SATB1 binding. Using a 615 bp fragment (-581 bp to +34 bp) of the *IL-5* proximal promoter as a probe (*Report III, Figure 4A*) in EMSA, SATB1 formed a characteristic complex of increasing size in a dose-dependent manner (*Report III, Figure 4B, upper left panel*). Such binding pattern suggested the presence of several binding sites for SATB1, and therefore the nucleotide sequence was screened for consensus SATB1 binding sites (SBSs) based on the findings by Purbey and coworkers (Purbey *et al.* 2008). We identified four putative SBSs and prepared suitable deletions and truncations of the *IL-5* proximal promoter (*Report III, Figure 4A and Table S3*). Binding of SATB1 to the probe A (-190 bp to +34 bp) but not to the probe B (-151 bp to +34 bp) confirmed the presence of an SBS in the 5' end of the probe A (*Report III, Figure 4B, lower panels*). However, the high affinity and progressively bigger dose-dependent complex formed by SATB1 with probe C (-581 bp to -172 bp) suggested presence of multiple SBSs in this region of the promoter (*Report III, Figure 4B, upper right panel*). Serial truncations of the probe C showed that the ultimate 5' end of the promoter region used in this study is devoid of any SBSs as SATB1 failed to bind with the probe D (-581 bp to -426 bp; *Report III, Figure 4C*). In contrast, SATB1 formed specific complexes with probe E (-444 bp to -301 bp) and probe F (-318 bp to -172 bp), suggesting that they harbor strong SBSs (*Report III, Figure 4C*). Probe F contains two putative SBSs and deleting both of them in the probe F substantially decreased the affinity of SATB1 (*Report III, Figure 4C)*. SATB1 also formed specific complexes with probes G (-556 bp to -301 bp), H (-444 bp to -172 bp) and I (-318 bp to +34 bp; *Report III, Figure 4C*). Probe G has only one SBS and binding of SATB1 with this probe was decreased upon removal of the SBS. The binding of SATB1 with the probes H, I and C was severely affected by deleting the newly identified SBSs (*Report III, Figures 4C and 4D*), confirming the absence of any further SBSs in the *IL-5* promoter used in this study. Similarly, removing all the four SBSs from the full-length *IL-5* probe resulted in a substantially weaker binding of SATB1 (*Report III, Figure 4D*). We also determined the relative binding affinities of

SATB1 to the wt and mutated full-length and truncated *IL-5* probes (summarized in *Report III, Table S4*). Binding of SATB1 was strongest with wt *IL-5* probe and probe I, whereas deleting the putative SBSs abrogated the binding activity drastically. Thus, using *in vitro* binding analysis four novel SBSs in the *IL-5* promoter were identified.

To investigate whether these SBSs are occupied by SATB1 from cell extracts of CD4+ T cells, we performed EMSA using nuclear extracts from polarizing Th1 and Th2 cells. Such analysis showed that the proteins of Th2 cells gave rise to a substantially stronger complex with probe A than that of Th1 cells (*Report III, Figure 4E*), corroborating the earlier finding that the Th2 cells express more SATB1 than Th1 during their early differentiation (Lund *et al.* 2005). In addition, both complexes were supershifted in presence of anti-SATB1 confirming the presence of SATB1 both in Th1 and Th2 nuclear extracts (*Report III, Figure 4F*). The complex is SATB1-dependent as nuclear extracts from SATB1-siRNA nucleofected cells cultured in Th2 conditions for 24 h failed to form complex in EMSA whereas control siRNA treated cells formed a complex (*Report III, Figure 4G*).

To elucidate the functional role of the identified SBSs on the expression of *IL-5*, we performed luciferase assay using the pGL3 reporter construct containing the 615 bp fragment (-581 bp to +34 bp) of the *IL-5* proximal promoter. Mutant reporter constructs were created from the wt *IL-5* reporter construct by deleting all individual SBSs, deleting SBSs one by one or deleting three SBSs at a time leaving one SBS intact (*Report III, Figure 5A*). Buffy coat CD4+ T cells were nucleofected with either wt or mutated reporter construct and pSUPER-scramble-H-2K$^k$ vector that enabled measuring the transfection efficiency (27-45 %). Cells were cultured under Th2 polarizing conditions for 24 h and harvested for the transactivation assay. The luciferase activity was increased compared to wt *IL-5* promoter construct when SBSs S1 or S2 were deleted separately (del1, del2) or they were both deleted together with S4 (del1+del2+del4; *Report III, Figure 5B*). In contrast, the luciferase activity was markedly decreased when SBS S3 was deleted alone (del3) or in combination with SBSs S2 and S4 (del2+del3+del4), S1 and S4 (del1+del3+del4) or S1 and S2 (del1+del2+del3). Furthermore, the reporter activity was also decreased compared to wt construct by deleting all the SBSs. These results indicate that SBSs S1 and S2 are repressive sites whereas S3 is a strongly activating site. This was further supported by the estimated coefficients of the individual SBSs in the linear model: S1 -3.165 (p=0.068), S2 -4.232 (p=0.017), S3 13.374 (p=0.000) and S4 2.512 (p=0.142).

The 5' flanking region of the human *IL-5* gene has been reported to interact with a variety of transcription factors that play important roles on *IL-5* expression. The conserved lymphokine element 0 (CLE0; -42 bp to -56 bp) acts as an on/off switch for *IL-5* transcription. This crucial element binds the transcription factor AP-1 members JunD and Fra-2 as well as Oct-1 and Oct-2 factors (Mori *et al.* 1997, Thomas *et al.* 1999). GATA3 binds to at least three sites in the *IL-5* promoter. Positive regulation is mediated by -70 and -152 sites whereas binding of GATA3 to -400 site results in strong inhibition of *IL-5* expression (Yamagata *et al.* 1995, Blumenthal *et al.* 1999, Schwenger *et al.* 2001). In addition, NFAT, ETS1, ETS2, YY1, C/EBPβ and glucocorticoid receptor are involved in the complex regulation of *IL-5* transcription (Prieschl *et al.* 1995, Blumenthal *et al.* 1999, De Boer *et al.* 1999, Mordvinov *et al.*

1999, Schwenger *et al.* 1999, Cousins *et al.* 2000, Li-Weber *et al.* 2001, Wang *et al.* 2006). Our *in vitro* binding assay demonstrated that SATB1 binds several sites on the human *IL-5* promoter. The SATB1 binding sites S1 (-162 bp to -152 bp) and S2 (-333 bp to -322 bp) map to previously identified negative regulatory elements (Gruart-Gouilleux *et al.* 1995, Stranick *et al.* 1997) while S4 (-254 bp to -243 bp) is located in a region shown to function as a positive element (Gruart-Gouilleux *et al.* 1995). The SATB1 binding site S3 does not have any previous annotation. Our data indicates that S1 and S2 are repressive sites, which is concurrent with the previous observations. Our finding that S3 is a strongly activating site provides new information on the regulatory elements in *IL-5* promoter. However, the specific use of different SBSs at different developmental or functional cell states needs further investigation. In addition, the *IL-5* promoter might harbor few other SBSs upstream of the region we have studied, as SATB1-siRNA increases *IL-5* mRNA expression by 6000 % whereas deletion of all four SBSs did not increase IL-5 reporter activity by the same amount. Nevertheless, this study demonstrates direct role of SATB1 in IL-5 expression.

#### 5.3.5.2 Occupancy of *IL-5* promoter by SATB1 is required for suppression of IL-5 expression during Th2 cell differentiation

We next studied the *in vivo* binding of SATB1, GATA3 and HDAC1 to the *IL-5* promoter during early Th cell differentiation. GATA3 mediates positive regulation of *IL-5* transcription via -70 and -152 sites (Yamagata *et al.* 1995, Schwenger *et al.* 2001), while overexpression of HDAC1 has been shown to augment the repression of *IL-5* (Jee *et al.* 2005). Furthermore, phosphorylated SATB1 has previously been demonstrated to recruit HDAC1 to its targets, and such recruitment leads to downregulation of gene expression (Kumar *et al.* 2006). Cord blood CD4+ T cells were cultured in Th1 and Th2 conditions for 24 h and subjected to ChIP assay to monitor the occupancy of SATB1, HDAC1 and GATA3 at the proximal *IL-5* promoter. ChIP-PCR analysis revealed that SATB1 and HDAC1 were bound to *IL-5* proximal promoter both in Th1 and Th2 conditions whereas GATA3 bound the *IL-5* promoter specifically in Th2 polarizing cells (*Report III, Figures 6A and 6B*). Quantitative PCR revealed more than 2-fold increase in the occupancy of SATB1 in Th2 cells (*Report III, Figure 6A*) corroborating with its higher expression in Th2 cells (Lund *et al.* 2005). Interestingly, the occupancy of HDAC1 was also increased by ~2-fold in Th2 cells suggesting a possible role of SATB1 in its recruitment. GATA3 occupancy was highest in Th2 cells, about 7-fold higher than the minimal occupancy observed in Th1 cells, which is in concordance with its preferential expression in Th2 cells. Similar occupancy profile was observed at the distal region of the *IL-5* promoter corresponding to probe C that also contains SBSs and GATA3 binding site (data not shown). In conclusion, our ChIP data suggests that SATB1 could repress IL-5 expression by recruiting the HDAC1 corepressor to *IL-5* promoter both in Th1 and Th2 cells during their early differentiation. In addition, binding of GATA3 to *IL-5* promoter in cells cultured in Th2 conditions presumably poises the *IL-5* gene for transcription later upon activation of Th2 cells.

SATB1 has previously been shown to coordinate the expression of Th2 cytokines by organizing the folding of chromatin (Cai *et al.* 2006). We performed matrix loop

partitioning assay using cord blood CD4+ T cells that were nucleofected with SATB1-siRNAs or scramble control siRNA oligonucleotides and cultured in Th2 conditions for 0, 24 and 48 h. The presence of *IL-2* and *IL-5* promoters in matrix-bound and loop-associated DNA was quantified by RT-PCR. The promoter of *IL-2* gene was used as a positive control as SATB1 binds to it *in vivo* (Kumar *et al.* 2005) and regulates its chromatin 'loopscape' (Purbey and Galande, *manuscript in preparation*). Our results show that downregulation of SATB1 expression increased the relative amount of *IL-2* promoter in loop fractions compared to matrix fractions whereas the looping of *IL-5* promoter region was not affected (Figure 8A). To study whether the looping of *IL-5* promoter changes during the Th2 differentiation, cord blood CD4+ T cells were cultured in Th2 polarizing conditions for 0, 24 and 48h to analyze the looping at the early differentiation and for 14 d to analyze the looping at the late differentiation. Interestingly, in the course of Th2 differentiation the occurrence of *IL-5* gene in matrix fraction increases as the relative amount of *IL-5* promoter in matrix fraction of 14 d Th2 cells was in average 2.7-fold increased compared to that of early Th2 cells (0-48 h; Figure 8B). Thus, looping of *IL-5* gene changes during Th2 differentiation but it is not SATB1 dependent during the early differentiation of Th2 cells and therefore cannot explain the induction of IL-5 expression upon SATB1 knockdown.

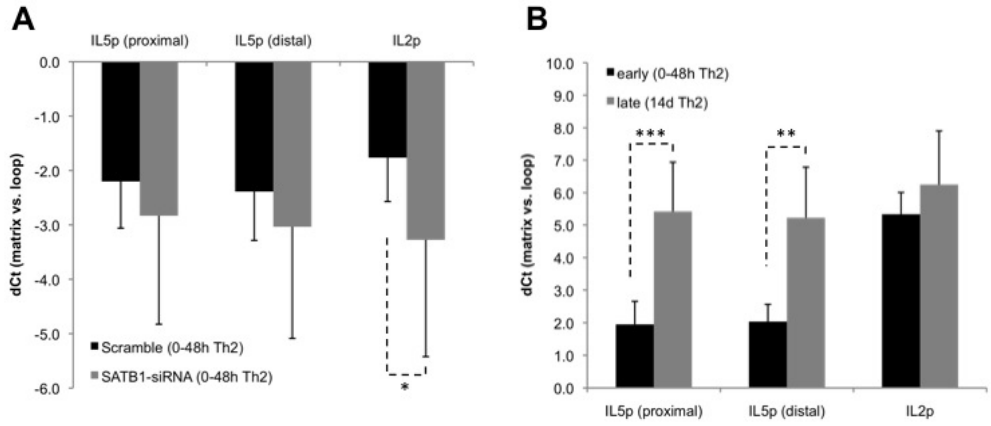


**Figure 8. Loopscape of *IL-5* promoter during early and late differentiation of Th2 cells.** (A) Loopscape of *IL-5* promoter is not SATB1 dependent during the early differentiation of Th2 cells. Matrix-loop partitioning assay was performed with cord blood CD4+ T cells which were nucleofected with SATB1-siRNA oligonucleotides (black bars) or scramble control siRNAs (grey bars) and cultured in Th2 polarizing conditions for 0, 24 and 48 h (time points pooled for the analysis). Matrix-bound and loop-associated DNA was quantified for the presence of *IL-5* and *IL-2* promoter sequences using qRT-PCR. The mean (± s.d.) difference of Ct values of matrix and loop fractions of three independent experiments is presented in the figure. *$p<0.05$. (B) *IL-5* promoter is enriched to matrix during the differentiation of Th2 cells. Matrix-loop partitioning assay was performed with cord blood $CD4^+$ T cells cultured in Th2 polarizing conditions for 0, 24 and 48 h (black bars; time points pooled) and 14 d (grey bars). Matrix-bound and loop-associated DNA was quantified for the presence of *IL-5* and *IL-2* promoter sequences using qRT-PCR. The mean (± s.d.) difference of Ct values of matrix and loop fractions of three independent experiments is presented in the figure. **$p<0.01$, ***$p<0.005$.

GATA3 has previously been shown to bind at least three sites (-70, -152 and -400 bp) in the *IL-5* promoter and regulate *IL-5* transcription (Yamagata *et al.* 1995, Blumenthal *et al.* 1999, Schwenger *et al.* 2001). Interestingly, one of the repressive SATB1 binding sites (S1) juxtaposes an activating GATA3 site (-152 bp) in the *IL-5* promoter, which led us to study the reciprocal role of SATB1 and GATA3 in the regulation of IL-5. IL-5 is not expressed during the differentiation of Th2 cells but induced in fully differentiated effector Th2 cells by restimulation (Cousins *et al.* 2002). Notably, *in vivo* upregulation of IL-5 during the priming of the CD4+ T cells in lymph nodes or soon after that could lead to aberrant recruitment and activation of eosinophils and development of allergic disease or asthma (De Monchy *et al.* 1985, Leung 1998, Kurowska-Stolarska *et al.* 2008). We hypothesized that in normal Th2 polarizing cells, binding of SATB1 and corepressors recruited by SATB1 to the *IL-5* promoter would block binding or function of GATA3 and result in repression of *IL-5* transcription whereas in SATB1 knockdown cells, GATA3 would freely act on SATB1-devoid promoter and induce aberrant *IL-5* expression. Interestingly, SATB1 and GATA3 binding sites are colocalized at the 3´ end of the human *CD8B* gene, a region suggested to function as a regulator of CD8 expression (Kieffer *et al.* 2002). Furthermore, SATB1 and GATA3 proteins were demonstrated to colocalize in activated mouse Th2 clone cells (Cai *et al.* 2006). However, any competition between SATB1 and GATA3 has not been previously observed.

We studied the binding of GATA3 with the probes described above to elucidate whether any of the identified SBSs affects the binding of GATA3. GATA3 formed complex with the full-length *IL-5* promoter and probes A, B, C (*Report III, Figure SA*), D, E, G, H and I, but not with probe F (*Report III, Figure SB*). Furthermore, EMSA showed that GATA3 has two binding sites in the promoter regions represented by probes B, D and E, resulting in at least six binding sites in the 615 bp fragment of the *IL-5* promoter used. Interestingly, deletion of any or all of the newly identified SBSs did not change GATA3 binding, indicating that SBSs do not affect the affinity of GATA3 for the *IL-5* promoter (*Report III, Figure SC*). This was also confirmed by calculating the relative binding affinities of GATA3 to the wt and mutated full-length and truncated IL-5 probes (summarized in *Report III, Table S4*), which showed that binding of GATA3 to all probes used in this study was relatively constant.

As knockdown of SATB1 did not induce GATA3 expression in developing Th2 cells, which could explain the increased expression of IL-5 (*Report III, Figure 7A*), we studied whether in case of SATB1 downregulation, GATA3 is the protein responsible for the aberrant induction of *IL-5* expression. Cord blood isolated CD4+ T cells were nucleofected with siRNA oligonucleotides targeting SATB1, GATA3 or both these gens and cells were induced to polarize to Th2 direction. Each specific siRNA inhibited substantially their target gene expression in each sample (*Report III, Figure 7A and 7B*). SATB1 knockdown strongly induced *IL-5* (over 4000 %) whereas downregulation of GATA3 did not decrease *IL-5* expression presumably as the basal expression of *IL-5* is negligible during early Th2 differentiation (*Report III, Figure 7C*). Intriguingly, the induction of IL-5 expression in cells where both SATB1 and GATA3 had been knocked down was only roughly ¼ of the induction resulting from knocking down SATB1 alone. We also measured using Bio-Plex assay the secreted IL-

5 from the culture media of SATB1-siRNA and/or GATA3-siRNA or scrambled control siRNA treated cells. In concordance with the results on mRNA level, the production of IL-5 was significantly more increased upon SATB1 knockdown compared to cells where both SATB1 and GATA3 were simultaneously downregulated using siRNA oligonucleotides (*Report III, Figure 7D and Table S5*). Collectively, these results indicate that GATA3 plays a role in the upregulation of IL-5 in the absence of SATB1 and thereby suggest that SATB1 is presumably required to block GATA3 mediated induction of IL-5 expression during early Th2 cell differentiation.

The mechanism how SATB1 regulates IL-5 expression probably includes (1) repression or prohibiting binding of *IL-5* expression activating transcription factors, such as GATA3 or NFATs (Yoshida *et al.* 1998, Blumenthal *et al.* 1999, Schwenger *et al.* 2001, Rengarajan *et al.* 2002b); (2) regulation of the chromatin loopscape of *IL-5* locus and/or (3) recruitment of histone modifying factors and changes in the histone modification status. These hypotheses are supported by several findings, as follow. Even if our results indicated that the expression of GATA3, NFATs or any other known IL-5 regulating transcription factor was not affected by SATB1 knockdown on mRNA level, alterations in their stability, localization or interaction partners might favor increased IL-5 expression (Yoshida *et al.* 1998, Blumenthal *et al.* 1999, Schwenger *et al.* 2001, Rengarajan *et al.* 2002b). Moreover, SATB1 has been shown to play an essential role in packing the mouse Th2 cytokine locus (Cai *et al.* 2006). Even if our results indicated that the looping of *IL-5* promoter changes in course of Th2 differentiation, the loop arrangement was not SATB1-dependent during early differentiation of Th2 cells. However, SATB1 might be required for the formation of three-dimensional chromatin configuration of the whole Th2 cytokine locus as observed in a mouse Th2 cell clone (Cai *et al.* 2006). Further studies are required to define the specific role of SATB1 in the loopscape of Th2 cytokine locus during Th2 cell polarization. Furthermore, histone hyperacetylation of the *IL-5* gene in CD4+ T cells has been shown to be Th2-specific and to occur in a STAT6- and GATA3-dependent manner (Yamashita *et al.* 2002). The overexpression of HDAC1 has been demonstrated to repress IL-5 (Jee *et al.* 2005). SATB1 can recruit corepressors (HDACs) and coactivators (HATs) directly to promoters (Kumar et al. 2005, Kumar et al. 2006). In the case of human *IL-2* and *IL-2Rα* genes, SATB1 has been shown to directly bind to their promoters and recruit HDAC1 *in vivo* causing a suppression of gene expression (Kumar *et al.* 2005). Since SATB1 has been shown to recruit HDAC1 to the regulatory sites (Kumar *et al.* 2005, Kumar *et al.* 2006, Purbey *et al.* 2009), it is plausible that a similar mechanism may operate during repression of IL-5 by SATB1 during early Th2 differentiation, as supported by the occupancy of *IL-5* promoter by both SATB1 and HDAC1.

To conclude, in light of our findings, we propose that the transcriptional regulator and chromatin organizer SATB1 plays an important role in T helper cell lineage decision since it coordinately regulates a large number of IL-4 target genes involved in Th2 cell differentiation and function. Our results suggest that a competitive mechanism involving SATB1 and GATA3 regulates IL-5 transcription, and provide new mechanistic insights into the stringent regulation of IL-5 expression during human Th2 cell differentiation.

# 6 CONCLUSIONS

The immune system, composed of innate and adaptive parts, protects the host from microbial attacks and malignant transformation. APCs, of which the most important subtype is DCs, present captured and processed antigens to lymphocytes and thereby start the specific immune response. Naïve CD4+ T cells differentiate to functionally distinct effector T cell subsets including Th1, Th2 and Th17 cells, which while being responsible for specific immune functions have also been implicated in pathological responses, such as autoimmunity, asthma and allergy. Detailed characterization of the signaling pathways and regulatory networks leading to the development of Th cell subsets is essential for understanding the pathogenesis of immune-mediated diseases. This thesis focuses on dissecting the IL-4 induced gene regulatory circuits leading to differentiation of DCs and Th2 cells. The main results of the thesis are as follows:

Gene expression profiling lead to identification of over 200 genes that are differentially expressed during cytokine induced differentiation of human monocytes to DCs or macrophages. These genes include several transcription factors that are likely to orchestrate crucial cell type specific gene expression patterns in DCs and macrophages. Furthermore, differentially expressed genes include receptors and other cell surface molecules, which regulate interactions of DCs or macrophages with other cells, microbes or their components, as well as extracellular soluble mediators, such as cytokines and immunoglobins, which transmit intercellular communication. These genes most probably play a key role in establishing the proper biological functions of DCs and macrophages.

Transcriptome analysis demonstrated the dynamic regulation of gene expression by IL-12 and IL-4 during the initiation of Th cell differentiation as only a subset of genes was preferentially expressed by the polarizing Th1 or Th2 cells throughout the early differentiation. Moreover, the gene expression pattern of several genes was changed by adding TGFβ, an immunosuppressive cytokine, to the cells cultured in Th1 or Th2 conditions, indicating the putative role of these factors in Th cell differentiation process. Similarly, IL-10 was potent in downregulating the expression of the key regulators of Th1 and Th2 cell differentiation, including T-bet and GATA3. Thus, the immediate target genes of IL-12 and IL-4 are likely play an important role in forming the gene regulatory networks needed for development of Th1 and Th2 cells.

RNAi mediated gene knockdown experiments and global gene expression analysis indicated that SATB1, a T cell enriched transcriptional regulator and chromatin organizer, coordinately regulates a large number of genes important for Th cell polarization or function. In Th2 lineage IL-4 induces SATB1 expression via STAT6. Furthermore, SATB1 directly binds the promoter of *IL-5*, recruits HDAC1 corepressor and may compete with GATA3 for the reciprocal regulation of *IL-5* transcription. These findings suggest that SATB1 plays an important role in Th1 and Th2 cell differentiation. Understanding the molecular basis of the putative SATB1 and GATA3 competition will provide important insights into global gene regulation by these crucial Th2 enriched factors.

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