Transcriptional Control of CD4+ T Lymphocytes

Peter C.J. Janson
TRANSCRIPTIONAL CONTROL OF CD4+ T LYMPHOCYTES

Peter C.J. Janson

Stockholm 2009
“I have opinions of my own, strong opinions, but I don't always agree with them.”

George W. Bush
ABSTRACT

The immune system can respond to various types of pathogens such as bacteria, viruses, tumors and parasites. The ability to provide protection against different types of pathogens partly relies on development of alternative T effector cells with separate effector functions. The discovery of new T helper subsets, provide insight to the great flexibility of the immune system, and raises questions about how alternative cell fates are regulated. Epigenetic regulation of genes, i.e. modulations to chromatin that affects transcription in a heritable manner without affecting primary DNA, has proven essential for the regulation of alternative T helper cell development. Many aspects of epigenetic regulation in T cells is however still unexplored, especially in the human immune system as most studies regarding T cells and epigenetics are performed with murine T cells.

The aim of this thesis was to explore epigenetic and transcriptional mechanisms involved in regulating differentiation and homeostasis of CD4+ T lymphocytes. Furthermore, the aim was to investigate if altered epigenetic regulation can be observed in clinical situations associated with dysregulated CD4+ T lymphocytes.

Initially, we investigated the methylation profile of cytokine and transcription factor loci known to be important for CD4+ cell differentiation and/or effector function. We show that complete demethylation of the FOXP3 promoter is only observed in committed T regulatory cells, whereas non-regulatory T cells exhibiting transient FOXP3 expression are partially methylated at the FOXP3 promoter. Furthermore we describe interspecies differences in regulation of IFN-γ expression, where the human IFNG gene is not ‘poised’ for rapid onset of IFN-γ expression. When investigating IFNG methylation in tumor infiltrating lymphocytes, we show that these cells are subjected to immunosuppression mediated by CpG hypermethylation of the IFNG promoter. This thesis also includes development of a method for analysis of commitment to any of the known CD4+ T cell subsets; Th1, Th2, Treg and Th17. When using this method to profile the CD4+ T cell population in autoimmune diseases we observe deviations from normal immune homeostasis, demonstrating how epigenetic regulation of T cell effector fates has great relevance for clinical immunology. Finally, we show that a micro-RNA, miR-155, regulates T cell proliferative responses by targeting the cytotoxic T lymphocyte antigen 4 (CTLA-4), a negative regulator of immune responses.

In conclusion, the work presented here highlights the importance of transcriptional control in development and homeostasis of CD4+ T lymphocytes.
LIST OF PUBLICATIONS


III. Peter C.J. Janson, Ludvig Linton, Emma Ahlén, Per Marits, Michael Eberhardson, Rolf Ohlsson, Fredrik Pichl, Vivianne Malmström, Ola Winqvist. Profiling of Autoimmune CD4+ T Cells by Epigenetic Immuno Lineage Analysis. *Submitted Manuscript


Publications not included in the thesis

### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activating protein 1</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
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<tr>
<td>CNS</td>
<td>Conserved noncoding sequence</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>CTLA</td>
<td>Cytotoxic T lymphocyte antigen</td>
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<tr>
<td>CTCF</td>
<td>cAMP response element binding protein</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FOXP3</td>
<td>Forkhead /winged-helix protein</td>
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<tr>
<td>GATA3</td>
<td>GATA-binding protein 3</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HAT</td>
<td>Histone acetyl transferase</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>LCR</td>
<td>Locus control region</td>
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<tr>
<td>MBD</td>
<td>Methyl-CpG-binding domain</td>
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<tr>
<td>miRNA</td>
<td>Micro-RNA</td>
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<tr>
<td>NF</td>
<td>Nuclear factor</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>Ms-SNUPE</td>
<td>Methylation-sensitive single nucleotide primer extension</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cell</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>SFMC</td>
<td>Synovial fluid mononuclear cell</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>SLN</td>
<td>Sentinel lymph node</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>T-bet</td>
<td>T-cell specific T-box transcription factor</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TGF</td>
<td>Tumor growth factor</td>
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<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocyte</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>TSS</td>
<td>Transcription start site</td>
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<td>UTR</td>
<td>Untranslated region</td>
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1 INTRODUCTION

The immune system mediates protection against potentially harmful pathogens and can be divided into the innate and the adaptive arm. The innate immune system is found in all classes of plant and animal life, and provides the first line of defense through physical barriers, antimicrobial peptides and germ-line encoded receptors. Receptors of innate immunity recognize evolutionarily conserved molecules and patterns in microbes (pathogen-associated molecular patterns; PAMPs), and cells of the innate arm respond quickly upon receptor engagement by phagocytosis, or secretion of inflammatory mediators.

The adaptive immune system, provided by lymphocytes, evolved in jawed vertebrates and mediates specificity and memory to the immune response. Through the diversity of T- and B-cell receptors, virtually any foreign peptide and/or structure can be recognized. The relatively few T- and B- cells, capable of recognizing any unique antigen, present a problem in achieving sufficient magnitude to the adaptive immune response. This problem is solved through the clonal expansion of antigen recognizing lymphocytes, during which long-lived memory cells are generated, capable of responding rapidly and efficiently upon antigen re-exposure.

As an additional level of flexibility to the immune system, clonal expansion is associated with development into alternative T cell effector lineages with unique cytokine expression profiles which subsequently triggers a unique set of effector mechanisms. The known set of T cell subtypes increases steadily, and brings the questions of what factors control alternative T cell fate differentiation, and how these factors are related to postulated T cell imbalances in situations such as allergy and autoimmunity? Gene expression patterns are maintained in differentiated cells by epigenetic processes, i.e. modifications to chromatin that regulate gene expression without affecting the primary DNA sequence, thus the riddles of alternative T helper cell fates are likely to be answered using an epigenetic approach.

Whereas epigenetic processes control DNA accessibility and cell fate integrity, additional mechanisms serve to regulate gene expression at a post transcriptional level. Here, micro-RNAs have emerged as important regulators of cell development and function, with great implications for control of gene expression during development and activation of immune cells.

Epigenetic regulation of gene expression and control of gene expression by micro-RNAs (miRNA) is the general theme of this thesis. My work has been focused on DNA methylation involved in controlling cytokine and transcription factor expression in CD4+ T cell effector lineages, and miRNA mediated control of immune homeostasis. Using epigenetic tools, I have also analyzed clinical situations which could be attributed to an imbalance in CD4+ T lymphocyte homeostasis.

In the following pages I will present an overview of T lymphocyte development and function, followed by an introduction to the mechanisms involved in epigenetic control, as well a miRNA mediated gene expression in T cell immunity.
1.1 MATURATION OF T LYMPHOCYTES

T cells originate from a common lymphoid progenitor cell in the bone marrow. The common lymphoid progenitor also gives rise to B cells as well as natural killer (NK) cells. Whereas NK cells are considered to belong to the innate immune cells, B cells and T cells are mediators of adaptive immunity. The principal function of a B cell is antibody production, which provides humoral immunity and recognition of soluble antigens. T cells instead recognize antigen derived peptides presented by cells in association with the major histocompatibility complex (MHC). There are two types of T cells, CD8+ cytotoxic T cells, and CD4+ T helper cells. Cytotoxic T cells destroy virally infected cells and tumor cells whereas T helper cells provide “help” to B-cells, macrophages and cytotoxic T cells by augmenting antibody production and increasing the cytotoxic capacity.

1.1.1 Intrathymic selection and TCR rearrangement

T cell maturation occurs in the thymus in a step-wise, highly ordered manner. The fundamental basis of adaptive immunity lies in the diversity of T cell receptors. The magnitude of T cell receptor diversity is immense, with approximately $10^8$ unique specificities. High diversity of T cell receptors is accomplished by the joining of V, D and J gene segments in the TCR locus during T cell maturation. To further increase the level of diversity the individual segments are imprecisely joined, and random nucleotides are inserted in the junctions between the different segments. A fully rearranged T cell receptor (TCR) consists of either an $\alpha$ and a $\beta$ chain, or a $\gamma$ chain together with a $\delta$ chain. The rearranged TCR chains are non-covalently associated with a CD3 polypeptide which together constitutes the intact T cell receptor complex.

T cell precursors arriving to the thymus lack most of their cell surface receptors, and their T cell receptors are un-rearranged. In the thymic environment, the arrangement is initiated almost simultaneously at the $\beta$, $\gamma$ and $\delta$ loci. A successful rearrangement of the $\gamma$ and $\delta$ loci leads to expression of an assembled $\gamma\delta$-TCR which delivers signals to the cell to continue along the $\gamma\delta$-pathway. A rearranged $\beta$-chain pairs with a pre-$\alpha$-chain, and signals through this complex is required for the subsequent rearrangement of the $\alpha$-locus. At this point, CD4 and CD8 co-receptors are expressed, and the TCR specificity for MHC-antigen binding is tested. Interaction with MHC class I molecules generates CD8+ cytotoxic T cells whereas MHC class II interaction gives rise to CD4+ T helper cells.

The process of TCR recombination also generates potentially harmful self-reactive T cells. Therefore, another crucial step in T cell maturation involves the removal of these auto-reactive cells. This is achieved through the process of negative selection where T cells that recognize self-peptide MHC with high affinity are eliminated. T cells that manage to avoid negative selection emigrate to the periphery as mature naïve T cells, capable of recognizing foreign antigens presented by antigen presenting cells (APCs) (1).

Naturally occurring T regulatory cells (Tregs) are generally believed to constitute a separate line of CD4+ T cells whose fate is already decided during the thymic maturation process. Tregs suppress immune responses and are
crucial in protection from autoimmunity. The signals that direct an immature T cell into Treg development in the thymus are not yet fully explored, however high affinity (but not high enough to result in elimination) of the TCR-MHC-self peptide interaction during negative selection is a potential mechanistic signal for Treg commitment (2-5).

1.2 CD4+ T CELL ACTIVATION IN THE PERIPHERY

In the periphery, CD4+ T cells encounter their antigens within the highly specialized environment of the lymph node. The most potent activator of T cells in the lymph nodes is the dendritic cell (DC). DCs capture antigens at the site of infection, and migrate to lymph nodes downstream of site of infection. Along the road towards a draining lymph node, the DC mature into a highly efficient antigen presenter, expressing co-stimulatory molecules which together with MHC molecules containing foreign peptides can activate a naïve T helper cell. The maturation process of DCs is enhanced by inflammatory mediators of innate immunity.

1.2.1 The pluripotent naïve CD4+ T cell

The naïve T helper cell has some level of pluripotent capacity, i.e. it can develop into one of several distinct T helper effector lineages, each with a separate cytokine secretion profile that promotes different effector functions. T helper type 1 (Th1) cells produce IFN-γ and provide protection against intracellular pathogens and cancer. T helper type 2 (Th2) cells are characterized by the cytokines IL-4, IL-5 and IL-13 which stimulate B cell antibody production and are involved in host defense against parasites (6). The recently discovered Th17 cells are involved in neutrophil mediated protection against extracellular bacteria and produce the cytokines IL-17A, IL-17F, IL-21 and IL-22.

The effector lineage choice of a naïve T cell is dependent on a series of events starting with the local cytokine environment and conditions of the TCR-MHC interaction during antigen recognition. At the site of infection, tissue specific factors, and pathogen-associated molecular patterns (PAMPs) shape the cytokine secretion profile of the APC during the migration of APC to secondary lymphoid organs. In this manner, both the type of pathogen involved and the site of infection influence the lineage commitment of the naïve T helper cell. Upon activation, a naïve T cell transcribes low levels of both Th1 and Th2 cytokines (7), and several rounds of replication are required before the transcription becomes entirely selective of Ifng or the cytokine genes of the Th2 locus. This initial latency represents the time required for upregulation of cell lineage specific transcription factors and chromatin remodeling of the cytokine loci. These events and the factors involved will be discussed in greater detail in the following sections.
1.3 CD4+ T LYMPHOCYTE LINEAGES

Cell lineage decision in T helper cells is influenced by the surrounding cytokine milieu at the site of antigen encounter. The intracellular signaling pathways downstream of cytokine receptors induce the expression of cell lineage specific transcription factors, with the ability to induce chromatin remodeling within their DNA binding regions. This section outlines the principal events and signaling pathways involved in T helper cell lineage decisions.

1.3.1 Th1

Th1 development is promoted by the cytokines IL-12 and IFN-γ, secreted by dendritic cells and macrophages in response to ingesting of bacteria. In response to binding of cytokines to cell surface receptors, Janus kinases (JAKs) become activated and phosphorylate tyrosine residues on the intracellular part of the cytokine receptors and thus create binding sites for Signal Transducers and Activators of Transcription (STAT) proteins. Upon binding, the STAT proteins are activated, dimerized and transported to the nucleus where they initiate transcription of cytokine inducible genes. IFN-γ receptor engagement activates STAT1, which in turn promotes transcription of T-bet – a central transcription factor that represents a developmental switch for the Th1 lineage (Figure 1) (8).

T-bet is considered a master regulator of Th1 development. This originates from the ability of T-bet to induce a Th1 phenotype in Th2 differentiated cells upon retroviral expression (9). T-bet executes several important functions including chromatin remodeling of the Ifng locus, upregulation of the IL-12 receptor and inhibition of Th2 development (9). Upregulation of IL-12Rβ2 by T-bet further reinforces the commitment to Th1 through IL-12 dependent activation of STAT3 and STAT4, which maintains T-bet expression and enhances IFN-γ production (8). In a recent report aiming at investigating the relationship between T-bet and STAT4 in Th1 development, it was shown that T-bet and STAT4 regulate individual subsets of genes, and sometimes regulates genes in a synergistic manner. Complete Th1 transition requires both STAT4 and T-bet, thus the relationship between these two transcription factors is not strictly linear (10). Furthermore, Th1 development also requires activation of Jak3 and STAT5, a signaling pathway independent of STAT1 and STAT4. Jak3 is required for signaling via the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Thus, at least three cytokine signals are required for proper Th1 development (11).

1.3.2 Th2

Development toward a Th2 phenotype is initiated by the presence of IL-4 during T helper cell activation. The source of this IL-4 is still unknown, but is believed to be either eosinophils or mast cells. IL-4 production from the T helper cell itself has been proven sufficient to drive Th2 effector responses in vivo. This observation, and the absence of known Th2 inducing cytokine release from DCs, suggests Th2 as default pathway with spontaneous development toward Th2 when levels of Th1 inducing signals are insufficient (12). However, non-cytokine instructive signals also exists which can induce a Th2 phenotype,
such as interaction with the Notch ligand Jagged present on DCs (13). IL-4 receptor engagement leads to activation of STAT6, which in turn upregulates the zinc finger transcription factor GATA3 (14). GATA3 is regarded as the Th2 counterpart to the Th1 transcription factor T-bet and it augments the production of the Th2 cytokines, IL-4, IL-5 and IL-13 (15). The basic-region leucine-zipper protein c-Maf is another transcription factor important for Th2 development. C-Maf promotes IL-4 expression by binding to the \( Il4 \) promoter region and mice lacking c-Maf are deficient in IL-4 production (16, 17).

### 1.3.3 Th17

Even though IL-17 production from T helper cells was observed many years ago, it was not until recently that Th17 cells were accepted as a separate T helper lineage, distinct from Th1 and Th2. Their existence as a unique T helper subset became evident in mouse models of experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA). In these models, deletion of IL-12 did not protect from disease development, whereas selective disruption of the subunits which constitute the cytokine IL-23 did. Thus, these autoimmune models, initially believed to be Th1 dependent, demonstrated IL-12 independency (18, 19). Since then, further evidence has demonstrated that Th17 cells comprise a functionally distinct population. In mice, Th17 development is not dependent on the “cell lineage switches” T-bet, GATA3 or FOXP3, nor does it require any of the Th1/Th2 associated STAT proteins (20). Instead, Th17 development is promoted by the transcription factor ROR\( \gamma_t \) (21). ROR\( \gamma_t \) is regulated by STAT3, and over expression of either STAT3 or ROR\( \gamma_t \) promotes IL-17 transcription (21, 22). It was recently shown that another orphan nuclear receptor, ROR\( \alpha \), also promotes Th17 differentiation in a similar STAT3 dependent manner (23). Although IL-23 is required for the expansion and activation of the memory/effector Th17 pool, it is not required for the initiation of Th17 development. Instead, IL-6 in combination with TGF-\( \beta \) induces Th17 development in mice (24, 25).

Distinction of the Th17 population in humans is less defined. Co-expression of IFN-\( \gamma \) and IL-17 has been observed in CD4+ cells, and although IL-22 and IL-26 expression is more prominent in Th17 cells, expression of these cytokines can also be detected in Th1 polarized human cells. In addition, the cytokines required for induction of IL-17 transcription in humans are not the same as in the mouse setting. In humans, IL-1\( \beta \) in combination with IL-23 induces IL-17 transcription, rather than IL-6 and TGF-\( \beta \) (26, 27).
Figure 1 Signaling pathways involved in CD4+ effector cell development. Th1 development is promoted by IFN-γ and IL-12, which activates STAT1 and STAT4 respectively. STAT1 induced T-bet promotes IFN-γ expression through chromatin remodeling of the IFNG locus. T-bet also induces expression of the IL-12R, which further promotes Th1 commitment through IL-12 induced STAT4 signaling. Runx3 and Hlx cooperate with T-bet to promote optimal IFNG transcription and to suppress Th2 development. Th2 development is induced by GATA3, activated by STAT6 in response to IL-4. GATA3 subsequently induces expression of C-maf and promotes IL-4 transcription by chromatin modulation of the Th2 cytokine locus. In addition, interaction with the notch ligand Jagged on DCs provides Th2 instructive signals, where the intracellular domain of the notch-receptor activates RBPJκ. RBPJκ binds directly to the IL-4 gene, and also activates transcription of GATA3. Th17 development is induced by TGF-β in the presence of IL-6. This causes activation of STAT3, which subsequently cooperates with TGF-β signaling to induce transcription of RORγt. In differentiated Th17 cells, IL-23 reinforces the IL-17 expression through STAT3 signaling. FOXP3 transcription in Tregs is induced by TCR ligand binding and co-receptor activation, which activates transcription factors such as NFAT, AP-1, CREB and ATF. TGF-β signaling and cytokine signaling through the γc-chain also induces FOXP3 expression when SMAD3 and STAT5 binds to regulatory elements in the FOXP3 locus.
1.3.4 Tregs and FOXP3

The most widely used marker for the Treg population today is the transcription factor Foxp3. Foxp3 expression is required for the development of Tregs and its expression is closely linked to a suppressive phenotype. Retroviral transduction or ectopic expression of Foxp3 in conventional murine T cells converts them to a regulatory phenotype (28, 29). Mutations in the human FOXP3 gene is responsible for the disease Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX) (30) and the Foxp3 mutant Scurfy mouse model displays a similar pathology involving dysregulated CD4+ T cell infiltration and activation (31).

1.3.4.1 Treg instructive signals

As mentioned in section 1.1, the Treg lineage is believed to constitute a separate T helper cell population whose fate is determined by the TCR specificity for self-antigens during thymic development. The observation that T cells of the effector arm and naturally occurring T regulatory cells have virtually non-overlapping TCRVβ repertoires in mice supports this theory (32, 33). However, other signals also contribute to the Foxp3+ Treg development in the thymus including CD28 co-stimulation and cytokine signaling via the IL-2Rβ receptor or the common γ-chain (γc) (34-36). Interestingly, whereas IL-2 is required for maintaining the Treg transcriptional identity in the periphery, it is dispensable for the induction of FOXP3 in the thymus (34), suggesting other γc signaling cytokines as FOXP3 inducers in the thymus. The requirements of co-stimulatory signaling for FOXP3 induction can be replaced by constitutive expression of STAT5, which signals downstream of γc. In addition, STAT5 over expression can override TCR instructive signals and initiate the Treg developmental program in T cells destined to become naïve T cells (37). Thus in the current model of thymic Treg development, TCR signals first induce cytokine responsiveness and the Treg developmental program is completed by STAT5-dependent cytokine signaling (37, 38). Tregs can also be induced from the same peripheral naïve T cell pool as the effector T helper cells. Presence of TGF-β during antigen recognition or exposure to the vitamin A metabolite retinoic acid can convert naïve T cells to Tregs (39, 40). Retinoic acid increases the expression and phosphorylation of SMAD3, thereby enhancing the Treg inducing effects of TGF-β (41). Stimulation under sub-immunogenic conditions has also been demonstrated to convert naïve T cells into Tregs (42). Tregs generated from naïve T helper cells are the so called induced T regulatory cells (iTregs), and manipulation and transfer of iTregs has been suggested as a potential therapy in the treatment of autoimmune conditions (43).

1.3.4.2 Human vs. murine Tregs

The origin of human T regulatory cells is debated. Long life span compared to mice, and continuous antigen exposure throughout life does not support a theory of Tregs generated in the thymus with a lifelong persistence without any de novo generation or expansion. Human in vivo experiments found Tregs to be highly proliferative compared to CD4+ memory or naïve cells. In addition, they
were highly susceptible to apoptosis and had short telomeres which would indicate a terminal state of differentiation. In contrast to the murine setting, TCRVβ repertoires of human Tregs share homology with the memory T cell pool. Thus, a subset of highly proliferative and differentiated memory T helper cells could be the source of the “naturally occurring T regulatory cells” in the human setting (44, 45).

In the murine setting, Foxp3 seems to be valid as a marker for the Treg population, both in the case of the thymically derived Tregs as well as peripherally induced Tregs. In humans however, there is accumulating evidence that conventional T helper cells undergo transient FOXP3 expression during activation (46, 47). The reason for this interspecies difference, and whether transient expression is associated with suppressive capacity, has been a matter of debate. The last year’s intensive research in the epigenetic regulation of FOXP3 in humans and mice has elucidated some of these disputes.

1.3.5 CD4+ effector lineage antagonism

The antagonistic properties of Th1 and Th2 development are well documented. Besides reinforcing their own expression, T-bet and GATA3 repress the development of the reciprocal effector lineage through interference of signaling pathways. T-bet directly interacts with GATA3, thereby inhibiting the binding of GATA3 to target regions in the Th2 locus (48). It was recently shown that T-bet requires co-expression of the transcription factor Runx3 to efficiently silence IL-4 production. T-bet itself induces the Runx3 expression in Th1 differentiating cells, and their cooperate binding to a silencer in the If4 locus represses Th2 differentiation, whereas binding to the Ifng locus further enhances Ifng transcription (49). T-bet also induces the expression of the homeobox transcription factor Hlx, and the two transcription factors promote optimal Ifng transcription in a synergistic manner (50). Hlx over expression results in IL-4Rα down-regulation, thus one possible mechanisms by which Hlx promotes Th1 differentiation is by interference with the IL-4 signaling (51). GATA3 is able to repress Th1 differentiation, but instead of binding to the Ifng locus, GATA3 acts through down regulation of STAT4 (52, 53). In addition to the intranuclear tug o’ war of the transcription factors, the paracrine effects of Th1 or Th2 cytokine release from the cells promote differentiation of neighboring T helper cells to form a corporate response.

Recent progress have begun to untangle the interplay in the transcription factor network of the more recently identified CD4+ T cell populations; Tregs and Th17. GATA3 expression is a potent inhibitor of Treg development (54), where GATA3 binds directly to the FOXP3 promoter thereby reducing the transcriptional activity. Th1 promoting cytokines such as IFN-γ and IL-27, however, do not oppose induction of FOXP3 but promotes FOXP3 transcription through binding of STAT1 to the FOXP3 promoter (55). Furthermore, FOXP3 inhibits Th17 development, through interaction with RORγt (56, 57).
1.4 EPIGENETIC MODIFICATIONS

The eukaryotic nucleus harbors genetic information consisting of several billion nucleotides. In order to fit all this genetic information into a nucleus, a few microns in size, DNA is tightly packed onto nucleosomes. The nucleosome consists of ~146 base pairs of DNA wrapped around four histone pairs (H2A, H2B, H3 and H4). Nucleosomes are further subjected to a higher order of packing into dense chromatin fibers. This tightly packed structure is however virtually inaccessible for the transcriptional machinery and a prerequisite for transcription of a certain gene segments is the structural alteration of the gene loci in question. Nucleosome repositioning, posttranslational modification of histone tails and methylation of CpG dinucleotides constitute different levels of chromatin modifications, which ultimately affect DNA accessibility. These structural alterations occur in a highly ordered fashion, where selective modifications allow for tissue specific expression and heritability; two hallmarks of epigenetic regulation. The following subsections discuss the general principles and effects of epigenetic modifications.

1.4.1 Posttranslational modifications to histones

Posttranslational histone modifications include acetylation, methylation; ubiquitylation, phosphorylation and SUMOylation. To further increase the complexity, many of the modifications occur multiple times at the same residue of a histone tail. The complex language of histone modifications is a subject of continuous research, and is not yet fully understood. However, certain types of modifications are more frequently observed in either silent (heterochromatin) or active chromatin (euchromatin) respectively.

Acetylation is the most frequently studied histone modification and is usually localized to active chromatin. Methylation, on the other hand, plays a dual role as it is important in both transcription and repression, depending on where the methyl group is situated. Trimethylation of lysine four in histone H3 (H3K4me3) for instance, is associated with transcriptional activity. By contrast; trimethylation of lysine nine (H3K9me3) or lysine 27 (H3K27me3) is a modification found in heterochromatin (58).

Two mechanisms by which histone tail modifications influence transcriptional accessibility have been suggested. Acetylation brings negative charge to histones, thereby increasing the repelling forces between the nucleosomes and the DNA which opens up the chromatin structure. Secondly, other modifications, such as methylation permit binding of protein complexes with chromatin modulating properties without affecting the charge.

Histone acetylation is flexible, where histone acetyltransferases (HATs) and histone deacetylases (HDACs) catalyzes the attachment and removal of acetyl groups. Histone methylation, on the other hand, has been interpreted as a more stable modification than acetylation. The variable nature of certain histone modifications has raised questions as to their true epigenetic nature - i.e. whether they are truly heritable and not just a result of transcriptional activity (58).
1.4.2 DNA methylation

DNA methylation occurs predominantly on CpG dinucleotides and is associated with transcriptional repression. CpG dinucleotides are often found accumulated in conserved regulatory regions (CpG islands) demonstrating their functional importance. (59). DNA methylation as a preserver of cellular identity is more rigid than histone modifications. Methylation pattern is maintained with high fidelity through cell divisions by the recruitment of maintenance methylase 1 (Dnmt1) to the DNA replication fork, where methyl groups are added to the newly synthesized daughter strand, using the mother strand as a template.

Demethylation occurs either by passive demethylation during replication if the level of maintenance methylase is insufficient to preserve the methylation pattern of the parental cell, or through the process of active demethylation where a not yet identified enzyme demethylates DNA independently of cell division. Two situations are known to involve active demethylation in T cells. At the onset of IL-2 production from T cells, the IL-2 promoter becomes demethylated within 20 minutes of stimulation even in the presence of agents that block cell cycle progression (60). Similarly, memory CD8^+ T cells rapidly produce large amounts of IFN-γ and, in contrast to T helper cells, do not require cell division to demethylate the Ifng promoter region (61). Silencing of previously active DNA regions is mediated by the de novo methylases Dnmt3a and Dnmt3b. This process does not require cell division.

Methylation of CpGs can repress transcription either directly by inhibiting the binding of transcription factors and/or co-activators to regulatory elements, or by secondary effects through the recruitment of histone modifying complexes to methyl binding proteins (62). MBD2 and MeCP2 are two such proteins that recognize methylated DNA, and interact with one of the co-repressor complexes NuRD/Mi-2 or mSin3A. Subsequently, these co-repressors recruit histone deacetylases (HDACs) that prevent the accumulation of permissive histone modifications. Chromatin modulating complexes can also recruit DNA methyltransferases, thus there exists a bidirectional interplay between DNA methylation and histone modifications (63).

1.4.3 Distal regulatory elements

Transcriptional activity of a gene is influenced by neighboring regulatory gene elements. Cis acting regulatory elements include enhancers, silencers, insulators and locus control regions (LCRs). These regulatory regions can exert their regulatory effects over long distances by chromatin loops, thereby coming in close proximity to the promoter region.

Enhancers positively regulate gene expression and contain binding sites for transcription factors. Enhancers can mediate tissue specific expression to a gene, through the binding of transcription factors sites required for maximal transcriptional efficiency. Silencers have opposite effects as compared to enhancers by binding repressors; transcription factors with negative effects on transcription. Silencers and enhancers share many features, such as the ability to mediate effects over long distances, independently of orientation (64).

Insulators, or boundary elements, are required for the transcriptional integrity of a gene segment. By blocking the communication between enhancers
and promoter regions or by preventing the spread of suppressive histone marks from nearby chromatin, insulators protect genes from the influence of neighboring gene segments. CCCTC-binding factor (CTCF) is a major protein involved in insulator and boundary element activity, and is so-far the recognized protein with enhancer blocking properties (65).

Finally, locus control regions (LCRs) are clusters of regulatory elements containing enhancers, silencers, insulators etc. These gene segments can, independently of orientation and location, enhance the transcription of specific genes in a copy dependent manner (64, 66).

1.5 EPGENETIC REGULATION OF CD4+ T EFFECTOR LINEAGES

Following sections addresses specific epigenetic modifications at genes involved in development of Th1, Th2, Tregs and Th17 cells. Since my work has mainly revolved around the epigenetic regulation of the IFNG and FOXP3 genes, the main focus of the following section will be on the Th1 and T regulatory cells.

1.5.1 The Ifng locus

Throughout the course of evolution, non-coding DNA regions with functional significance are preserved. Thus a general approach to identify potential cis regulatory elements is to find conserved noncoding sequences (CNSs) and characterize these regions in reporter constructs, in terms epigenetic modifications and/or binding of transcriptional complexes. The CNS regions are usually given suffixes that indicate their position in kilobase pairs relative the transcription start site (TSS).

The first reports describing regulatory elements in the Ifng region dates back more than 10 years, when a minimal region upstream of the Ifng TSS displayed reporter activity in the CD4+ leukemia Jurkat cell line (67, 68). This promoter region was found to be differentially methylated in murine Th1 and Th2 clones and treatment of Th2 clones with the demethylating agent 5-azacytidine (5-aza) resulted in IFN-γ expression (69). The proximal promoter region was shown to contain binding sites for several transcription factors including nuclear factor of activated T cells (NFAT), nuclear factor κB (NF-κB) and cAMP response element-binding protein activating transcription factor 1 (CREB-ATF1) (reviewed in (70)). However, no elements were yet identified that conferred selective expression of IFN-γ to Th1 cells. In 2002, Suotto et al demonstrated Th1 selective expression of a promoter construct stretching -565 bp upstream of the Ifng TSS, where the Th1 specificity was assigned a T-bet binding site flanking the 5’end of this region (71). It is now well established that more distal elements are crucial for potent IFN-γ transcription.

The CNS1 region, also referred to in the literature as the 5’CNS or CNS-6, is located approximately 6 kb upstream of the Ifng TSS. This region binds NFAT as well as T-bet. During Th1 differentiation the CNS1 region becomes susceptible to cleavage by the endonuclease enzyme DNAse I. DNAse I
hypersensitivity is a characteristic feature of accessible gene segments and is often used to identify regulatory elements. During Th1 differentiation, permissive histone modifications appear at CNS1 along with DNAse I hypersensitivity, indicating this region as more accessible in Th1 cells (72). CNS2 (or CNS+18) is another evolutionarily conserved sequence element located ~18 kb downstream of Ifng (73), which also demonstrates Th1 associated DNAse I hypersensitivity and permissive histone modifications.

The CNS-22 region is thought to be involved in both activation and silencing of IFN-γ expression since the chromatin structure of this region is permissive in both Th1 and Th2 cells. This region shows strong T-bet dependent enhancer activity as compared to other upstream elements in reporter assays, and deletion of this region in a transgenic reporter system resulted in loss of Ifng reporter signal (74, 75).

Schoenborn et al (75), performed a large scale epigenetic profiling of the Ifng locus and identified several additional conserved noncoding sequences (CNS-56, -54, -34, +29, +46 and +55). CNS-6 and CNS+29 displayed T-bet independent enhancer activity, whereas other regions only enhanced expression in the presence of T-bet (CNS-22 and CNS-34). The distal elements (CNS-54 and +46) function mainly as boundary elements, probably in order to protect the Ifng gene from the influence of neighboring genes and/or their regulatory elements. The more proximal regions (CNS-34, CNS-22, CNS-6, and CNS+29) exhibit enhancer properties, and some possibly have dual enhancer and boundary element function (CNS-34 and CNS-22).

1.5.1.1 Opening the Ifng locus in Th1 cells

CNS elements of the Ifng gene are subject to epigenetic modifications that further enhance the transcription and increase accessibility of the locus in Th1 cells. DNAse I hypersensitivity sites appear at the CNS regions during lineage commitment to Th1 (72, 73, 75, 76), whereas the hypersensitivity sites that appear in Th2 cells are more diffusely distributed and found adjacent to, but not within the CNS regions. This could reflect nucleosome sliding to expose important regulatory elements in Th1 developing cells, whereas uneven and diffuse distribution of nucleosomes results in an inaccessible chromatin structure in Th2 cells. The nucleosome repositioning at the Ifng promoter requires STAT4, but not T-bet (77). Furthermore, permissive histone modifications accumulate at the conserved regions during Th1 differentiation, (72-75, 78, 79) and the low levels of repressive modifications present in naïve T cells are lost (75). The establishment of histone acetylation requires both STAT4 and T-bet, which partially explains why mice deficient of these transcription factors fail to mount a proper Th1 response (78).

During commitment toward the Th1 phenotype, some of the distal elements such as CNS-6, CNS-54, CNS+18 as well as the introns of Ifng becomes demethylated. This process is believed to amplify IFN-γ expression at later stages during Th1 commitment. The regions CNS-6 and CNS+18 are of special interest since they are positioned proximally to the Ifng promoter in differentiated Th1 cells (80), presumably through the formation of chromatin loops.
1.5.1.2 Silencing of Ifng in Th2 cells

Th2 development requires silencing of IFN-γ expression, where establishment of repressive marks at the Ifng gene play a crucial role. The initially hypomethylated CpGs of the promoter are rapidly subjected to de novo methylation, where the conserved -53 bp position (relative the TSS) has rendered special interest (81). Methylation of the -53 bp site causes dislocation of the Activating Transcription Factor 2 (ATF2)/C-Jun and CREB transcription factors from the promoter, and subsequently silences Ifng transcription. In contrast to the promoter region, distal elements of the Ifng locus remain hypomethylated in Th2 differentiated cells, including CNS-34, CNS-22, CNS+29 and CNS+46, thus DNA methylation at these sites is not likely to be of significance for the silencing of Ifng transcription in Th2 cells.

After about 2-3 cell divisions in Th2 polarizing conditions, a developing T cell loses its ability to express IFN-γ, even if the exterior milieu is changed to a Th1 promoting one (7). This inability to express IFN-γ is explained by loss of permissive and introduction of repressive chromatin modifications across the Ifng locus (75, 82). STAT6 dependent establishment of methylated H3K27 is one such repressive marker (79). Even though repressive marks have been established, imposed expression of T-bet in terminally differentiated Th2 cells can induce re-expression of IFN-γ (9). This phenomenon can be explained by T-bet binding to the IFNG promoter in collaboration with C/EBPβ, which causes dislocation of the co-repressor mSin3a (83).

1.5.2 The Th2 locus

The Th2 cytokine locus, containing the Il4, Il5 and Il13 genes, has been extensively characterized with respect to regulatory sequences and epigenetic mechanisms involved in the control of gene transcription. As in the IFNG locus, regions of regulatory importance within the Th2 locus have been identified both through analysis of sequence conservation and DNAse hypersensitivity as well as functional analyses.

The conserved noncoding sequence CNS1, situated between the Il4 and Il13 genes 5' of the Il4 TSS, contains the Th2 specific DNAse hypersensitivity sites HSS1 and HSS2 (84, 85). This region binds GATA3 and has established enhancer properties (86). Deletion results in reduced numbers of Th2 cytokine producing cells (84, 87) demonstrating its importance for Th2 development. Another evolutionary conserved sequence, CNS2, is located 3' of Il4 (76). It overlaps with the Th2 associated DNAse hypersensitivity site (DHS) HS5, which together with the adjacent site, HS5a, show strong enhancer properties (86, 88). HS5a is a Th2 specific, activation dependent site which binds the transcription factors GATA3 and NFAT (88). Deletion of these sequences results in reduced IL-4, IL-5 and IL-13 expression in Th2 and mast cells (89).

Upstream of HS5 and HS5a lies HS4, a hypersensitivity site present in naïve, Th1 as well as Th2 cells (76). This site coincides with HM1, a highly conserved region, which reduces Il4 promoter activity in reporter assays. This region therefore is believed to function as a repressor or silencer of IL-4 expression (86, 90). In addition, Th1 differentiated cells from HS4−/− mice
displayed aberrant IL-4 expression and were more susceptible to *Leishmania major* infection than their HS4<sup>wt</sup> littermates, despite unaffected IFN-γ production (90).

The Th2 LCR is located at the 3’ end of the *Rad50* gene. In coherence with the definition of an LCR discussed above, this region confers copy number dependent, orientation independent and tissue-specific expression (91). Four DHSs have been identified within this region, of which the RHS7/LCR-C site has been most extensively studied.

The regulatory sequence elements above are subject to epigenetic control on several levels during T helper differentiation. Similar to the *IFNG* locus during commitment towards Th1, permissive histone marks are established at the Th2 cytokine locus during differentiation towards Th2 (92-94).

DNA methylation also plays an important role in the control of Th2 cytokine expression during helper T cell development. When murine naïve T cells are activated under Th2 polarizing conditions, two phases of demethylation occur at the *Il4* locus. Initially, the 5’ end of the *Il4* gene and areas within the *Il4-Il13* intergenic region become demethylated, followed by extended demethylation of the *Il4* gene over 1-2 weeks (95).

Studies of the human Th2 cytokine locus have revealed several differences in epigenetic regulation compared to the murine setting. The DNA of the *IL4* promoter in human T cells is predominantly methylated in naïve, Th1 as well as Th2 differentiated cells, in contrast to mice where this region instead is demethylated (95, 96). Furthermore, the demethylation that occurs across the locus during Th2 polarization is not as widespread as in mice and localized to the DHSs in humans. Th2 associated demethylation at CNS1 is not pronounced, however a partial Th2 specific demethylation of the CpGs within intron 2 of *IL4* and the *IL13* promoter region has been observed (96).

### 1.5.3 The poised formation theory

Naïve T cells produce low amounts of IFN-γ and IL-4 shortly after TCR engagement (7). The ability to rapidly initiate cytokine transcription is believed to be mediated by a ‘pre-poised’ chromatin architecture of the *Ifng* and Th2 loci in naïve T cells. The *Ifng* and Th2 loci in naïve CD4<sup>+</sup> T cells lack the histone modifications associated with transcribing gene segments (75, 92, 93). However, the DNA of the promoter regions of *Ifng* and *Il4* is demethylated (75, 81, 94, 97), and readily accessible for the transcriptional machinery. Additional regulatory elements of the *Ifng* locus are also demethylated in naïve CD4<sup>+</sup> cells (CNS-34, -22, +29 and +46), which would implicate involvement in the early events of IFN-γ gene expression. The *Ifng* gene and Th2 locus are also juxtaposed, which might allow the regions to compete for a limited supply of transcription factors during initial cell lineage decisions. This pre-set chromatin architecture is believed to facilitate the choice of Th1 vs. Th2 at the initiation of antigen encounter (80).
1.5.4 Long range interactions at the cytokine loci

During Th1 differentiation of naïve T cells, the interchromosomal interaction between the \textit{Ifng} and Th2 loci is lost and replaced by intrachromosomal interactions. At the \textit{Ifng} gene, loops are formed which mediate interaction between the promoter and the downstream enhancer CNS+18. CNS+18 binds T-bet and this communication could serve as a mechanism to increase IFN-\(\gamma\) transcription in a tissue specific manner (80).

The genes of the Th2 locus also participate in loop formations. The Th2 LCR plays a crucial role in this loop formation. The promoter regions of \textit{Il4}, \textit{Il5} and \textit{Il13} are found in close spatial proximity in CD4+ T cells, NK cells, B cells and fibroblasts, whereas the Th2 LCR only participates in NK cells and CD4+ T cells. The interactions of the promoters and the Th2 LCR become stronger in effector T cells and is dependent on the presence of GATA3 and STAT6 (98). In this manner, a ‘poised’ structure is formed where transcription is immediate upon recruitment of Th2 specific factors.

RHS7 knock-out mice have reduced promoter-LCR interaction in the Th2 locus and the expression of IL-4, IL-5 and IL-13 in T helper cells is severely impaired (99). Interestingly, the Th2 LCR is also required for the interchromosomal association between the Th2 and \textit{Ifng} loci, and loss of RHS7 causes delayed expression of IFN-\(\gamma\) in Th1 stimulated cells. Thus the transcriptional components directed from the Th2 LCR also seem to influence transcription of remote genes located on other chromosomes (80).

1.5.5 The \textit{FOXP3} locus

Considering the importance of FOXP3 in tolerance, surprisingly little is known about the molecular mechanisms underlying FOXP3 induction. There is great inconsistency in reports regarding FOXP3 expression in mice vs. men where human conventional T cells transiently express FOXP3 upon stimulation. In addition, there is disagreement in human and murine reports regarding the ability TGF-\(\beta\) to induce a Treg phenotype. These differences raise questions of which components that regulate FOXP3 expression in different settings.

The human \textit{FOXP3} promoter region was first described by Mantel and colleagues. The promoter was found to contain binding sites for NFAT and activator protein 1 (AP-1) (100), and disruption of these elements reduced the \textit{trans} activation of the promoter after TCR stimulation. Histone H4 acetylation analysis revealed that the promoter region is accessible for the transcriptional machinery in naïve and activated CD4+ cells as well as CD25\textsuperscript{hi} T regulatory cells. The observation that a minimal promoter construct, containing binding sites for well established mediators of T cell activation, showed reporter activity when transfected into CD4+ cells is in agreement with the observation of FOXP3 transcription in human CD4+CD25\textsuperscript{lo} T cells upon activation. The promoter region also binds STAT5 in murine Tregs, which agrees with the finding that STAT5 expression promotes Treg development in the thymus (36).

The TGF-\(\beta\) sensor, or CNS-2, is located approximately 2 kb downstream of the transcription start site. The link between TGF-\(\beta\) and Foxp3 induction is well established, and evidence that SMAD3 binds to CNS2 upon TGF-\(\beta\) receptor engagement provided mechanistic insight to the effects of TGF-\(\beta\) on
FOXP3 induction (101). Whereas SMAD3 binding is required for chromatin remodeling of CNS2, maintenance of activity in this region is dependent on the binding of NFAT.

Mice studies of the epigenetic regulation of Foxp3 have focused on a conserved element in intron 1 of the Foxp3 gene, the Treg-Specific Demethylated Region (TSDR), which shows transcriptional activity and differential methylation between CD4+CD25− and T regulatory cells (102). This region binds the cyclic AMP response element binding protein (CREB) in a methylation dependent manner, and it also binds STAT5 in Tregs (103). Furthermore, demethylation occurs at intron 1 in CD25hi cells in the presence of TGF-β (104). However, the TGF-β induced demethylation was not as pronounced as in the freshly isolated CD25hi population (102).

The importance of methylation dependent control of FOXP3 expression has also been demonstrated in experiments using drug-mediated DNA methylation. 5-aza treatment induces expression of Foxp3 in murine CD25hi cells, similar to the presence of TGF-β (104). In a recent report the effects of 5-aza and TGF-β in terms of demethylation and Foxp3 expression stability was compared (105). Interestingly, cells treated with demethylating agents showed greater stability in their Foxp3 expression profile compared to the cells stimulated with TGF-β. This suggests that additional chromatin modulating agents apart from the ones downstream of TGF-β signaling are needed for the complete conversion of conventional T cells to committed Tregs. The Foxp3+ fraction treated with 5-aza also displayed more selective demethylation at the regulatory region located in intron 1 compared to the TGF-β treated cells, which suggests that demethylation of the TSDR region determines stability of FOXP3 expression.

1.5.6 The IL-17 locus

Epigenetic events that lead to selective expression of the cytokines secreted from Th17 cells are just beginning to be explored. There are reports that Th17 polarization is accompanied by permissive histone modifications at the Il17 promoter and intronic regions, implicating epigenetic regulation as an important factor also in Th17 differentiation (106). Interestingly, Th17 differentiated cells requires continuous exposure to TGF-β in order to retain IL-17 expression. Upon removal of TGF-β, or addition of IL-12, T-bet and STAT4 is re-expressed, even in long term polarized Th17 cells (107), thus there seems to be a high level of plasticity in the Th17 population. The ability of Th17 cells to shift phenotype could be explained by the presence of bivalent histone modifications in the promoters of T-bet and STAT4. STAT4 and T-bet are marked by both permissive (H3K4me3) and suppressive (H3K27me3) histone modifications in Th17 differentiated cells, and this type of bivalent modification is thought to be associated with a ’poised’ formation, allowing rapid induction upon change of environmental stimuli (108). These observations suggest a weak epigenetic integrity of the Th17 population, at least in terms of histone modifications. As previously mentioned, DNA methylation is considered a more stable epigenetic modification compared to histone modifications, and the DNA methylation status of IL-17 producing cells has not yet been investigated.
1.6 TRANSLATIONAL CONTROL BY MICRO-RNAs

Whereas histone and DNA modifications serve to regulate DNA accessibility, and thereby transcriptional activity, additional mechanisms regulate gene expression at a post transcriptional level. Micro-RNAs (miRNAs) function through binding to 3′ untranslated regions (3’UTRs) of transcripts, thereby target them for degradation or translational repression. Today, more than 700 miRNAs have been identified in the mammalian genome, and they are known to regulate a wide array of physiological processes such as development, differentiation and metabolism. MiRNAs are processed from endogenous transcripts by the enzymes Drosha and Dicer into mature ~22 nucleotide RNA duplexes. In the cytoplasm a mature miRNA associates with the RNA-induced silencing (RISC) complex and a single strand of the miRNA acts as a target sequence for mRNA 3’UTR’s.

![Figure 2: Primary miRNAs (pri-miRNA) are transcribed from endogenous genes by RNA polymerase II or III. The pri-miRNA is cleaved by Drosha into precursor miRNAs (pre-miRNA) and exported to the cytoplasm by Exportin 5. Here, the pre-miRNA is further processed into a ~22 nt double-stranded miRNA duplex by the cytoplasmic RNase III enzyme Dicer. One strand is subsequently incorporated into the RNA-induced silencing complex (RISC). The mature miRNA can bind to the target messenger RNA by base pairing, causing inhibition of protein translation or degradation of the target messenger RNA.](image-url)
1.6.1 Micro-RNA mediated control of immune function

The importance of a proper miRNA mediated control in lymphoid cell lineages has been demonstrated in lineage specific knock out of the Dicer nuclease, responsible for the processing of endogenously derived pre-miRNAs into their mature form. Absence of dicer in B-cell progenitors, for instance, results in a developmental block at the pro- to pre-B cell transition (109). When dicer is deleted in T-cells, the net effect is a block of peripheral CD8+ cell generation, reduced proliferative capacity and increased apoptosis of the CD4+ T cell pool (110). Individual miRNAs and their target genes are today known to be involved in many aspects of immunity, including innate as well as adaptive responses. MiR-146, for instance is expressed by macrophages in response to lipopolysaccharide (LPS) and interferes with signaling from Toll-like and cytokine receptors (111). MiR-181 regulates B-cell development (112), as well as the sensitivity of T cell receptor signaling during the development and effector phase of T cells (113).

1.6.1.1 MiR-155

Several miRNAs have shown to be differentially expressed upon immune activation (114). One such miRNA, miR-155 is processed from the non-protein coding transcript of the bic-gene. MiR-155 expression becomes elevated upon B- and T-cell activation and is highly expressed in various types of B-cell lymphomas including diffuse large B-cell and Hodgkin lymphomas (115-119). In agreement with this, miR-155 transgenic mice develop B-cell malignancies (120). An intensive research during the last years has highlighted miR-155 as a central molecule in adaptive immune responses required for proper function of several immune-cells including B and T cells as well as dendritic cells. The bic deficient mice have a reduced T cell dependent class switched Ig-response, and fewer germinal centre B cells. In addition, T helper cells activated by dendritic cells from bic-deficient mice display limited proliferation and effector cytokine secretion, suggesting dysfunctional antigen-presentation and/or co-stimulation from DC’s (121, 122).

The observed reduction in germinal centre response and low level of class-switched immunoglobulins in bic-deficient mice can be explained by two identified targets of miR-155, PU.1 and activation-induced cytidine deaminase (AID) (123-125). PU.1 overexpression negatively regulates the IgG-switch response whereas AID is required for somatic hypermutation and affinity maturation of the B-cell receptor. Bic-deficient T helper cells show a bias towards Th2 when cultured under neutral conditions. This bias results from miR-155 binding to C-maf, which acts as a chromatin modulator of the Th2 cytokine locus (122). Furthermore, miR-155 seems to be important for maintaining homeostasis of the Treg population by downregulating SOCS1 protein expression (126). Elevated SOCS1 expression in miR-155 deficient Tregs is believed to result in an impaired IL-2 signaling pathway, and thereby a reduced proliferative potential of the Treg pool.

The recently verified targets of miR-155 have begun to explain some of the severe effects that are observed upon loss of bic expression. However, the substantially reduced T helper cell cytokine production and inability to induce T
cell dependent B cell responses in the absence of bic suggests additional, yet unidentified miR-155 regulated genes in T helper cells.

1.7 CLINICAL APPLICATIONS

The increasing evidence for epigenetic involvement in the immune system raises questions of how epigenetic changes contribute to immune dysregulation and immune diseases. Autoimmunity and allergy are two situations where the pathogenesis can (at least in part) be attributed to an imbalance of the T helper cell responses. Th1 and Th17 cells have for example been implicated in autoimmunity whereas allergic conditions are connected to the effector cytokines of the Th2 lineage. This section describes some clinical situations where epigenetic regulation of T helper cells is known to play a role in disease development.

1.7.1 Allergy

The prenatal environment is biased away from a potentially harmful cytotoxic Th1 milieu. The Th1 effector arm does not mature until early childhood, which suggests failure of Th2 silencing during maturation of the immune system as a mechanism of Th2 mediated disease development (127). To our knowledge, there are still no direct evidence of epigenetic dysregulation in CD4+ cells from atopics or allergic patients, however alterations in CpG methylation patterns has been observed in CD8+ cells from atopic individuals. Elevated IFN-γ levels have been reported in atopic individuals, especially among children, where hyperproduction of IFN-γ from CD8+ cells has been linked to a more severe symptomatology. Consistent with this, CD8+ T cells from atopic children display a hypomethylated IFNG promoter compared to healthy controls (128).

1.7.2 Autoimmunity

Dysregulated DNA methylation of CD4+ cells has been implicated in the pathogenesis of autoimmune disorders (reviewed in (129)). CD4+ T cells lose their ability to distinguish MHC-self peptides from non-self when treated with the demethylating agent 5-aza. The reason for this involves increased expression of LFA-1, an adhesion molecule that stabilizes the MHC-TCR interaction at the immunological synapse. Mice injected with isogenic CD4+ T cells treated with 5-aza develop a symptomatology similar to patients with SLE (Systemic lupus erythematosus), including anti-DNA antibodies and nephritis due to immune complex formation (130). Demethylated CD4+ T cells kill autologous macrophages in a MHC restricted manner. The cytotoxicity is mediated by heightened levels of LFA-1 and perforin (otherwise only used by CD8+ cytotoxic T cells and NK cells). This results in release and accumulation of apoptotic material, which triggers the production of anti-DNA antibodies from B cells (131). The B cells are activated due to over expression of CD70 and CD40 ligand as well as hyperproduction of IFN-γ and IL-4 by the CD4+ T cells (132, 133). Thus, demethylation of the promoter regions of adhesion molecules, co-stimulatory molecules and cytokines in CD4+ T cells results in
auto-reactivity that triggers release of apoptotic material. Failure of clearance due to the absence of macrophages induces autoantibody production that is further enforced by the elevated levels of co-stimulatory molecules and cytokines release from T cells. Interestingly, T cells from lupus patients display genome wide demethylation, elevated expression of LFA-1 and perforin as well as B cell activating properties, suggesting that similar mechanisms are involved as in the experimentally induced murine model of this disease. This is further supported by the observation that patients with active lupus show deficiency in ERK signaling, known to regulate the methyltransferase Dnmt1 (129).

1.7.3 T cells and tumors

The human immune-system has the capacity to identify and eliminate cancerous cells before they cause us any harm. As a result of genomic instability during malignant cell formation, tumors express mutated proteins which will be recognized by the immune-system as “non-self”. The concept of tumor recognition by the immune-system is referred to as the immune-surveillance hypothesis and there are several clinical observations in humans in favour of this. Patients receiving immunosuppressive drugs have an increased incidence of cancers (134), in addition; infiltration of lymphocytes is frequently seen in tumors and is also correlated to a favourable prognosis (135). Presence of Th1 associated cytokines and expression of Th1 promoting transcription factors is generally associated with a favourable prognosis (135), suggesting rejection of tumors as a Th1-mediated process. This is further demonstrated by the ability of STAT6 deficient mice to reject poorly immunogenic tumors (136), whereas STAT1 deficiency and thus absence of Th1 cells leads to tumor progression (137).

Tumors have several strategies to overcome the intrinsic immune defence which explains why tumors do develop also in individuals with functional immune-systems. Downregulation of MHC molecules by tumors represents one well documented immune-escape mechanism (138). MHC class I molecules are normally used by CD8+ cytotoxic T cells to recognize foreign peptides and kill infected cells, thus the absence of these molecules leads to impaired tumor recognition. In addition, the tumor microenvironment has immunosuppressive effects. High levels of TGF-β and IL-10 are found in malignancies and both cytokines are associated with inhibition of lymphocyte proliferation and effector cell function (139, 140). Moreover, a suppressive microenvironment can be achieved through the recruitment and upregulation of Tregs. Increased numbers of Tregs with immuno-modulatory functions have been observed in peripheral blood from cancer patients and among tumor-infiltrating lymphocytes (141, 142).
2 AIM OF THE THESIS

The overall aim of this thesis was to investigate epigenetic and transcriptional mechanisms involved in governing pro- and anti-inflammatory responses among CD4+ T lymphocytes.

The specific aims were:

1. To investigate the methylation status of the \textit{FOXP3} promoter and its relation to FOXP3 expression in Tregs and conventional T helper cells.

2. To investigate if silencing of the \textit{IFNG} gene by methylation CD4+ T lymphocytes could serve as a mechanism of tumor induced immunosuppression in CD4+ T lymphocytes.

3. To develop a method suitable for clinical samples to analyze epigenetic commitment of Th1, Th2, Th17 and T regulatory cells.

4. To investigate the function of miR-155 as a modulator of inflammation in CD4+ T lymphocytes.
3 METHODOLOGY

This section summarizes the methods used in paper I-IV. For additional details, please refer to Material and Methods in each paper.

Bisulphite sequencing (I, II, III)
DNA was bisulphite converted, and PCR amplification was performed using region specific primers. PCR products were cloned into a pCRH4-TOPOH vector, and transfected into E.coli bacteria. DNA isolated from individual clones was sequenced by sanger sequencing.

Combined Bisulphite Restriction Enzyme Analysis (COBRA) (I, II)
Genomic DNA was isolated and bisulphate converted. The FOXP3 promoter or IFNG 5’CNS enhancer were amplified by PCR using nonconjugated forward primers and 6-FAM conjugated reverse primers. PCR products were digested by restriction enzymes and analyzed by Gene Scan fragment analysis.

DNA isolation (I, II, III)
DNA was isolated from cells using either phenol:chloroform extraction or DNA isolation kit.

Enzyme-linked immunosorbent assay (II, IV)
Protein levels of IFN-γ, IL-4 and IL-5 in supernatants from cell cultures was detected by enzyme conjugated antibodies.

Flow cytometry (I, II, III, IV)
Intra and extracellular protein levels were measured using fluorochrome conjugated antibodies.

In vitro differentiation of T cells (II, III, IV)
Isolated naïve T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of cytokines and neutralizing antibodies that promote Th1, Th2 or iTreg differentiation.

Methylation sensitive Single Nucleotide Primer Extension (Ms-SNuPE) (III)
PCR products generated from bisulphite converted DNA were mixed in equal volumes. Ms-SNuPE reaction was performed with primers directed towards differentially methylated sites within the IFNG, IL-13, FOXP3 and IL-17A gene. Products from the Ms-SNuPE reaction were analyzed by capillary electrophoresis.
Molecular cloning (IV)
DNA fragments for cloning were generated by annealing of oligo nucleotides. Annealed DNA fragments were subsequently ligated into luciferase reporter constructs.

Quantitative Real-time PCR (I, II, III, IV)
RNA was isolated with Trizol reagent (Invitrogen) and subsequent cDNA synthesis was performed by reverse transcription. FOXP3, IFNG, IL-4, T-bet, GATA-3, IL-17A and miR-155 transcript levels were measured by PCR amplification using transcript specific primers or probes.

PCR amplification (I, II, III, IV)
Genomic DNA was amplified by region specific primers.

Statistical analysis
Mann-Whitney U-test or independent two sample t-test was used to compare populations of unpaired samples. Dependent t-test was used for the comparison of paired samples and Fisher’s exact test was used to calculate level of significance from bisulphite sequencing data.

T cell isolation (I, II, III, IV)
Peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) were isolated from patient whole blood or knee joint effusions by gradient centrifugation. CD4+ T cells were subsequently isolated from PBMC by magnetic sorting using bead conjugated antibodies.

T cell proliferation assay by [3H] incorporation (I, IV)
[3H] thymidine was added to cultures of activated T cells. Incorporation of thymidine was measured using a beta scintillation counter.

T cell proliferation assay by CFSE labeling
T cells were incubated with media containing Carboxyfluorescein succinimidyl ester prior to stimulation. Proliferation was evaluated by FACS.

Transfection (IV)
Hela cells or Naive CD4+ T cells were transfected with luciferase reporter constructs and/or miRNA precursors. Luciferase activity was measured using a luminometer.
4 RESULTS AND DISCUSSION

4.1 THE FOXP3 PROMOTER IS DEMETHYLATED IN T REGULATORY CELLS

T regulatory cells are crucial for maintaining peripheral tolerance and preventing chronic inflammation. The suppressive ability of Tregs is dependent on the transcription factor Foxp3, where loss of expression or mutations leads to fatal autoimmunity (28, 29). FOXP3 can also be expressed by conventional non-regulatory human CD4+ T cells upon stimulation, an observation which makes FOXP3 expression alone a questionable marker for the Treg population in humans. In order to see if epigenetic markers of the FOXP3 gene could be responsible for the various expression profiles of FOXP3 in regulatory vs. non-regulatory cells, we investigated the methylation status of the human FOXP3 promoter in sorted lymphocyte populations.

When performing bisulphite sequencing on freshly isolated CD4+CD25<sup>hi</sup> cells, we found them to be demethylated at the FOXP3 promoter. By contrast, CD4+CD25<sup>lo</sup> cells, which transiently expressed FOXP3 upon stimulation, displayed a semi-methylated pattern at the FOXP3 promoter (Figure 3). These results suggest that promoter demethylation mediate stability to the FOXP3 expression in T regulatory cells.

![Figure 3](image)

*Figure 3* Results from bisulphite sequencing of the FOXP3 promoter in (A) CD4+CD25<sup>hi</sup> T regulatory cells, (B) CD4+CD25<sup>lo</sup> T cells, and (C) CD19<sup>+</sup> B-cells. Bars indicate the percentage of methylated (black) and unmethylated (white) cells at each CpG position (specified on x-axis as relative the transcription start site).

Next, we wanted to investigate what happens with the methylation status in CD25<sup>lo</sup> T helper cells during transient expression of FOXP3. During activation of CD25<sup>lo</sup> T helper cells, the methylation status was monitored in both the CD25<sup>hi</sup> and CD25<sup>lo</sup> fraction. Initially, we observed a higher level of demethylation in the CD25<sup>hi</sup> (CD25<sup>lo</sup> derived) fraction. Over time, the methylation level of the CD25<sup>hi</sup> and CD25<sup>lo</sup> derived cells gradually converged at about 60%. Stimulated T regulatory cells however, maintained a completely demethylated profile (Figure 4). Sustained FOXP3 expression within a small portion of stimulated CD25<sup>lo</sup> cells have been interpreted as a subpopulation of antigen experienced cells capable of converting to the Treg phenotype in the periphery (46). This is in accordance with the findings discussed earlier, that memory T cells and Tregs are most likely generated from
the same peripheral source in humans (44, 45). If the subpopulation with sustained FOXP3 expression is associated with Treg commitment it would be expected to see demethylation of regulatory elements within the FOXP3 locus among these cells. However, at day 16 post stimulation, the CD25hi population did not display higher level of demethylation compared to the CD25lo population. Thus, complete conversion into Tregs does not occur upon in vitro stimulation of conventional CD4+ T helper cells, at least not during experimental conditions used in our study.

![Figure 4](image)
Figure 4 Methylation status of the FOXP3 promoter region during activation of CD4+CD25hi cells and CD4+CD25lo T regulatory cells. Both the CD25hi and CD25lo fraction derived fraction from stimulated CD25lo cells was analyzed. Shown are results from three separate donors.

Reports on suppressive capacity of induced FOXP3+ cells are conflicting, probably due to different protocols regarding time points and/or isolation purity of the activated potential suppressants. When we isolated the CD25hi and CD25lo fraction, 5 days post stimulation of non-regulatory T helper cells, we observed a higher level of suppressive capacity among the CD25hi fraction. Thus in our hands, a clean population of induced FOXP3+ cells seems capable of suppressing autologous activated responder cells. This transient suppressive ability could serve as an intrinsic speed brake during immune responses, in order to control excessive proliferation and restrain inflammation.

Presence of TGF-β during activation or stimulation under sub-immunogenic conditions is able to convert non-regulatory murine cells into Tregs in the periphery (40, 42). With this in mind we tested if various concentrations of anti-CD3, presence of TGF-β and/or IL-10, or repeated stimulations with anti-CD3 and anti-CD28 coated beads could induce FOXP3 promoter demethylation in human cells. Although high levels of FOXP3 expression was observed during all types of stimuli, a Treg-like methylation profile was not established in any of the culturing conditions, suggesting that the stimuli applied here does not fully convert CD25lo T helper cells into Tregs. Interestingly, TGF-β induces substantial demethylation in the promoter region during activation of murine naïve T cells and partial demethylation in the intronic region described by Floess et al (102, 104), suggesting TGF-β as a more potent inducer of Tregs in the murine setting. The differing effects of
TGF-β on methylation status in humans vs. mice agree with functional experiments where TGF-β induces functional Tregs in mice (40, 143) but only transiently upregulates FOXP3 expression in humans.

In contrast to our findings, Foxp3 promoter methylation increases upon activation of murine non- regulatory cells (104). This probably functions as a mechanism that prevents induction of Foxp3 upon stimulation of conventional murine T helper cells. Thus human FOXP3 induction in effector T helper cells seems to be regulated in a more promiscuous fashion compared to mouse.

**Conclusion:**
Complete FOXP3 promoter demethylation and stable FOXP3 expression is only seen in freshly isolated CD4+CD25hi Tregs. CD25lo non regulatory T cells with a semi-methylated promoter pattern transiently express FOXP3 and retain a partially methylated promoter pattern. Established culturing protocols for the conversion of non-regulatory cells into Tregs do not induce a Treg methylation profile at the human FOXP3 promoter. We conclude that methylation analysis of the FOXP3 promoter allows the distinction of truly committed Tregs.

### 4.2 IFNG HYPMETHYLATION IN TUMOR INfiltrATING LYMPHOCYTES MEDIATES IMMUNOSUPPRESSION

Members in our laboratory have previously shown that tumor infiltrating lymphocytes (TILs) are poor producers of IFN-γ, and have a low proliferative rate, suggesting altered Th1 effector mechanisms, induced by the immunosuppressive tumor micro-environment (144). Considering the importance CpG methylation in controlling the IFN-γ production in Th1 cells, we investigated the methylation pattern of the 5′CNS enhancer in tumor infiltrating CD4+ lymphocytes. Interestingly, we found TILs to be hypermethylated, with a similar pattern to in vitro differentiated Th2 cells (Figure 5A). By contrast, CD4+ lymphocytes isolated from the sentinel node were significantly less methylated at the IFNG enhancer. The sentinel node is the first lymph node to receive lymphatic drainage from the tumor and therefore also the location for tumor recognition and the initial clonal expansion of tumor reactive T cells. These results prompted us to investigate if inducing demethylation in TILs would restore the expression of IFN-γ. 5-aza treatment induced substantial IFNG expression from TILs (Figure 5B). Finally, we cultured Th1 differentiated cells with the colon cancer cell line CCL-221, and investigated IFNG and T-bet expression upon restimulation. Here, we saw that co-cultured cells expressed IFNG at lower levels compared to cells cultured alone. These observations suggest an altered cytokine profile of TILs, induced by the tumor, and regulated by methylation of cytokine loci as an additional tumor immune escape mechanism.
Most epigenetic analyses of the cytokine loci have been performed on murine cells. The demethylated status of the IFNG and IL-4 promoters in T cells from mice has added fuel to the theory of a ‘poised’ formation of the IFNG and Th2 loci, where cytokine transcription is immediate upon TCR engagement. In contrast to the mouse setting, we found the promoter region of IFNG in human naïve CD4+ T cells to be hypermethylated. In addition, IFNG expression was initiated simultaneously to DNA demethylation in Th1 stimulated cells which suggests that human naïve T helper cells are not poised for rapid onset of Th1 responses.

**Conclusion:**
CpG hypermethylation of the IFNG locus among tumor infiltrating CD4+ lymphocytes limits their ability to produce IFN-γ upon stimulation, and thereby contributes to immunosuppression. In addition, hypermethylation of the IFNG promoter in naïve human T helper cells reveal interspecies differences in the way by which IFN-γ production is regulated.

### 4.3 PROFILING OF AUTOIMMUNE CD4+ T CELLS BY EPIGENETIC IMMUNO LINEAGE ANALYSIS

The observation of CD4+ T cell specific demethylation of key cytokine genes and transcription factor loci in paper I and II inspired us to develop a method to profile the CD4+ effector lineage composition based upon epigenetic markers. For this purpose we adapted a protocol of the Snapshot Multiplex kit from Applied Biosystems, originally designed for single nucleotide polymorphism detection. In our method design, a single nucleotide extension reaction is performed to determine if a template strand contains methylated or...
unmethylated CpG dinucleotides (Figure 6). Probes were designed for differentially methylated sites in the FOXP3 and IFNG locus, identified in paper I and II. For the identification of Th2 committed cells, we designed a probe directed to one differentially methylated CpG in the IL-13 promoter (96). In order to determine if Th17 committed cells could be identified by methylation analysis, we performed bisulphite sequencing and compared the methylation status of the IL-17A promoter in IL-17A producing effector/memory cells naïve T helper cells. Interestingly, we found IL-17A producing cells to be demethylated compared to non-producing naïve T helper cells. In addition, adding 5-aza to cultures of CD4+ cells induced IL-17A transcription, demonstrating that IL-17A transcription is directly regulated by CpG methylation.

Figure 6 Experimental design of Epigenetic Immuno Lineage Analysis (EILA). Bisulphite treated DNA from CD4+ cells is amplified by locus specific PCR amplification. PCR products are mixed in equal volumes and used as template in a Methylation Sensitive Single Nucleotide Primer Extension (Ms-SNUPE) reaction. In the Ms-SNUPE reaction, a single nucleotide is incorporated to determine methylation status of the original DNA template. By using different lengths of the probes for each locus, individual products can be distinguished in the subsequent capillary electrophoresis.

Next, we used the EILA method to profile the CD4+ lymphocyte composition in PBMC’s of patients with Rheumatoid Arthritis (RA) and Multiple Sclerosis (MS) and compared the results to that of healthy controls. In patients with MS, we did not observe any significant difference in methylation level at any of the loci analyzed. However, patients receiving Natalizumab therapy displayed elevated methylation levels at the IL-17, FOXP3 and IL-13 loci, suggesting a silencing effect on cytokine loci of circulating CD4+ cells. Natalizumab inhibits the transmigratory capacity of CD4+ and one possible explanation to our observation is the reduced access to site of inflammation which follows Natalizumab treatment. When investigating CD4+ T helper cells from synovial fluid of patients with RA we found them to be committed towards Th1 and Tregs. Interestingly, in in vitro differentiated Th1 cells we also observed a substantial demethylation of FOXP3. Thus Th1 commitment seems to be accompanied with a certain level of iTreg co-commitment, an observation
which most likely reflects the ability of Th1 promoting conditions to induce FOXP3 expression and suppressive ability of iTregs in humans (55).

Given the strong link between Th17 cells and autoimmunity in experimental models of Rheumatoid Arthritis, we were surprised not to see a general Th17 phenotype in synovial infiltrating T cells. Commitment towards Th17 rather occurred on individual basis and in concert with Th1. Thus it is possible that the current mouse models used to mimic Rheumatoid Arthritis not accurately reflect the in vivo situation of human disease.

Upon analysis of ex vivo isolated effector/memory T cells, we observed substantial demethylation of the IL-13 gene, suggesting that circulating T helper cells are predominantly of a Th2 phenotype. Strikingly, in naïve T helper cells the IL-13 promoter was already partially demethylated compared to other analyzed loci, possibly reflecting a T helper cell default programming which is tilted towards Th2.

Conclusion:
The method that we used in paper III allows for accurate epigenetic profiling of CD4+ T cell effector lineages, and is useful to further illuminate T cell involvement and interplay of T effector subpopulations in clinical situations. In addition we found promoter methylation to regulate the expression of IL-17A in Th17 cells.

4.4 MIR-155 REGULATES T CELL PROLIFERATIVE RESPONSES

Paper I, II and III focused on heritable modifications to DNA which determines the transcriptional activity, i.e. the prerequisites for transcriptional activity and maintenance of cellular identity. However, there are additional levels of gene expression control, acting downstream of the transcriptional process. Gene expression control by miRNAs has received much attention during the past years and is involved in controlling gene expression during development and activation of immune cells. One micro-RNA, miR-155, has received a special interest in T cell immunology since it seems to be involved in T cell dependent immune responses and T helper effector cell polarization (122).

When we investigated miRNA expression levels in skin from atopic eczema patients compared to healthy controls using miRNA arrays, we found miR-155 to be elevated in lesional skin samples from the atopic eczema patients. Extrinsic triggers of skin inflammation also induced the expression of miR-155, suggesting an involvement in the process of inflammation. Upon miR-155 expression analysis of 21 human organs and tissues, miR-155 was found to be highly expressed in organs infiltrated by immune cells, such as the thymus, spleen and lung. Furthermore, miR-155 was expressed at a strikingly high level by activated T helper cells.

In order to identify putative target transcripts of miR-155 coupled to T cell activation and/or differentiation, we performed computational target prediction. One of the top targets predicted by several algorithms was CTLA-4. The crucial role of CTLA-4 in controlling T cell proliferation and in the maintenance of immune homeostasis inspired us to further investigate the relationship between
miR-155 and CTLA-4 during T helper cell activation. CTLA-4 as a target of miR-155 was verified in luciferase constructs where co-transfection of miR-155 precursor and a luciferase construct containing a wild type CTLA-4 3’UTR significantly reduced the luciferase activity. A plasmid containing a mutated miR-155 target recognition sequence, on the other hand, was not subjected to miR-155 suppression. Finally, transfection of miR-155 precursor into naïve T helper cells significantly reduced the protein levels of CTLA-4 (Figure 6A) and increased the proliferative rate upon activation (Figure 6B).

![Figure 7](image)

**Figure 7** MiR-155 downregulates CTLA-4 protein expression and increases the proliferative rate of CD4+ cells. (A) Naïve T helper cells were transfected with either a miR-155 precursor, or a control precursor. 3 days post stimulation with ConA, intracellular CTLA-4 levels were measured with flow cytometry. (B) Cells were cultured for 3 days in the presence of recombinant B7-1/Fc Chimera protein and anti-CD3 antibodies, and proliferative rate was analyzed day 2-3 of culture. Shown are results from four individual transfections, where error bars represent mean values ±SEM. P-values derive from Mann-Whitney U tests.

Our results show that miR-155 regulates the intrinsic T cell proliferative response by targeting the CTLA-4 transcript, thereby modulating the protein levels of CTLA-4. Apart from autonomously regulating cell cycle progression and IL-2 production, CTLA-4 has also been proposed to have non-autonomous inhibitory effects. These are perhaps best demonstrated in T regulatory cells whose suppressive potential is dependent on the constitutive expression of CTLA-4 (145, 146). B-7 is required on activated effector cells for the susceptibility to Treg mediated suppression, confirming the potential of CTLA-4 to deliver inhibitory signals in a paracrine manner (147). CTLA-4 on Tregs downregulates co-stimulatory molecules and increases the tryptophan metabolism of DC’s (148, 149), thereby also reducing the potency by which they can activate effector T cells. Thus the malfunctioning of DC’s reported from miR-155 knock-out mice (122) could potentially be explained by T helper cells overexpressing CTLA-4 and inhibiting DC function.

The miR-155 knock out mouse is reported to have a reduced homeostasis and proliferative capacity in the Treg population. However, the Tregs of miR-155 deficient mice are still functional in their suppressive potential (126). In agreement with this, our findings suggest that absence of miR-155 would not result in loss of CTLA-4 expression, but rather over expression of CTLA-4. It
is possible that abnormally high level of CTLA-4 could contribute to the failure of Treg homeostasis, without having any impact on the suppressive nature of the T regulatory cells.

MiR-155 is processed from the non coding *bic*-transcript. In a genome wide target scan analysis for putative FOXP3 regulated genes, the *bic*-promoter was identified as a target for FOXP3. Accordingly, miR-155 expression was elevated upon FOXP3 binding to the *bic*-promoter region in T regulatory cells (150). Whereas FOXP3 is exclusively expressed by thymically derived murine Tregs, it is transiently expressed by all activated human T helper cells (discussed in section 1.2.4.2). FOXP3 expression by all activated human T helper would therefore also imply a more general expression of FOXP3 induced miR-155 expression in the human setting, possibly acting to regulate the effector phase of proliferation in conventional cells.

**Conclusion:**
MiR-155 regulates T helper cell proliferative responses by modulating the expression of CTLA-4. Furthermore, miR-155 expression is elevated in lesional skin of patients with atopic eczema, and is expressed by non-lesional skin upon exposure to extrinsic triggers of inflammation, suggesting miR-155 regulation as an important component of inflammation in vivo.
5 FUTURE PERSPECTIVES

The results generated from my work highlight the importance of epigenetic regulation during differentiation and homeostasis of human CD4+ lymphocytes. Most reports on epigenetic regulation of T helper cells are hitherto performed on murine cells, and emerging evidence from studies on human cells reveal interspecies differences in regulation of cytokine and transcription factor expression. Further work is needed, particularly on human cells, to clarify to what extent the systems are interchangeable and how they work individually.

Regulation of FOXP3 represents one situation where interspecies differences have become apparent. Despite extensive efforts, questions remain concerning differences in stability of FOXP3 expression and origin of regulatory T cells in mouse and humans. In our results, we noted how activated human cells, that transiently express FOXP3, exert suppression in their transient FOXP3 expressing state. This raises questions regarding the relevance of this suppressive activity in vivo. Transient FOXP3 expression and suppressive ability was observed when cells were cultured in presence of human serum, which suggests transient expression/suppression most relevant also during physiological conditions. The transient suppressive ability of proliferating cells also raises another important question. Whereas recently activated cells in suppression assays are highly suppressed by activated conventional T cells, the potential suppressants themselves proliferate vigorously. The reason for this insensitivity to signals derived by neighboring cells is not known, and will be of great interest to study further.

The Th1 and Treg population have traditionally been regarded as having separate origin, and antagonistic properties with Th1 cells promoting autoimmunity, and Tregs mediating tolerance. However, in our results from in vitro differentiated Th1 cells we observed a simultaneous demethylation of the IFNG gene and FOXP3, suggesting co-regulation of FOXP3 as more important for Th1 cells compared to Th2 cells, potentially acting as an intrinsic regulator. This cooperative demethylation was also observed among CD4+ cells from synovial fluid, suggesting IFNG-FOXP3 co-regulation as important also in in vivo situations. STAT1 mediated induction of FOXP3 provide mechanistic insight to FOXP3 and IFNG co-regulation (55), but questions still remain regarding the function of FOXP3 in Th1 cells.

An increased understanding of activation, maturation and lineage commitment of CD4+ T lymphocytes also renders extending studies into the clinical setting possible. Our results from methylation analysis of tumor infiltrating lymphocytes demonstrate how CpG methylation is responsible for the absence of high IFN-γ production from these cells, however the mechanisms which ultimately “close down” the Th1 response remain to be elucidated. Our co-culture experiments suggest the presence of soluble mediators of suppression within the tumor micro-environment. A putative candidate with potent immunomodulatory functions for further analysis is TGF-β. TGF-β is known to have suppressive effects on IFN-γ production from differentiated Th1 cells, but if this suppression involves chromatin modulation remains to be investigated.
The importance of CTLA-4 in clinical situations is demonstrated by the successful treatment of several autoimmune diseases with CTLA4–Ig fusion protein (151). Our results demonstrate miRNA mediated modulation of CD4+ T cell proliferative responses through CTLA-4, and that this is highly relevant for inflammation. MiR-155 regulation of CTLA-4 is likely to play a crucial function in all aspects of T helper cell proliferation and also involve other clinical situations than atopic eczema. Myasthenia gravis and rheumatoid arthritis have been associated with polymorphisms within the 3’UTR of the CTLA-4 locus (152-154), where longer AT-repeats is associated with decreased mRNA stability and reduced expression of CTLA-4 (153). The reason for this increased turnover rate of CTLA-4 is not known, but it is tempting to speculate that the polymorphisms in the 3’UTR affect miRNA binding and CTLA-4 transcript degradation.

Epigenetic modifications, induced by signals from the local cytokine milieu, actively control and modify gene expression in all T helper lineages including regulatory T cells and Th17 cells. As such, epigenetic changes can also be used to predict active or silenced genes, and as markers of lineage commitment. The method described in paper III allows profiling of CD4+ cells in clinical situations where CD4+ dysregulation have been implicated. It would be of great interest to expand this study to involve other clinical situations and/or profiling of disease progression.
Kroppens immunförsvar skyddar oss mot virus, bakterier och parasiter (patogen) och även mot tumörer. Förekomsten av olika slags patogen kräver att immunförsvaret är flexibelt, så att det kan stå emot infektioner av olika typer. För att vi skall kunna bli immuna mot infektioner krävs även att immunförsvaret kan komma ihåg vilka patogen som immunförsvaret tidigare har varit i kontakt med och snabbt kunna eliminera detta patogen om det skulle infektera oss igen. Immunförsvarets förmåga till flexibilitet och minne medieras delvis av T cellerna. T cellerna har unika receptorer, som kan känna igen främmande proteiner, som visas upp för T cellen via s.k. antigen presenterande celler. Presentationen av främmande proteiner för T-cellerna sker i lymfkörtlarna. När en T cell känner igen ett främmande protein börjar den snabbt att dela sig. Under denna expansionsfas genomgår även T cellen differentiering, dvs. den ändrar karaktär. Det har visat sig att en T cell kan differentiera mot olika riktningar, beroende på typ av infektion. Th1 celler uppstår då kroppen infekteras av virus, medan Th2 celler differentierar till följd av en parasit-infektion. Th1 och Th2 celler utsöndrar olika uppsättningar av cytokiner, signaleringsproteiner som i sin tur har olika effektormekanismer som syftar till att eliminera bakterie- eller parasitinfectionen. Genom denna process bidrar flexibiliteten hos immunförsvarets T celler till att effektivt kunna eliminera olika typer av patogen.

Att en T cell kan anta olika identiteter har länge varit känt, däremot har det varit en gåta hur dessa olika identiteter bibehålls under celldelningen som följer efter infektion. Svaret finns att finna i de s.k. epigenetiska mekanismerna som styr genuttryck och genomgår genom celldelning. Epigenetiska modifieringar av arvsmassan är modifieringar som styr genuttryck utan att ändra den primära DNA sekvensen. Genom att dessa kemiska modifieringar kan läggas till eller tas bort från arvsmassan uttrycks antigen gener, eller så tystas dem. De kemiska modifieringarna skapar på så vis förutsättningar för genuttryck. Dessa modifieringar på arvsmassan kopieras då en cell genomgår celldelning, och därför bibehåller en delande cell sin identitet. Epigenetisk reglering av genuttryck är på så vis orsaken till varför t ex en muskelell är så olika en hudcell, trots att de innehåller identisk genetisk information. Epigenetisk reglering har på senare tid visat sig vara mycket viktig för immunförsvar, inte minst för T cellerna som kan anta olika identiteter under en immunreaktion.

I denna avhandling har jag undersökt epigenetisk reglering av gener som är viktiga för T cellens funktion. Först undersöktens metyleringsmönster hos FOXP3-genen. Metylering är en typ av modifiering hos arvsmassan som negativt reglerar uttrycket av gener. FOXP3-genen är särskilt intressant att titta på eftersom denna gen är viktig för att förhindra tillväxten och produktion av inflammatoriska signalmolekyler hos T cellerna. Vi fann att regulatoriska T celler som negativt reglerar tillväxten hos andra T-cellers, har en unikt metyleringsmönster i FOXP3 genen som kännetecknades av tillgänglig arvsmassa. Vi fann även att vanliga T celler, som inte har till uppgift att
förhindra inflammation, har ett metyleringsmönster som inte tillåter ett stabilt uttryck av FOXP3 genen. Denna upptäckt förklarar varför regulatoriska T celler är så unika i sin förmåga att uttrycka FOXP3-genen stabilt. Det unika metyleringsmönstret som vi upptäckte hos regulatoriska T celler kan även användas för att identifiera regulatoriska T celler i blodprover från patienter.

Vi fortsatte med att undersöka metyleringsmönster i T celler som återfinns inuti tjocktarmcancer. Vid cancer har det tidigare visat sig att det är fördelaktigt att ha T celler av Th1-typ. Th1 cellerna utsöndrar bland annat signaleringsprotein IFNG som i sin tur kan medverka till att döda tumörceller. När vi undersökte metyleringsmönstret hos IFNG genen i T celler från tjocktarmcancer så fann vi intressant nog att denna gen var nedtystad. Denna upptäckt skulle kunna förklara varför celler som återfinns i tumörer är så dåliga producenter av IFNG.

Eftersom metyleringsmönster hos arvsmassan kan förutsa genuttrycket i T cellerna utarbetade vi en metod för att kunna kartlägga T cellernas identitet utifrån deras epigenetiska förutsättningar för genuttryck. Genom att ta reda på vilka celler som finns närvarande i inflammerad vävnad hos patienter, kan man få ökad förståelse för de mekanismer som ligger till grund för sjuksom, och på så vis kunna utarbeta bättre behandlingar. Med detta i åtanke använde vi vår metod för att kartlägga T celler från patienter med autoimmuna sjukdomar (reumatoid artrit och multipel skleros). Vi undersökte både T celler från blodprover, och även T celler från inflammerade leder. Vi fann att T cellerna inuti inflammerade leder från patienter med reumatism är av Th1 och T regulatorisk typ. I djurexperimentella modeller av reumatism och multipel skleros har man visat att T celler av Th17 typ är orsaken till inflammationen, men dessa celler återfinns inte i de prover som vi undersökte med vår metod. Detta skulle kunna bero på att djurmodellerna inte korrekt återspeglar det inflammatoriska förloppet hos sjuka patienter.

I denna avhandlings sista projekt undersöckes hur små molekyler som uttrycks från arvsmassan i sin tur kan påverka uttrycket av andra gener. Vi upptäckte att mikro-RNA molekylen miR-155 kan styra uttrycket av proteinet CTLA-4. CTLA-4 är ett mycket viktigt protein som styr T cellernas delningshastighet. Det har bland annat visat sig att man kan behandla autoimmuna sjukdomar med mediciner som hårmar CTLA-4 proteinets egenskaper. Eftersom miR-155 molekylen sty r uttrycket av CTLA-4 kan man tänka sig att även denna molekyl kan utgöra en målmolekyl i syfte att behandla sjukdomar.

Sammanfattningsvis kan man säga att de projekt som är inkluderade i denna avhandling har bidragit till ökad förståelse för de mekanismer som ligger till grund för genuttryck hos T celler. Vi har hittat bidragande orsaker till varför immunförsvarset ibland inte kan bekämpa uppkomsten av tumörer. Vi har utarbetat en metod som kan kartlägga T cellernas identitet i patientprover, samt funnit ett mikro-RNA som styr delningshastigheten hos T cellerna.
7 ACKNOWLEDGEMENTS

Many people have contributed to the content of this book, not only the people directly involved in laboratory work and/or writing of manuscripts. I would like to send special thanks to the following people:

My supervisor Ola Winqvist, a visionary, a never ending source of inspiration and a wonderful person with contagious enthusiasm. Thank you for accepting me as a PhD student in your group and for being the best supervisor one can imagine. It never seize to amaze me how you always have a few extra minutes for a conversation, even though I know you are one of the busiest men on the planet.

Rolf Ohlsson, my co-supervisor, for introducing me into the epigenetic field of research, and for valuable education in your lab.

Per Marits, for being my teacher when working on my masters-thesis. I have learned so much from our discussions, and I am truly impressed by your immense knowledge in immunology.

Thank you Emma Lundgren, for being a great friend during these past years. I am so happy that we signed up for the ITG group at the same time.

Former and present colleagues of the ITG group; Signe Hässler and Mona Karlsson, for helping me out in the beginning. A special thanks to Mona for many laughs and op material arriving at my front door by taxi at 2 am. Johan Brännström, for nice company and for being my side-kick when harassing “Milton”. My co-author Malin Winerdahl, for being more careful and deliberate than me during our mutual experiments, something I am very grateful for. Thanks to Petra Jones, for being such a gentle and kind person and for being a great company at morning coffee. Martina Jones, for nice company and for your spirited personality. Ludvig Linton, for great collaboration, those clown-school lessons really payed off!!! Emma Ahlén, for keeping track of things such as primer sheets etc that tend to disappear from me, and thank you for great collaboration. Michael Eberhardson, for an eccentric and entertaining personality, and for amusing stories about the suburban lifestyle on Lidingö. The rest of the ITG group, including Evelina Lindmark, and Ali Zerakzadeh for creating a great atmosphere at work.

I would also like to thank my collaborators and coauthors Vivianne Malmström and Fredrik Piehl, for fruitful discussions and for your expertise in autoimmune diseases. Andor Pivarsci, for rewarding scientific collaboration and interesting discussions.
A special thanks to my mentor Hans Grönlund, for encouraging small talks and guidance in my scientific career.

All the rest of the staff at L2:04 for making every day at work enjoyable.

Ett alldeles speciellt tack till mina vänner och mina båda familjer:

Mina underbara systrar Barbro och Anna-Karin, för att ni alltid finns där. Min mamma Monica och min pappa Lennart för allt stöd och för att ni är så snälla och generösa. Jag vill även tacka Gunilla för all vänlighet och generositet.

Mina älsklingar Nadja och lilla Julia, som betyder allt för mig och som jag är så stolt över.
8 REFERENCES


