From DEPARTMENT OF MEDICINE, SOLNA
Karolinska Institutet, Stockholm, Sweden

LOCUS SPECIFIC DNA METHYLATION IN HUMAN IMMUNOLOGICAL RESPONSES

Emma Ahlén Bergman

Stockholm 2018

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Published by Karolinska Institutet.
Cover design: Emma Ahlén Bergman
Illustrations made by Sofie Önnestam
Printed by Universitetsservice US-AB

ISBN 978-91-7831-205-4

Karolinska Institutet

Institutionen för medicin, Solna

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AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Inghesalen, Widerströmska huset.

Fredagen den 9 november, 2018, kl 10.00

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Stockholm 2018

ABSTRACT

All cells in the human body contains the same genome. Yet, there are hundreds of different cell types, with widely different phenotype, and function. The differential gene expression leading to this diversity is tightly regulated by epigenetics, i.e. modifications to the genome without interfering with the sequence. There are a number of epigenetic modifications. In this thesis, I have studied DNA methylation as a readout for lineage specificity or effector function in cells from the immune system, in clinical settings.

In paper I we develop a method based on DNA methylation analysis to determine the lineage commitment of the $CD4^+$ T helper cells. First, we establish a regulatory site in the promotor of the *IL17A* gene. Next, we use signature genes *IFNG*, *IL13*, *IL17A* and *FOXP3*, to determine lineage commitment towards corresponding T helper cell subsets Th1, Th2, Th17 and Tregs. We call this method EILA (Epigenetic immune lineage assay), and demonstrate that it is usable in clinical samples from rheumatoid arthritis and multiple sclerosis.

In paper II, we take advantage of the same $CD4^+$ T cell specific *loci* to investigate the adaptive immune response in and around the tumour microenvironment in specimens derived from patients with urinary bladder cancer (UBC). By sorting and examining $CD4^+$ T cells from blood, tumour and regional lymph nodes, we conclude that patients with higher degree of lineage commitment have a better prognosis. Furthermore, we demonstrate that patients responding to neoadjuvant chemotherapy have a larger proportion of commitment cells, post treatment, compared with the non-responders.

In paper III we further examine the same patient material from UBC patients, but instead focus on the cytotoxic features of $CD8^+$ T lymphocytes. We establish a methylation pattern in the perforin gene *PRF1* predictive for protein expression and deploy this *locus* as a readout for cytotoxic functionality. We demonstrate that the tumour infiltrating $CD8^+$ T cells are pre-dispositioned to be cytotoxic through PRF1 demethylation, but that they lack corresponding protein expression, and show signs of exhaustion. The cells demonstrating a T_{RM} phenotype, can still be woken anew, upon *in vitro* re-stimulation, demonstrating that they are not terminally exhausted.

In paper IV we investigate whole blood leucocytes and the DNA methylation status of the glucocorticoid gene *NR3C1*. In contrast to literature studies on healthy volunteers, our cohort of surgical patients demonstrate a homogeneous pattern of demethylation in the previously described CpG island of *NR3C1*. As opposed to our hypothesis, we found no correlation between methylation and clinical outcome post-surgery in this patient cohort. Nevertheless, when employing multifactorial analysis to investigating the impact of genotype we found four single nucleotide polymorphisms that influenced the outcome.

In conclusion, this thesis demonstrate multiple ways in which DNA methylation analysis can be used to read the immune system, but also that the *loci* selected for investigation has to be carefully chosen following thorough functional investigations. The results presented herein can contribute to further development of treatments to a variety of clinical conditions.

Pain is inevitable, suffering is optional

Unknown

LIST OF SCIENTIFIC PAPERS

I. Janson PC, Linton LB, Bergman EA, Marits P, Eberhardson M, Piehl F, Malmström V, Winqvist O.
Profiling of CD4+ T cells with epigenetic immune lineage analysis.
J Immunol. 2011 Jan 1;186(1):92-102.

II. Ahlén Bergman E, Hartana CA, Johansson M, Linton LB, Berglund S, Hyllienmark M, Lundgren C, Holmström B, Palmqvist K, Hansson J, Alamdari F, Huge Y, Aljabery F, Riklund K, Winerdal ME, Krantz D, Zirakzadeh AA, Marits P, Sjöholm LK, Sherif A, Winqvist O.
Increased CD4+ T cell lineage commitment determined by CpG methylation correlates with better prognosis in urinary bladder cancer patients.
Clin Epigenetics. 2018 Aug 3;10(1):102.

III. Hartana CA, Ahlén Bergman E, Broomé A, Berglund S, Johansson M, Alamdari F, Jakubczyk T, Huge Y, Aljabery F, Palmqvist K, Holmström B, Glise H, Riklund K, Sherif A, Winqvist O.
Tissue-resident memory T cells are epigenetically cytotoxic with signs of exhaustion in human urinary bladder cancer.
Clin Exp Immunol. 2018 Oct;194(1):39-53.

IV. Emma Ahlén Bergman, Truls Gråberg, Louise K Sjöholm, Per Marits, Lovisa Strömmer, Ann-Charlotte Wikström, Ola Winqvist, Max Winerdal.
Genetic variability in exon 1 of the glucocorticoid receptor gene NR3C1 is associated with post-surgical complications.
Manuscript.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

I. Hartana CA, Ahlén Bergman E, Zirakzadeh AA, Krantz D, Winerdal ME, Winerdal M, Johansson M, Alamdari F, Jakubczyk T, Glise H, Riklund K, Sherif A, Winqvist O.
Urothelial bladder cancer may suppress perforin expression in CD8+ T cells by an ICAM-1/TGFβ2 mediated pathway.
PLoS One. 2018 Jul 2;13(7):e0200079.

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Urinary Bladder Cancer Tregs Suppress MMP2 and Potentially Regulate Invasiveness.
Cancer Immunol Res. 2018 May;6(5):528-538.

III. Zirakzadeh AA, Kinn J, Krantz D, Rosenblatt R, Winerdal ME, Hu J, Hartana CA, Lundgren C, Bergman EA, Johansson M, Holmström B, Hansson J, Sidikii A, Vasko J, Marits P, Sherif A, Winqvist O.
Doxorubicin enhances the capacity of B cells to activate T cells in urothelial urinary bladder cancer.
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CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
ACT	Adoptive cell transfer
CpG	Cytosine-phosphate-guanine
DC	Dendritic cell
EILA	Epigenetic Immune lineage analysis
GC	Glucocorticoid
GR	Glucocorticoid receptor
HLA	Human leucocyte antigen
LN	Lymph node
MHC	Major histocompatibility complex
MS	Multiple Sclerosis
PBMC	Peripheral blood mononuclear cells
RA	Rheumatoid Arthritis
Tc	T cytotoxic/cytotoxic T
TF	Transcription factor
Th	T helper
TIL	Tumour infiltrating lymphocyte
Treg	Regulatory T cells
UBC	Urinary bladder cancer

1 INTRODUCTION

1.1 EPIGENETICS

Every (somatic) cell in the human body contains the same DNA, yet the diversity of cell types, tissues and structures is enormous. This is conducted through a tight regulation of the genome, so that only certain genes are expressed in a specific cell, at a certain time point, leading to its individual properties. The regulation is accomplished by epigenetic modifications, which include histone modifications and DNA methylation, among others. Epigenetic modifications are heritable traits that do not change the nucleotide sequence. Whereas histone modifications determine the accessibility of the chromatin by controlling the unwinding of the DNA helix to increase or decrease the steric accessibility of the nucleotide sequence [1], DNA methylation regulates gene expression by fine-tuning the expression (Figure 1). In the work within this thesis we have used DNA methylation at specifically selected loci to determine the phenotypic stability of immune cells.

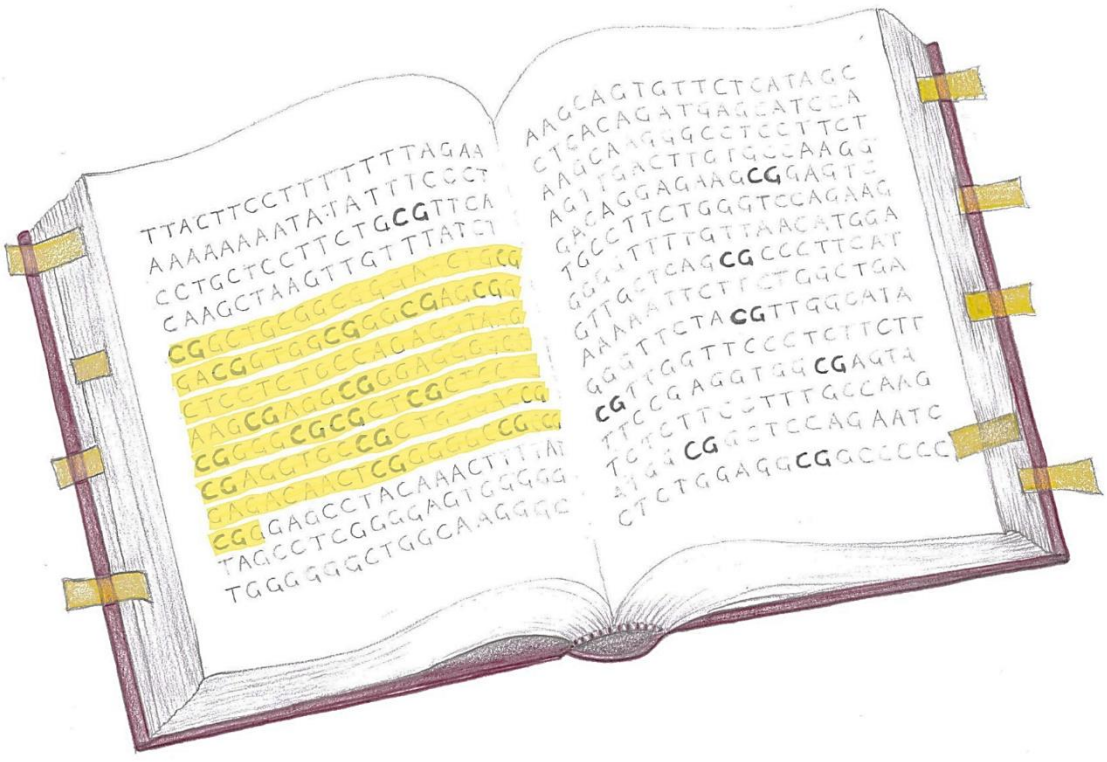

Figure 1. A popular scientific picture conveying a simplified version of the general idea of epigenetics. The book represents the whole genome. The yellow tags would be the open histone marks, enabling access to the region. Every page presents the DNA sequence. DNA methylation sites are bold, and CpG islands are highlighted within the sequence.

1.1.1 DNA methylation

DNA methylation in human cells occurs almost exclusively at CpG sites, i.e. at a cytosine (C) residue that is followed by a guanine (G). CpG sites occur in diverse frequencies along the genome, and when multiple CpGs are situated in close proximity to one another, it is considered a CpG island. The definition of CpG island differs, but is based on the ratio between expected and observed C and G content, where the ratio should be between (0.4 and 0.7). These islands often harbour regulatory features, including binding sites for transcription factors and other regulatory proteins, as well as being involved in three-dimensional formations of the chromatin [2]. *De novo* DNA methylation is conducted by DNA methyltransferases (DNMTs) DNMT3A and B, whereas DNMT1 in responsible for maintenance of methylation to daughter cells during replication [3]. Demethylation can occur both through passive demethylation during replication and by active demethylation initiated by TET-enzymes [4-6].

DNA methylation has proven to be of outermost importance during embryogenesis and cell lineage differentiation [7] and the impact of dysregulation has been demonstrated both during development and in a vast number of diseases [8-11]. Furthermore, locus specific DNA methylation has been investigated in a number of different cell types, in order to scrutinize both methylation signatures of cells at different stages, as well as its association with dysregulation in different clinical disease phenotypes and abnormalities [12].
It has become increasingly evident that our "genes are not our destiny" and that there are other mechanisms connecting genetics with the environment. This is obvious, when comparing monozygotic twins, since although genetically identical, they become gradually more different throughout life. The concordance rate of multifactorial diseases, including autoimmune diseases and cancer, is ≠1, leaving the difference gap to be explained by other means [13-15]. This phenomenon can, at least in part be explained by epigenetic changes. Thus, the era of epigenetic studies in health and disease has just begun.

1.2 THE IMMUNE SYSTEM

The human body has exquisite ways to protect itself from dangers, external as well as internal. The immune system functions as an army that patrols the body to detect pathogens and abnormalities harmful to our health. The two parts of the immune system, innate and adaptive, work in synergy to mount the most appropriate response to the encountered threat. The innate arm is rapid, robust and rough, but is restricted to recognition of a few, common antigens, shared by many microbes and pathogens. The adaptive immune system, though considerably slower, has the capacity to mount an antigen specific response and allows for long term memory [16].

Inflammation has since the days of Celsus (the first century AD) been described by five hallmarks: tumour (swelling), rubor (redness) dolor (pain), calor (heat) and function laesa (loss of function), where ≥3 of these symptoms would be sufficient for diagnosis [17]. Although 2000 years of research has revealed the underlying complicated mechanisms, the physiological features still holds true.

In this thesis I have explored different parts of the immune system, to investigate the possibility of using DNA methylation at specific loci as readouts of immune responses. This chapter will focus on the parts of the immune systems that I have explored, in the clinical settings I have investigated and leave out vast parts of the immune system, and its functions in the healthy and diseased human body.

1.2.1 Innate immunity

The innate branch of the immune system is the body's first line of defence. Beyond the skin, and other epithelial barriers, there is a soluble defence system present in extracellular body fluids. It consists of enzymes and other peptides that can destroy microbes immediately upon contact. Part of this response is called the complement system. It involves a series of proteins present in the plasma and on the surface of haematopoietic cells. Simplified, the complement system is triggered by foreign surface structures and immune complexes and subsequently initiates an enzymatic cascade of reactions leading to a pore formation and consequent lysis of the foreign cell. In addition, the complement also facilitates phagocytosis of microbes by macrophages, granulocytes and dendritic cells, the key cells of the innate immune response. The cells belonging to the innate immune response express a variety of different receptors, recognizing common molecules expressed on pathogens. These molecules, collectively called pathogen-associated molecular patterns (PAMPs) include peptides, polysaccharides and nucleotide chains. The receptors are collectively called pattern recognition receptors (PPRs), and upon encounter of these molecular foreign patterns the receptor-bearing cell is activated and start to produce inflammatory cytokines and phagocytose the intruding pathogen. Phagocytosis has two major purposes: to degrade and eliminate the microbe, and to process the foreign material into peptides suitable for antigen presentation to the adaptive immune system [18].
Together, the parts of if the innate immune response protects the body in a rapid and robust,

although non-specific way. The link between innate and adaptive (i.e. specific) immune response is the antigen presenting cells (APCs), relocating to secondary lymphoid organs to engage lymphocytes.
Inflammation is initiated at the moment when innate cells are activated by a danger signal, as described above. This will lead to accumulation of fluids through vasodilation and capillary leakages (swelling, reddening and pain). These fluids harbours inflammatory proteins (e.g. chemokines) that both act locally, but also function as chemoattractants to recruit immune cells. Through the combination of these events, inflammation itself affects the tissue in a way to further fortify the sense of pain, which in itself makes impaired functionality, to variable extents. When the cause of the inflammatory reaction is cleared, the inflammation has to be resolved and tissue damage repaired. This is mediated by feed-back signalling by already engaged neutrophils, and recruitment of resolution-phase macrophages, a process is described in detail by Ortega-Gomez et al [19]. Sufficient resolution is key to restoration of healthy, functional tissue. Failure in revoking the inflammation can lead to scarring, tissue damage and pathological states.

1.2.1.1 Sterile Inflammation

Sterile inflammation is defined by inflammation that occurs without any non-self agent present. This could occur in acute situations, including trauma or major surgery of large tissue areas or reperfusion of ischemic tissue. It can also occur as a result of chronic conditions such as chronic arthritis and asbestosis [20, 21]. Despite lack of foreign molecules, the inflammation is triggered by endogenous structures. One of the main reasons for this is cell death through necrosis, as opposed to apoptosis. Necrosis is an uncontrolled way of cell-death leading to leakage of otherwise contained molecules. This includes intracellular and intranuclear molecules and mitochondrial contents. In addition to intracellular molecules, tissue damage also induces degradation of extracellular molecules and structures. All these endogenous inflammation triggers are collectively termed damage associated molecular patterns (DAMPs). DAMPs can consequently trigger sterile inflammation [22]. Like with any inflammation, resolution, as mentioned above, has to ensue, or immunopathology will follow. In the case of acute systemic inflammation, the situation can be greater than the body can manage. Subsequently, an inflammatory storm that is impossible to revoke, may lead to system failure and death. In clinical settings, administration of glucocorticoids pre- and post-surgery is the main line of treatment.

1.2.1.2 Glucocorticoids in inflammation

Glucocorticoids (GCs) are expressed by the adrenal gland and belong to the endocrine system HPA (hypothalamus, pituitary adrenal) axis. GC binds to the glucocorticoid receptor (GR), which is expressed in almost all cells in the human body. GR has a classic structure of a nuclear receptor, featuring an amino-terminal domain, a DNA binding domain and a ligand binding domain. It is encoded by the gene *NR3C1*. This gene has multiple isoforms, both transcriptional and translational. The regulation and function of these are not yet fully understood, but studies conducted mainly in the neurobehavioral field have demonstrated

implications in neuropsychological conditions [23]. GC affects many biological systems, but for the sake of this thesis, only the effects on inflammation and the immune system are described.
GC is soluble and inactive in the circulation. It diffuses through cell membranes and can bind to the GR in the cytoplasm. Once bound, GC/GR complex can either interact with cytoplasmic proteins and mitochondria, or translocate into the nucleus. Once there, the GC/GR complex acts to directly or indirectly modulate gene expression.
When inflammation is initiated, glucocorticoids reduce the inflammatory cytokine releases and dampens the signalling of PAMPs and DAMPs. This decreases the signalling and recruitment of the adaptive immune response. During the course of inflammation GC further inhibit the adaptive immune system, both by inhibiting the expression of extravasation receptors, which hinders the tissue re-localisation of lymphocytes to the inflamed area, but also through decreased chemokine expression. Once the triggering agent is cleared, GC facilitates the resolution by enhancing the differentiation of macrophages to become more efficient in clearance of apoptotic cell debris and secretion of anti-inflammatory cytokines. By contrast, GC disturbs processes of tissue healing. This is contradictory, as is most data concerning GC signalling, and the effect of GCs seems to be dose-dependent [24]. Glucocorticoids can also modulate the skewing of the T helper cell pool, promoting Th2 and Treg differentiation, but inhibiting Th1 and Th17. GCs play a role in the interface between APCs and T cells where GR signalling interferes with MHC expression as well as other receptors necessary for T cell co-stimulation. The role of GCs in inflammation is reviewed in detail by Cain et al. [24].

1.2.2 Adaptive immunity

When inflammation is not sufficiently taken care of by the immediate response of the innate immunity, APCs migrate to secondary lymphoid organs, such as lymph nodes, where they present processed antigens to lymphocytes. Lymphocytes are key players of the adaptive immune system. The common lymphoid progenitor cells from the bone marrow give rise to a number of immune cells, including T and B lymphocytes. B cells are masters of the humoral response, creating antigen specific antibodies to target extracellular and soluble pathogens encountered. T cells recognize antigens presented on a major histocompatibility complex (MHC) molecule, situated on all nucleated cells, including antigen presenting cells [18].

1.2.2.1 T LYMPHOCYTES

There are two types of T cells; $CD8^+$ Cytotoxic T lymphocytes, which respond to intracellular pathogens such as viruses and tumour cells, and $CD4^+$ T helper cells that direct the immune system into a humoral or a cellular response [16].

1.2.2.1.1 Development and maturation

T cell maturation occurs in the thymus, a highly organised process where immature thymocytes are selected upon their successful T cell receptor (TCR) and their specificity.

This is done in two steps. First, thymocytes carrying a TCR that adequately recognize a MHC molecule are positively selected. The cells recognizing MHC class II will become $CD4^+$ T helper cells, whereas the T cells carrying a TCR recognizing MHC class I will become $CD8^+$ cytotoxic T cells. The second step, negative selection, is conducted in the medulla to ensure that the TCR does not recognize self-antigens, which could then contribute to autoimmunity. Thus, thymocytes expressing a TCR that recognize a self-peptide presented by MHC expressed on thymic medullary epithelial cells with high affinity is eliminated to avoid self-recognition and autoimmunity. Only a few percent of T cells undergoing this selection survive these two selection steps. The T cell receptor is highly variable, with $10x10^{15}$ possible variants of TCR specificity. This is conceived by genetic recombination of the V, D and J segment of the TCR locus during maturation in the thymus. This process, called somatic VDJ recombination, is unique to lymphocytes and establish the high diversity of pathogens that the adaptive immune system can recognize [25]. $CD4^+$ Natural T regulatory cells (nTregs) (also called thymic derived T regulatory cells; tTregs) are a subpopulation of T cells that are separately developed in the thymus. Their role, simplified, is to restrain immune responses at the right time to modulate the intensity of inflammation. They are developed through the same positive and negative selection as the other Th cells, but develop into Tregs if they encounter medium affinity self-peptides. This is to be able to suppress the immune system from attacking self-antigens [26]. The master regulator and phenotype inducer of nTregs is the transcription factor FOXP3. The nTreg can be distinguished from peripheral T cells with induced FOXP3 expression by their demethylated pattern in the FOXP3 gene [27]. This demethylation is an active process that takes place already in the thymus, thus determining the lineage commitment of the nTregs [28]. After passing through thymic selection T cells migrate to the periphery as naïve T cells.

1.2.2.1.2 T cell activation, circulation and memory formation

In the human body, T cells encounter antigens through presentation by the human leucocyte antigen (HLA) encoded by the major histocompatibility complex (MHC). Nucleated cells in the body express HLA class I, which present endogenous peptides to be inspected by the immune system. This to ensure that "all is well". If a cell is infected, the pathogen derived peptides are processed and presented, enabling antigen presenting cells (APCs) and $CD8^+$ T cells to recognize and act towards the infection [16].
HLA class II is expressed on APCs (dendritic cells, macrophages, monocytes, and B cells). APCs capture the antigen at the site of infection and migrate to the lymph node where the pathogenic peptides are presented on HLA I and II molecule to the T cell for recognition by the TCR. Upon APC migration maturation also occurs, where the APC adapts its features to the infecting pathogen as well as the inflammatory environment at the infectious site [18]. Depending on the environmental signals present upon T cell activation, as well as the co-stimulatory molecules expressed by the APC, a diverse range of effector and memory cells are formed [29].
Activation of lymphocytes requires tree signals for proper initiation; TCR-HLA recognition, binding of co-stimulatory molecules and immune-stimulatory environment (cytokines). When

activated, T cells mature into different phenotypes, both in terms of immunological directions and memory traits. The memory formation, in brief, constitutes of several properties, where tissue localisation and activation states are the major features. Thus, the cells are subdivided into the recirculating phenotypes; effector memory T cells (T_{EM}) and central memory T cell (T_{CM}), which are further distinguished by their ability to enter peripheral tissue or stay in blood- and lymphatic circulation, respectively. Contrary to these subsets are the tissue resident memory T cells (T_{RM}), which resides in the tissue, without possibility to re-enter the circulation. The partitioning of these memory subsets is, however, not rock solid [29].
Simultaneously, activated T cells differentiate in terms of immunological pathways, where there are several distinct routs depending on the pathogen encountered. Simplified, these can be divided into three types of immunity, depicted in figure 6 (page 26) [30, 31].
In the light of this, here follows a brief description of the $CD4^+$ and $CD8^+$ T lymphocytes investigated within this thesis, and their role in immunity.

1.2.2.1.3 $CD4^+$ T helper lymphocytes

The role of the T helper cell (Th) is to direct the subsequent immune response into an adequate attack depending on the nature of the pathogen/danger signal. This is conducted through lineage commitment during Th cell activation. Dysregulation of this process may lead to inadequate immune responses and cause disease, such as failure to distinguish pathogens, but also to autoimmune disease, allergy and cancer.
The naïve T helper cell will proliferate to become one out of several potential subpopulations of mature T helper effector cells, thereby directing the subsequent immune responses [16]. There are a number of different T helper cell lineages known to date, and during recent years more have been discovered. The most pronounced are T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17), as well as the thymic derived natural T regulatory cell (described above). In addition, there are more recently discovered lineages denoted Th9, Th22 and T follicular helper cells (Tfh cells) [32]. These are not within the scoop of this thesis. The subset that is established during activation and maturation acquire a number of signature features, in order to steer the subsequent immune response towards different paths. The two distinct branches are the cytotoxic response, featuring recruitment and activation of cytotoxic T cells, phagocytes and NK cells, and the humoral response, recruiting B lymphocytes as the key player [16].
All of the cell-cell interactions described above are through highly specific antigen recognition receptors (TCR, BCR, MHC), why this is called the specific immune system [33].
Here follows a short description of the T helper cell subsets and their signature features, such as master transcription factors, cytokine production and known epigenetic regulation, with emphasis on the human setting.

1.2.2.1.3.1 Th1 cells

Th1 lymphocytes are known to be the regulators of cellular immunity, i.e. activation of $CD8^+$ cytotoxic lymphocytes and enhancement of antigen presentation. The signature master

transcription factor (TF) T-bet collaborates with signal transducer and activator of transcription 1 (STAT1) and STAT4 to establish the expression of Th1 signature cytokine interferon-γ (IFNγ) [34]. Epigenetic studies performed in mice show permissive histone marks (H3K4me3) at the *Ifng* locus in Th1 cells specifically, whereas this site in naïve, Th2, Th17 and Tregs is repressed (H3K27me3) [35]. Consistently there are regulatory methylation patterns of the same locus in the corresponding human cells, where Th1 are demethylated and the other three lineages, and naïve cells are hypermethylated [36]. The master regulator T-bet show a more ambiguous pattern in mice, with both repressive and permissive histone patterns [35].

1.2.2.1.3.2 Th2 cells

The humoral response is initiated by Th2 cells when presented with antigens of extracellular pathogens and toxins. Upon stimulation, STAT6 and extrinsic interleukin (IL) 4 induce GATA3 expression that leads to production of signature cytokines IL-4 and IL-13 [34]. Similar to the Th1 setting, the master TF of Th2, *Gata3*, show both permissive and repressive histone marks in mice [35], while *Il4* is exclusively open in the Th2 setting. In humans, a number of studies have been conducted on the Th2 locus (IL-4, IL-13 and IL-5). Although it is not as uniform as in the *IFNG* locus, there are some differentially methylated CpG sites in Th2 cytokine locus that distinguish them from Th1/Th17/Treg/naïve cells [37, 38].

1.2.2.1.3.3 Th17 cells

T helper 17 cells play a role in the defence against helminths, fungi and extracellular bacteria [39], and have also been implicated in a number of autoimmune diseases as well as in tumour immunology [40]. It is of importance to notice the numerous differences between human and mouse generation of Th17 cells when reviewing the literature. In the human setting, transforming growth factor-β (TGFβ), together with IL-1β, (IL-6) and STAT3-inducing cytokines IL-21 and IL-23 elicit RORγt, the master transcription factor of Th17 cells, leading to the expression of IL-17. Epigenetically, Th17 cells have a demethylated pattern in *IL17A* locus and RORyt gene *RORC2* in human [41, 42] as well as permissive histone marks in mouse both restricted to this subset. *Rorc* locus also show a somewhat more lineage specific histone pattern than the master TFs of the other subset [35].

1.2.2.1.3.4 Natural T regulatory cells

As described above, natural Tregs are developed already in the thymus, and are therefore not properly belonging to the T helper cell pool. In humans these thymus derived cells are considered to be the only stable regulatory cells, even though other subset can express the master TF FOXP3 and exert regulatory functions. Unlike the nTregs, the FOXP3 expression by activated Th cells are transient, and doesn't seem to lead to acquisition of suppressive function, but rather have a self-limitation to control activation [43]. It has been shown that a way of distinguishing human nTregs from induced FOXP3-expressing Tregs is by the methylation pattern on the *FOXP3* locus. Only nTregs show a demethylated pattern, allowing

a stable expression [27]. In mice the permissive histone marks are also more restricted for *Foxp3* than some of the aforementioned TFs [35].

1.2.2.1.3.5 Lineage plasticity

The potential flexibility between the subsets is of vast interest due to possibilities to manipulate immune responses in a therapeutic manner towards various diseases. Current ongoing discussions on flexibility in-between lineages includes hypothesis on variation of intensity during primary TCR stimuli [44-46], accessibility of chromatin by master regulators, transcription factors and earlier factors [47, 48] as well as the impact on cytokine milieu during activation. However research in this field often lack discrimination between human and murine settings, a fact that might have immense impact [49, 50].
The stability and plasticity in-between these different lineages have been a subject for debate for more than two decades. Firstly, transitions between i.e. Th1 and Th2 was explored showing that time and repeated stimulus decrease the ability to switch lineage commitment [51-53]. More recently, the flexibility in-between Th1 and Th17 cells have been investigated, due to numerous reports on IFNγ/IL-17 double positive cells [42, 54]. These studies suggest partial flexibility and epigenetic modulation from Th17 cells to a subset called non-classic Th1 cells. It becomes obvious when reviewing the multiple lineages that the STAT family plays a crucial role in differentiation of these subpopulations. Numerous studies on this protein family in the context of Th cell regulations have been conducted [55] and it is clear that STATs affects both positive and negative regulation of transcription as well as the epigenetic landscape in T helper cells [56]. Genome-wide investigations of histone marks in a number of Th cells have been performed, revealing a much more multifaceted pattern then assumed [35]. Simplified, open patterns were revealed on the signature cytokines for every subset investigated, respectively, but when investigating histone methylations at loci of signature transcription factor a bivalent pattern occurred, insinuating a more flexible arrangement. Although it is worth keeping in mind that these studies were performed on murine, *in vitro* obtained subpopulations, examined at one single time point, it might shed some light on the complexity of the plasticity matter.

1.2.2.1.3.6 $CD4^+$ T cells in autoimmune diseases

The definition of an autoimmune disease is pathogenic outcome by the recognition of self-antigens by the adaptive immune-system [18]. In light of thymic selection, the etiology of most autoimmune diseases is unknown. There is immense evidence of multiple factors affecting the onset and duration of autoimmunity, including genetic variants, environmental factors and (prolonged) infection or tissue damage. On the immunological level, dysregulation of thymic selection would emerge as a starting point. However, the onset of autoimmune diseases usually does not occur until young adulthood. This would implicate other mechanisms, for which self-tolerance is broken. In view of this, regulatory T cells has been intensively studied [57]. Briefly, the conclusions to this day states that Tregs have demonstrated both plastic as well as instable phenotype in patients with autoimmune disorders, compared to healthy subjects. Dysregulation of Tissue resident Tregs have also

been implicated in autoimmune settings [57].
Within the scoop of this thesis, we have investigated samples from patients with Rheumatoid Arthritis (RA) and Multiple Sclerosis (MS). While many findings in autoimmune research demonstrate similar T cell dysregulation, the short subsequent comments will focus on the findings in these to diseases.
RA is characterised by systemic chronic inflammation of the joints, which results in degradation of cartilage and bone tissue. A number of cells from the immune system, most prominently T lymphocytes, infiltrate the synovial fluid of the joints, fuelling the chronic inflammation through constant expression of pro-inflammatory chemokines.
MS is caused by inflammation and demyelination of neurons in the brain and spinal cord. Both these diseases, as well as most other autoimmune diseases, has originally been thought to have Th1 skewing properties, although more recent research has demonstrated that Th17 cells seem to play a role in the maintenance of these two (as well as other autoimmune) diseases. In RA, synovial fluid contains high levels of IL-17, but not of IFNγ [58]. In MS, IL-17 has been demonstrated to negatively affect the integrity of the blood-brain barrier, as well as having Th cells co-expressing IFNγ and IL-17 [59].
Taken together, the research of autoimmune diseases has demonstrated that specific subsets of $CD4^+$ T helper cells are key players in the ingoing inflammation, that regulatory T cells are impaired in these situations, that tissue resident T cells might be implicated in initiation of disease, and also that IL-6 signalling is involved in driving the inflammation. All these features are sufficiently reviewed elsewhere [57, 60-63]. It is, however, clear that the different lineages of $CD4^+$ T cells matters greatly, and that ongoing studies and clinical trials are exploring ways of interfering in this part of the inflammation processes.

1.2.2.1.4 $CD8^+$ cytotoxic T lymphocytes

$CD8^+$ cells are the key player, ditto subsequent effector cells, of the Th1-initiated cellular cytotoxic response, i.e. type 1 immunity. The naïve $CD8^+$ T cells become activated through recognition of the antigen presented on a HLA class I molecule by an APC, a process called cross-presentation. The APC as well as T helper cells use chemotaxis to attract naïve $CD8^+$ T cells to the lymph node for priming. The signalling molecules, such as chemokines, also greatly enhance the efficacy of the activation. The $CD8^+$ T cells can also be helped in activation through direct contact with T helper cells via CD40-CD40L interaction [64, 65]. The primed $CD8^+$ T cell proliferate and acquire cytotoxic ability by expression of effector molecules. Through chemotaxis, cytotoxic T cells (CTLs) find their way to appropriate tissue, where they partake in elimination of the target antigen. Their mechanism of action is through signalling molecules like IFNγ and tumour necrosis factor (TNF), among others, and through killing of target cells, by means of their main effector molecules granzyme B and perforin [64]. This perfect duo of proteins are secreted when the TCR recognize the peptide for which it was primed, presented on HLA I on any somatic cell. Simplified, secreted perforin creates a pore in the cell membrane where granzyme B can enter into the cytoplasm. Membrane disruption along with enzymatic activity of granzyme B induces apoptosis [64, 66]. The regulation of these two signature proteins have been investigated in mouse and human. The

human *PRF1* gene (coding perforin) demonstrate regulation by DNA methylation in $CD8^+$ T cells [67], implicating that stable expression of perforin occurs after demethylation at this locus. The *GZMB* gene, coding for granzyme B, is in mouse epigenetically regulated by histone modifications [68] but have only been studied in $CD4^+$ cells for locus specific DNA methylation [69]. Nevertheless, these studies implicate that epigenetic regulation is highly involved in memory development and maintenance of $CD8^+$ T cells. The overall (bigger picture) transcriptional and epigenetic regulation of $CD8^+$ T cells is reviewed in [70, 71] and is not in the scoop of this thesis.

1.2.3 Tumour immunology

Cancer is an abnormal event where the cells of the human body transform and proliferate in an uncontrolled way. This is due to mutations that primarily affect the cell cycle, and has been thoroughly described in the literature, beautifully clarified by the famous "hallmarks of cancer" [72]. Throughout a lifespan of a human, mutations inevitably arise and therefore exquisite ways to identify and protect the body against cancer has been developed throughout evolution. Protein-altering mutations are promptly discovered by circulating leucocytes, such as macrophages and NK cells, exerting immunosurveillance, through e. g. abnormal peptides presented on MHC I molecules. When abnormalities are recognised the innate immune cells eliminate the transformed cells, as well as secrete pro-inflammatory signalling molecules, such as IFNγ, to recruit dendritic cells (DCs). DCs migrate to LNs to involve T lymphocytes and thus the adaptive immune system is engaged in the process of tumour elimination. In order for the tumour to survive this elimination phase, further properties have to be developed. Tumours successful in continues growth maintain proliferation, while the adaptive immune cells keep the outgrowth in check. For the tumour to propagate further, more aggressive features have to be acquired. These properties, called immune escape, involve expressing immunosuppressive signalling molecules, such as TGFβ and inhibitory cell surface markers, such as PD-L1. Tumours also recruit immunosuppressive cells, such as Tregs and myeloid-derived suppressor cells (MDSC) to transform the surrounding milieu. All these new properties inhibit the immune response, despite the fact that the adaptive immune response may have antigen specificity and reside in the surrounding lymph nodes. This last stage, immune escape, is a prerequisite for the tumour to evolve into a full-blown malignancy [73, 74].

Importantly, the tumour states summarized above is a simplified overview of solid tumours, and is not translatable to tumours derived from lymphoid cells, such as lymphomas.

In the last few decades, the potential of using the endogenous immune response in the battle towards cancer has been successfully explored. Opposite of chemotherapy and radiation, which primary goal was to eliminate rapidly growing cell populations, immunomodulatory strategies aim to block the immunosuppressive signals by the tumour and (re-)stimulate the silenced immune responses already present in the patient. The use of so called check-point inhibitors towards CTLA-4 and PD-1/PD-L1 has demonstrated a remarkable response (10-

35%). Several other blocking antibodies are currently being developed to minimize the tumour derived immunosuppressive effect. It is, however, important to point out that far from all patients respond to these treatments, and that there is no or poor pre-treatment assessment to elucidate which patients will be responders [75]. Nevertheless, we are only just starting to grasp the full potential of our own immune system.

Another method is adoptive cell therapy (ACT), which in the early 2000s' presented promising results. It was based on re-infusion of extracted and *in* vitro expanded T cells, derived from tumour or lymph node. Although somewhat successful, there was great inter-patient differences in success rate [76].

A newcomer on the ACT scene are the chimeric antigen derived T cells (CAR T cells). This is a development of the adoptive cell transfer, since autologous T cells are the starting material. Simplified, patient derived T cells are transfected with an engineered B cell receptor containing a kinase signalling construct to promote activation upon recognition of surface antigen. This therapy has been successful in liquid tumours, such as $CD19^+$ B cell leukemia. Its effect on solid tumours has, however, yet to be further explored [77, 78].

Collectively, the immune system plays an important role in cancer development, and is a promising target and tool in new treatment strategies.

2 AIM

The overall aim of this thesis was to investigate DNA methylation at specific loci as a method for interpretation of the direction of the immune responses in clinical settings.

Paper I

To examine DNA methylation in the *IL17A* locus and to design a DNA methylation assay to investigate the whole $CD4^+$ T lymphocyte population for lineage commitment.

Paper II

To study the $CD4^+$ T cell response in urinary bladder cancer using DNA methylation to establish methylation patterns and correlate with clinical outcome.

Paper III

To investigate the cytotoxic properties of tumour infiltrating $CD8^+$ cytotoxic T lymphocytes in urinary bladder cancer.

Paper IV

To scrutinize the involvement of DNA methylation in glucocorticoid receptor gene *NR3C1* in clinical outcomes after major surgical interventions.

3 METHODOLOGICAL CONSIDERATIONS

Here follows brief comprehensive descriptions of selected methods used in this thesis, along with comments with regards to their applications herein. Detailed information is found in the papers, respectively.

3.1 HUMAN TISSUE SPECIMENS

All papers within this thesis contain research experiments conducted on human tissues. To a great extent, the samples have been processed immediately after retrieval, to minimize the risk of introducing errors by unnecessary interfering with the tissues.

3.1.1 Peripheral blood (Paper I-IV)

Patient blood was collected in heparin tubes, and processed for PBMC extraction and subsequent methods as fresh samples in paper I-III. In paper IV whole blood was frozen in RLT buffer for later processing. For healthy donors, blood was either collected in heparin tubes or as whole blood-derived leukocyte enrichments (buffy coats), and processed as fresh samples.

3.1.2 Synovial fluid (paper I)

The specimens was extracted from knee joints and processed for $CD4^+$ T cell extraction.

3.1.3 Lymph nodes and tumour tissue (paper II-III)

During surgical procedures, specimens were collected in chilled serum-free medium and processed for cell extractions immediately after retrieval.

It would have added great value to have absolute number of cells from each tissue, however, due to technical and surgical reasons, it was impossible to interpret the number of cells and correlate in-between patients. For lymph nodes, each node detected was divided between research studies and clinical evaluation by pathologist. The tumour tissues obtained are largely macroscopically diverse, in density and size, and the cell count from blood is not only dependent on volume, but also chemotherapy and possibly other clinical interventions unknown to us.

3.2 CELL SORTING

Paper I-III features sorted $CD4^+$ and $CD8^+$ T cells as a readout to investigate the course of the adaptive immune response. $CD3^+$ cells were sorted from single cell suspensions derived from various tissues, through a magnetic antibody cell sorting method. The purified cells were then labelled with anti-CD4 and/or anti-CD8 fluorescent antibodies and sorted through flow cytometry. Although this two-step sorting strategy decreases the final yield, it was required to achieve desired purity of the final population from tissues other than peripheral blood.

3.3 DNA METHYLATION ANALYSIS

In order to investigate DNA methylation, extracted DNA was bisulfite converted. Treating DNA with sodium bisulfite makes all the cytosine (C) not carrying a methyl group convert into uracil (U) (which during PCR is translated into thymine (T)). When performing sequencing the only Cs left are those which were originally methylated. This treatment does, however, make the DNA less stable and more fragile. It also greatly complicates the process of primer design for downstream applications.

3.3.1 Sanger sequencing (paper I and III)

This method is based on random blunt termination of amplification during PCR reaction, through incorporation of fluorescently labelled dideoxynucleotides (ddNTPs). The PCR products is then sorted by size through (capillary) gel electrophoresis and sequence is decoded by fluorescence, differentiated by four different colours corresponding to the four bases A, T, C and G respectively.

When wanting to investigate a locus on a single cell level, a normal PCR is run and the amplicons are cloned into plasmids and transformed into bacteria. After culturing bacteria in medium they are plated on agar dishes. Individual clones from these dishes theoretically represents one single original PCR amplicon, derived from one single cell. By selecting a sufficient number of clones and proceed with sequencing it is possible to investigate the original cell population, from which the DNA was obtained, on a single cell level.

The Sanger sequencing method can render a sequencing size up to ~800 base pairs, depending on the PCR amplicon size. However, for bisulfite sequencing the amplicon size is greatly reduced due to instability of bisulfite converted DNA.

3.3.2 Bisulfite pyrosequencing (Paper II and III)

A locus specific PCR is carried out, where one of the primers has been biotinylated. Following denaturation and clean-up procedure of the PCR product, a sequencing primer is annealed to the single stranded, biotinylated strand from the PCR product. The sequencing reaction is then performed by releasing one dNTP at a time. If the nucleotide is incorporated ATP is released to catalyse the luciferase reaction, leading to a light signal directly proportional to the number of incorporated nucleotides. The left-over nucleotides are then degraded before a new nucleotide is added. The dispensation order of the nucleotides is based on a pre-set, target sequence where a CpG site, and a possible methylated C, is evaluated by adding the nucleotides C and T. The relative intensity of the C and T signals will give the methylation percentage at that particular site. To this end, bisulfite pyrosequencing can be used to evaluate the proportion of methylation in a multi-cell sample. In this thesis pyrosequencing is used to establish the percentage of methylation in loci that have previously been investigated at a single cell level, and in a cell type specific manner. This allows for interpretation on a cell population basis.

3.3.3 Methyl sensitive Single Nucleotide primer extension (MS-SNuPE) (paper I)

This method can be utilized when investigating methylation at a single CpG site. Briefly, a sequencing primer is designed to stretch to the base right before the C to be evaluated. A mixture of di-deoxyNTP is added to the previously performed locus specific PCR product for a single nucleotide extension and termination of elongation. The ddNTPs are coupled to fluorescent molecules to distinguish between the different nucleotides. The product is analysed by capillary gel electrophoresis, exiting the fluorescence and thereby revealing the incorporated nucleotide. The result discloses the ration between C to T fluorescence. In paper I this method is used on multiple sites, separated by different sizes of site specific sequencing primers. This allows for multiple site analyses in one read, although all previous steps need to be individually executed.

3.4 CELL CULTURES WITH 5-AZA-CYTIDINE (5-AZA) (PAPER I AND III)

5-aza is a nucleoside analogue that, after uptake of the cell, is metabolised into the nucleotide 5-aza-2-deoxycytidin-thriphosphate and can subsequently be incorporated into DNA during replication as a substitute for cytosine [79, 80]. This random substitution leads to a covalent binding of DNMT1 to the DNA, and hence the DNA methylation is discontinued. By adding 5-aza to *in vitro* cultures of proliferating cells it is possible to investigate downstream effects of random demethylation. Using cells that are naturally negative for the gene expression of interest might, upon proliferation, randomly begin to express the gene. This indicate the involvement of DNA methylation in regulation of the gene expression. It does not, however, convey if the outcome of 5-aza treatment has a direct effect on DNA methylation on the specific gene, or if it is indirect, through other genes that were demethylated. Hence, this is an indirect method of proving the involvement of DNA methylation on gene expression.

3.5 ETHICAL CONSIDERATIONS

The research in this thesis was conducted using a number of human tissues, all donated by patients and healthy volunteers. All tissues collected from patients were taken during clinical procedures, and all were approved by local ethical board (see papers for detailed information). Apart from blood and synovial fluid, that was taken *in addition* to clinical sampling, all other samples were extracted from the patient regardless of inclusion in our research. All patients have been well informed according to guidelines and appropriate ethical permissions are referred to in respective paper. For healthy donors, the same guidelines of informed consent apply, and the buffy coats, retrieved from voluntary blood donors are also an unused product of the blood components used by the clinic.
To this end, I consider no additional harm has been inflicted, and I feel confident that this research stand on solid ethical ground.

4 RESULTS AND DISCUSSION

The common denominator of my research was to use locus specific DNA methylation as a predictive indicator of the direction of immune response and of stable phenotype. The rational for this is that many genes can be transiently transcribed, with subsequent mRNA and protein expression. Hence, the time of measurement, the exact occasion that one retrieves and analyse the sample and the stage the overall biological process is in, has great effect on the levels of these molecules. On the contrary, DNA methylation is a stable modification that is only affected by active cell faith decisions during the course of a cells life [81].

The choice of gene to investigate in order to be able to draw conclusions on cell lineage, cell functionality or prediction of immune reaction is therefor of outermost importance. These features have to be investigated thoroughly, to establish the DNA methylation's specific connection to features. It is important however, to realise that these characteristic measures reflect the attributes researchers have decided to name and label as separate, individual traits, setting one population apart from another, or on the other hand, putting all cells as equals. Although this has been thoroughly studied in most areas discussed within this thesis, it is not for me to say that these divisions are rock solid, and a 100% true at all times. With this in mind I will discuss the results of my work, in a somewhat fused, comprehensive context.

4.1 THE TH17 LINEAGE

In paper I we sought to design an epigenetic method to investigate the lineage distribution of $CD4^+$ T cells. In order to do so, we first demonstrated that the *IL17A* locus could define Th17 lineage. The *in vitro* protocols for generation of Th17 cells from naïve $CD4^+$ T cells were not optimal (paper I, Figure 3B-C) when this research was performed, hence the Th17 cells analysed for DNA methylation in our paper derived from primary *in vivo* differentiated cells, present in peripheral blood.

The methylation percentage of our sorted IL-$17A^+$ cells is not irrefutable, and in hind sight I am convinced that the cells we sorted were contaminated in a number of ways. Firstly, the IL-17A positive gate was unnecessarily generous. Secondly, the cells derive from over-night stimulated bulk $CD4^+$ T cells from peripheral blood, which could give rise to a small portion of transient expression of IL-17A [82]. Thirdly, the gating strategy for sorting does not feature an exclusion gate for IFN-γ expression, which means that the cells sorted could possibly also contain a fraction of the reported Th1/Th17 cell subset. This, however, only support our conclusion that DNA methylation is a better way of defining $CD4^+$ T cell lineages, as compared to protein expression.

Newer studies on human Th17 cells have fortified our initial findings concerning DNA methylation being predictive of lineage commitment [42, 83]. Furthermore, it has been demonstrated that the non-classic Th1 cells (described in a number of papers with clinical settings) [84-87] derive from Th17 cells, as demonstrated by their demethylation in signature loci such as *IL17A* [42]. The non-classic Th1 cells are not in our sorted cell fraction, since

they have lost their IL-17A expression. These recent findings demonstrate a more flexible phenotype of the Th17 lineage, resulting in the immunological community becoming more liberal in their view on Th17 cells. In light of these new discoveries, the population we refer to as Th17 cells (based on DNA demethylation) should instead be referred to as Th17 derived cells (paper II). Consequently, this has to be taken into consideration for future analysis of *IL17A* DNA methylation. Nevertheless, since we do not aim to show the activity of the immune system, but merely the skewing, suggesting what direction the adaptive immune response has taken, DNA methylation is still a valid method for these means. Thus, the methylation *loci* presented within this thesis still holds the appropriate information (Figure 2), even when considering the non-classic Th1 cells. The new knowledge on these cells, regarding the plasticity properties they hold [42, 88], only add further dimensions in how treatments could be designed.

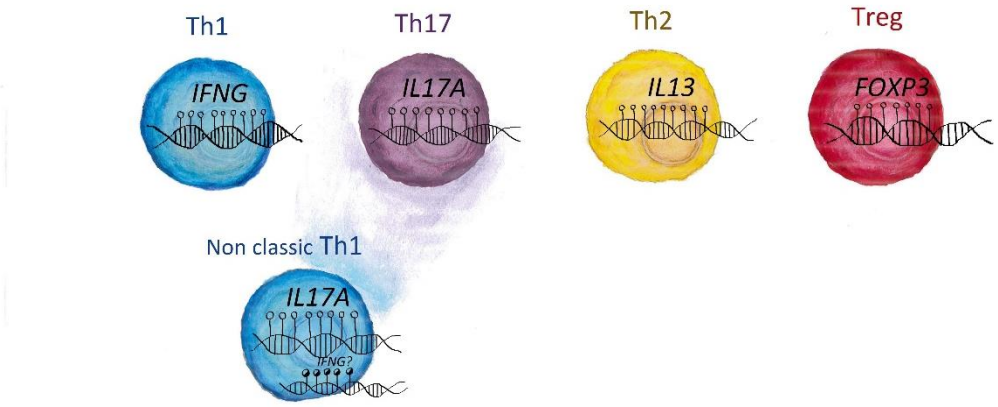

Figure 2. The CD4$^+$ T cells and their lineage specific genes employed in this thesis. The demethylation is depicted through empty "lollipops" on a DNA helix. The picture conveys the lineage "snap-shot" we acquire through the DNA methylation analysis perform. The non-classic Th1 cells demonstrate demethylation in IL17A locus, but the IFNG locus is yet to be investigated.

4.2 CD4$^+$ TH CELL LINEAGES IN AUTOIMMUNE DISEASES

In order to implement our method "Epigenetic Immune Lineage Analysis" (EILA) we examined clinical samples from patients suffering from rheumatoid arthritis (RA) or multiple sclerosis (MS). The classic assumption on autoimmune disease is that the immune system is skewed towards a Th1 lineage and potential lack of, or dysfunctional Tregs [30, 32]. This is in accordance with our results (paper I figure 4A) where there is a higher *FOXP3* methylation profile in circulating CD4$^+$ T cells from RA patients, compared to healthy controls. Furthermore, comparing paired patient samples we demonstrate that Th cells from synovial fluid have a lower methylation in *IFNG* and *FOXP3* compared to corresponding cells in peripheral blood. More recent investigations, demonstrate that non-classic Th1 cells constitute a significant proportion of the CD4$^+$ T cells in synovial fluid from RA patients, although on a protein level [89]. This is consistent with part of our cohort, where four patients

displayed demethylation in *IL17A*, without corresponding mRNA expression (Paper I Suppl. Figure 3). It would have been of immense interest to investigate the epigenetic status of *IL17A* in the cells from the paper from Basdeo et al [88, 89]. Overall, the patient cohort might be taken into consideration and should potentially be more carefully dissected, for further insight and clarification.

When investigating peripheral blood derived Th cells from patients with MS the methylation levels were significantly lower in *FOXP3* and *IL17A* compared to healthy donors. The patients treated with Natalizumab (α4-integrin blocking antibody, inhibiting extravasation of T cells) had a Th cell methylation profile like the healthy controls (paper I figure 5A). The discovery of Th17 cells being an important player in MS has since been further investigated and established, as reviewed in [59].

These results suggests that while patients with RA lean towards Th1 skewing, the MS patients have a more pronounced Th17 profile. More recent studies have, however, demonstrated Th1 and Th17, as well as non-classic Th1 cells in both these conditions, although different Th subsets seem to be involved in various stages of the diseases [84-87]. Of note, while RA patients was examined at the site of inflammation (joints) the samples from MS patients was restricted to peripheral blood. This in itself could potentiate for miss-match in read out and hence, the results demonstrated in these two disease states might not be straightforward to compare.

Our aim was to implement our method in a clinical setting, for proof of concept, and not dive deep into the pathogenesis of RA or MS as a specific disease. Accordingly, the examination of these two autoimmune diseases demonstrate that our method EILA is sufficient to make an accurate readout of the Th cell lineage proportions, and by extension the current direction of the adaptive immune system.

4.3 THE EILA CALCULATION

In paper I, we investigated the sorted $CD4^+$ T cell population. Due to suboptimal purity, we were obliged to find a way of excluding the contaminating cells by other means. To solve this, we made rigorous studies on the contaminating cell population of monocytes, to determine their background noise to our readout. We then developed a calculation method based on these investigations. By combining the contamination percentage with the corresponding background noise, we could subtract the influence of the monocytes, as well as that of naïve $CD4^+$ T cells.

A number of years passed from us publishing paper I until I started to investigate the $CD4^+$ T cell population in patients with urinary bladder cancer. During this time, the field of human CD4+ T cells expanded and the consensus of what were the true stable lineages changed. Not only were subsets proposed, such as Th22 and Th9, but there was also the division into tissue specific subtypes, such as Tfh cells. In the beginning of my research studies, the two isoforms of the surface protein CD45, were used to distinguish naïve $CD4^+$ T cells ($CD45RA^+CD45RO^-$) from antigen experienced memory CD4+ T cells ($CD45RA^-$

$CD45RO^+$). The RA/RO distinction was widely used until an effector subset, namely T_{EMRA} was discovered and other markers were established to distinguish between the two RA^+ populations [90]. This new knowledge, in combination with the fact that the distribution of different memory subsets varies in tissues made me decide to discard of the calculation developed in paper I. This decision was made because the calculation takes the proportion of $CD45RA^+$ cells into consideration and refers to this population as naïve. Worth pointing out is that all the initial studies made on the stable differentiated lineages (Th1, Th2, Th17 and Tregs) (Figure 2) that we base the data on in paper I is executed on cells derived from peripheral blood [36-38]. These papers must have included the $CD45RA^+$ cells (T_{EMRA}), and although they might not be a big proportion in the blood, when looking in other tissues, such as synovial fluid, LNs and tumour, it is hard to justify the use of this calculation. The methylation pattern is convincing in all the previous studies, and therefore I believe that the data presented herein is valid.

This means, however, that the results presented in paper II is not a direct product of the method we developed in paper I, but a new interpretation of the DNA methylation results referred to, and presented in paper I. We have not performed any further examinations into what type of $CD4^+$ memory cells are present in the different tissues. Hence, I cannot draw any conclusions as to what memory differentiation stage the cells are in. I can, however, make conclusions on what proportions of cells are stably committed to one or the other of the four lineages we have investigated. This is, in my opinion, sufficient to make interpretations on the skewing of immune response in the tissues investigated (regardless of number of cells, or proportion of naïve and memory cells).

4.4 ON TISSUE DISTRIBUTION AND DIFFERENTIATION STAGES OF T LYMPHOCYTES

In order to include the new knowledge of different memory stages of $CD4^+$ T cells we could have added more surface markers to discriminate different subsets, especially T_{EMRA} etc. Upon doing so, it would have been necessary to investigate possible differences in methylation pattern in-between these. This could potentially have been an interesting and highly informative study, had it shown any results. It is, however worth noticing that the investigations leading up to the *loci* studied herein did not take these subpopulations into consideration [27, 36, 37, 41, 91, 92]. The research my work is based on have demonstrated functional properties in correlation with methylation patterns and this is what we consider valid information for our investigation.

One might also speculate in the solidness of this localization-based subdivisions. For one, there are 2-5% T_{RM} cells in the blood of both patients and healthy controls (Paper III figure 3A-B, and data not shown), even though the subdivision/classification is based on markers such as epithelial adhesive proteins, (including CD103) that potentially should retain the aforementioned cells at its local site. Also, the impact that the subdivision has on our data is, in my opinion, a mere demonstration of different types off effector capacity. It is important to

clarify that the localisation subdivision has demonstrated clear differences in effector abilities [93], but within all these subsets, the skewing of the adaptive immune system is still present. Hence, these are two different layers of adaptive immunity, and it only becomes interesting when scrutinizing the T cells in a specific contextual setting.

4.5 USING DNA METHYLATION OF T CELLS TO INVESTIGATE IMMUNE RESPONSES IN URINARY BLADDER CANCER

In paper II and III we had the opportunity to examine T cells from different tissues collected from patients suffering from urinary bladder cancer. In paper II we take advantage of the results in paper I to explore the $CD4^+$ T cell distribution in and around the tumour microenvironment. We demonstrate the possibility to extract pure T cells from different tissues and conclude that overall greater lineage commitment is correlated with better prognosis.

In paper III we instead investigate the $CD8^+$ T cell pool to scrutinize the ability of cytotoxicity. Through previous studies, we identified perforin as being a key effector molecule suppressed by the tumour microenvironment [94]. Hence, we decided to further investigate the regulation of perforin in Tc cells.

In $CD8^+$ T cells, there is a significant lower level of perforin expression in tumour compared to blood, but the methylation in *PRF1* is the same in blood and tumour (Paper III fig 2A). This suggests that the tumour infiltrating cells are currently less activated, and we speculate that this might reflect the effect of tumour immunosuppression (Paper III figure 2A and 2B). When dividing the tumour derived $CD8^+$ T cells into memory subsets the methylation status reflects the protein expression (as seen in Paper III, Figure 5C and 5D).

The T_{RM} cells, the most prominent subset in the tumour, are more methylated in blood than in tumour (Paper III, figure 3E). T_{RM} cells are also T-bet low, on a protein level, which indicates that although demethylated in *PRF1*, the PD-1 expression is probably a sign of exhaustion, rather than that of activation, in these T_{RM} cells. This is also in line with the immunosuppressive environment.

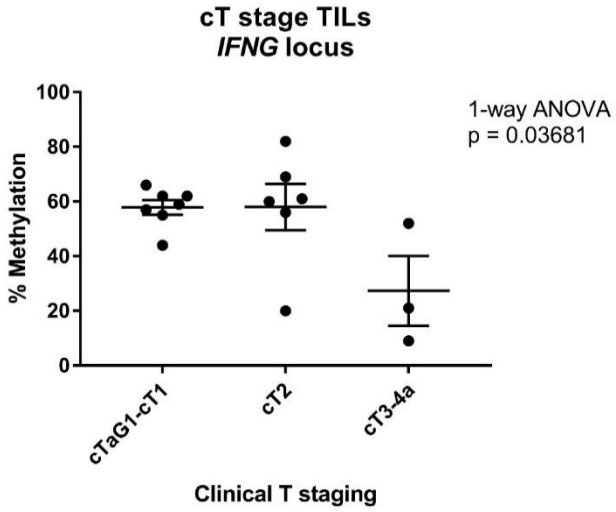

Figure 3. DNA methylation in IFNG locus of CD4$^+$ TILs reveals an increase in Th1 lineage at tumour cT stages ≥3.

On another note, concerning the T-bet expression, we show that upon *in vitro* stimulation of CD8+ TILs, the expression of T-bet is reinstated (Paper III, figure 4C and 4D). This leads me to hypothesis that the CD8$^+$ T cells retrieved from the tumour mainly are Tc1 cells.
To link the data from paper II and III, I compared the results from CD8$^+$ T_{RM} TILs with the *IFNG* methylation data from CD4$^+$ TILs, both sorted out at time of Tur-B and stratified on cT stage (Figure 4, and Paper III, figure 6). (*IFNG* demethylated cells are referred to as Th1 cells here onwards). In contrast to the T_{RM} proportion, Th1 cells from TILs increase in tumours staged ≥3 (Figure 4). We believe that the difference in stage 2 and 3, being the invasion of the perivesical tissue, is of importance for this increase in (and possibly renewed) Th1 differentiation (Figure 3), also seen at pT staging in paper II (Figure 6A). There was no change seen in the other three Th cell *loci* investigated when stratified for cT stage (data not shown). We speculate that the perivesical invasion, being another barrier breakage, is the cause of this increase. Since the same stage have the opposite effect for the T_{RM} pool, I'm once again speculating that the subdivision based on localisation is highly context dependent, and that stage 2 to 3 transformation is a game changer.
My interpretation of these results is that the subdivision of memory subsets based on localisation does not seem to define the phenotypical properties of the CD8$^+$ T cell pool. Also, the (relative) number of cells should be considered; not just the proportions within the CD8$^+$ T cell or CD4$^+$ Th cell pool, but in the whole local tissue.
The CD8$^+$ subset increasing in the *one* patient with stage 3 tumour was T_{EMRA} which only seems like an expected transition when the tumour moves out of the tissue in which the Tcs were once resident.

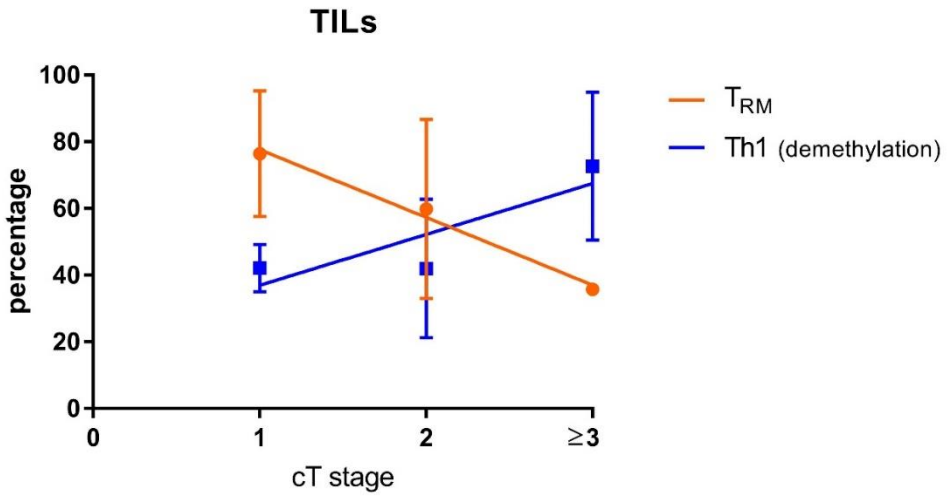

Figure 4. Trends in population proportions stratified on cT stage. TILs extracted from Tur-B specimens. Proportion of $CD8^+$ T_{RM} cells (orange) within the $CD8^+$T cell pool decreases with higher cT stage, but the Th1 cells (blue) increase. Th1 cells defined as IFNG demethylated.

Regardless of our findings in paper II and III, it is important to point out that we have not investigated the antigen specificity in our studies. Instead, we base our conclusions on tissue localisation. Hence, it is not straightforward to make assumptions that all lymphocytes found within or adjacent to the tumour are tumour-specific. However, it has previously been established that adjacent LNs holds tumour antigen-specific lymphocytes [95]. Moreover, we have convincingly demonstrated, in patient belonging to the same recruitment study, [94, 96] that cells derived from SNs can be activated and proliferated *in vitro* in response to autologous tumour homogenate. However, there has not yet been any epigenetic investigations on the $CD4^+$ or $CD8^+$ T lymphocytes following these proliferation experiments.

When combining the findings in Paper II and III it is plausible that a Th1/Tc1 response is of major benefit for prognosis. These speculations have support in the literature [97, 98] and hence, our data only further reinforce this consensus.

4.6 DNA METHYLATION AND GENOTYPE VARIATIONS IN *NR3C1* IN STERILE INFLAMMATION COHORT

In Paper IV we wanted to investigate the role of DNA methylation in the Glucocorticoid receptor gene *NR3C1* in the clinical setting of post-surgical sterile inflammation. This condition is a well-known complication following major surgery [99]. The first line of treatment is immunosuppression by corticosteroids, but this has an uneven effect between patients. The high variability in responses to the drugs cannot be correlated to pre-surgical endogenous cortisol levels, nor to the level of GR expression in peripheral blood cells [100, 101]. Hence, we hypothesised that the differences in response in-between patients could be due to differential methylation patterns in the *NR3C1* gene. It has previously been established

that DNA methylation pattern in the 3kb CpG island within this gene is a product of early life events and that this imprinting has an impact on neurobehavioral outcomes later in life [102, 103]. If our hypothesis was correct, the pre-operative blood samples should display a high variation of methylation level in this locus, as has previously been demonstrated [104], and that this would correlate to clinical outcome. This was however not the case. Instead, our small cohort of 24 patients showed no variation in methylation (Figure 5). Thus, the methylation levels did not reveal any differences in correlation to any clinical readouts of outcome/outcome parameters.

Figure 5: Heat map of all 319 CpG sites in NR3C1 locus chr5:143402.505-143.405.805. Apart from the last CpG, all sites are demethylated. Every row is one patient, every column a CpG site.

There could be a number of reasons for our lack of variable methylation levels. First, our cohort may not reflect a cross section of the general population, but is biased by the clinical implications, although pathologically different, for which they are enrolled in major surgery. Another important factor, is that the studies made on early-life imprinting is conducted in brain tissue. The disease states investigated are to a high extent also neuro-behavioural conditions. This makes me speculate that specific cell type is of immense importance when studying DNA methylation. Our rational for hypothesising that we would see differences in whole blood, was (apart from others demonstrating it in healthy donors [104]) that the GR is expressed by virtually all nucleated cells and since the sterile inflammation is systemic, the blood would be a natural tissue to use for readout in this setting. As it turned out, there was no variation in these pre-surgical samples, and it is hard to predict if sorted cell populations (e.g. neutrophils, which would stand for the major expansion upon acute inflammatory response) would have differential DNA methylation levels, compared to e.g. lymphocytes. It is, difficult to speculate to what extent this would have made a difference in our results. Nevertheless, investigations of DNA methylation should be carefully examined in dissected cell populations, and correlated to cellular and biological function, prior to establishing methods that can use DNA methylation solely to make conclusions.

Despite our rejected hypothesis, we further analysed the sequence data obtained. By multiple factor analysis we first ruled out that any of the clinical parameters available to us had a great impact on our model. Worth mentioning is that two variables emerged from this; sex and benign disease. The first two dimensions, however, only accounted for 7,1 % and 6,7 %,

respectively, of the variance in the material. Hence we concluded that none of these parameters had the power to significantly skew the model.

The multifactorial partial least square (PLS) regression based analysis integrated the whole sequence (recovered from the bisulfite sequencing) and correlated it to outcome, as defined by Clavien-Dindo Score. We identified four SNPs that had an impact on outcome.

The SNPs discovered in our analysis have all previously been identified and two of them have been studied in various settings (Paper IV, table 2). Although investigated in clinical settings different from ours, it is clear that genotype in the *NR3C1* gene has an impact on clinical implications [105-107].

Due to our small cohort, it is difficult to draw any conclusions on how these SNPs impact the outcome, but on the basis of our results, in coherence with other findings, it is likely that simple genotype investigations on a much larger cohort would be sufficient to make greater conclusions on clinical outcome, when it comes to post-surgical sterile inflammation.

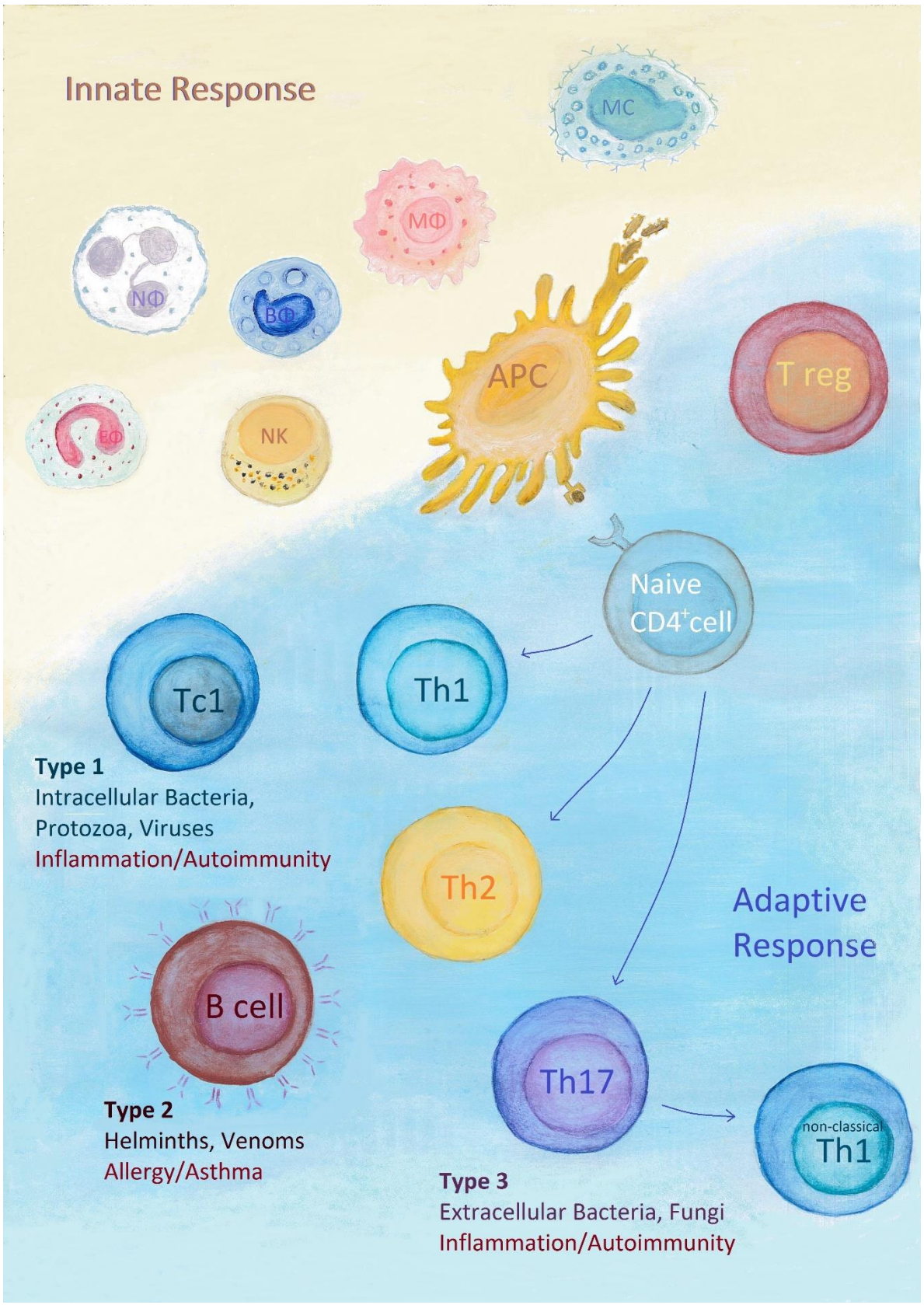

Figure 6. An artistic summery of the basic routs the adaptive immune system can take. With three major adaptive arms, type 1, type 2 and type 3 immunity sufficiently explains the different courses of immune responses seen in health and disease. In the middle is the APC, connecting the innate and adaptive immune system.

5 CONCLUDING REMARKS

The collected work in this thesis has used DNA methylation as a tool for readout of the (innate and adaptive) immune responses in different clinical settings, such as RA, MS, UBC and sterile inflammation. Although widely different in their manifestations, and underlying cause, these studies have helped to shed some light over the involvement of inflammation in these pathological states. In addition, the results presented herein demonstrate the possibility of using DNA methylation to interpret disease states, but also that it is of great importance to thoroughly investigate cell specific features. When properly scrutinized, simple assays could be developed and used for clinical assessments.

In the autoimmune diseases RA and MS, we investigated the $CD4^+$ T cell population in blood and synovial fluid. We conclude that in both of these autoimmune diseases, there seem to be a difference in immune response on an individual level, which possibly reflects differential cause and development. In the tumour setting, we demonstrate that an overall greater immunological response correlates with better prognosis. When it comes to patients developing sterile inflammation, we conclude that genotype investigations could be a way forward to elucidate individual reactions.

A major strength with the studies within this thesis is that primary human cells were used throughout. No tampering of cells, such as *in vitro* stimulation and differentiation, was needed to conduct most of these investigations [108]. This means that the pattern seen in the cells from the respective tissues is a snapshot of the true clinical picture. Although solely using DNA methylation only tells part of, and not the full story of how active the immune system actually is at present, it conveys in what direction and to what extent the body is leading the response.

My overall conclusions regarding the adaptive immune response is that the dissection into type 1, 2 and 3 immunity, as previously proposed by many, sufficiently depicts the overall immune responses mounted by the human body (Figure 6), and that DNA methylation can be used to interpret this.

The future goal is to use the results presented herein, on an individual basis, to evaluate the patient's probability of responding to treatment. Furthermore, these data should be taken into consideration upon development of new, cell-based immunotherapies, or immunomodulatory therapeutic approach.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Alla celler i kroppen innehåller samma DNA. Trots det har vi hundratals olika typer av celler som ser helt olika ut och kan göra helt olika saker. Det beror på att även om alla celler innehåller samma information, som en stor handbok, läser olika celler olika delar av boken (se Figur 1, sida 1). Vilka "kapitel" som är tillgängliga att läsa är reglerat av Epigenetik; "ovanför genetiken". Epigenetisk reglering är således det som gör DNA åtkomligt eller inte. Det finns olika nivåer av epigenetisk reglering. Vissa påverkar histoner, proteiner som rullar upp DNA på spolar, och på så vis omöjliggör avläsning av denna genetiska region. En mer specifik reglering av enskilda gener är DNA-metylering. Ofta finns det kluster av dessa metylgrupper i så kallade CpG-öar, och dessa utgör regulatoriska element. Genom tillförsel eller avlägsnande av metylgrupper från DNA-sekvensen kan uttrycket av gener kopplade till denna region regleras. I den här avhandlingen har jag studerat DNA-metylering i celler från immunförsvaret.

Immunförsvaret är uppdelat i det medfödda och det adaptiva immunförsvaret. Medan det medfödda ger ett snabbt men ospecifikt immunsvar tar det adaptiva upp till en vecka på sig för att skapa ett immunsvar som perfekt känner igen patogenet. Patogen kan vara allt från bakterier, virus eller annan infekterande agent, men även cancerceller och ibland den egna vävnaden, vilket resulterar i autoimmuna sjukdomar. Olika hot ger skilda adaptiva immunsvar (se Bild 6, sida 26).

I första arbetet sätter vi upp en metod för att studera olika T-hjälparceller för att kunna utvärdera i vilken riktning det adaptiva immunförsvaret är vridet. Genom att använda fyra gener som är specifika för vart och ett av flera olika potentiella immunsvaren analyserar vi celler från friska personer såväl som från patienter med reumatoid artrit och multipel skleros. Vår slutsats är att metoden fungerar bra och att det finns många individuella skillnader mellan patienter med samma diagnos. Det är en fördel att veta innan det bestäms vilken behandling som ska sättas in, men även för att följa hur behandlingen påverkar immunförsvaret.

I det andra arbetet tittar vi på samma gener i T-hjälparceller i patienter med urinblåsacancer. Genom att undersöka T-hjälparceller från både blod, tumör och lymfkörtlar får vi en bättre bild av vad som händer i och omkring tumören. Vår slutsats är att de patienter som har större proportioner av sina T-hjälparceller vridna mot ett starkt immunsvar har en bättre prognos. Vi visar också att de patienter som svarar på behandlingen med cytostatika har ett starkare immunsvar än de som inte svarade på behandlingen.

I det tredje arbetet fortsätter vi att studera samma patienter och material, men nu i cytotoxiska T-celler. Vår slutsats är att dessa celler är temporärt avstängda i tumörer, förmodligen till följd av att tumören sänder ut negativa signaler. Vi visar däremot att om vi ger ny stimulans till dessa cytotoxiska T-celler utanför kroppen, i laboratoriet, kan de aktiveras på nytt. Detta skulle kunna vara ett sätt att ta tillvara på de celler som redan finns i tumören och därmed förmodligen känner igen den som ett hot. Våra resultat pekar på att det skulle gå att behandla patienten med sina egna cytotoxiska T celler. Denna metod (kallad cellulär immunterapi) är tidigare utvecklad för ovan nämnda T-hjälparcellerna.

I det fjärde arbetet undersöker vi istället alla celler i blod från patienter som senare

genomgick en stor bukoperation. En känd komplikation vid stora operationer är utvecklandet av en steril inflammation. Det är en inflammation som är orsakad av själva traumat som operationen innebär. Människor reagerar väldigt olika på dessa operationer och vissa patienter utvecklar en steril inflammation, trots att de behandlas likadant. Behandlingen som ges är kortison som har en immunhämmande effekt. Kortison binder till Glukokortikoidreceptorn (GR) som finns på nästan alla celler i kroppen. Genen som styr GR (*NR3C1*) är tidigare känd för att vara reglerad av DNA-metylering. Därför utredde vi om olika metyleringsmönster kunde vara orsaken till de varierande svaren på kortison-behandling mellan patienterna. Det visade sig att så *inte* var fallet. Däremot hittade vi fyra SNPs (single nucleotide polymorphims)som kunde påverka utfallet av den sterila inflammationen. SNPs är en typ av variationer i genomet där endast en nukleotid (bas) i DNA-sekvensen skiljer sig åt och finns i varierande grad hos befolkningen. Vi summerar att vår studie är för liten för att kunna dra långtgående slutsatser kring dessa SNPs på ett tillförlitligt sätt, men vi tror att det skulle gå att utreda vikten av dessa sekvens-variationer om en större grupp patienter inkluderades.

Mina sammanfattande slutsatser är att det går att använda analys av DNA-metylering för att påvisa olika adaptiva immunsvar i specifika immunceller, vilket kan vara till stor hjälp inom sjukvården för att utvärdera prognos och hur bra kroppen svarar på behandling. Det går också att använda för att utveckla nya biologiska och immunologiska behandlingsmetoder. Däremot verkar det vara av stor vikt att titta på specifika celltyper, även när det gäller en gen som uttrycks i många celltyper.

Jag hoppas att min forskning kan komma till användning för utvecklingen av behandlingar och diagnostiska metoder för framtida bruk inom sjukvården.

7 ACKNOWLEDGEMENTS

I have spent a decade, nearly a third of my life, at **L2:04**[1]. During this time, a lot of great events have come to pass, in life and lab. To this end, there are numerous people whom I wish to acknowledge. As music is my greatest passion, I've also put together a playlist over the years, with music that says a lot.

First, and foremost, it is with gratitude I thank my main supervisor, **Ola Winqvist**[2] for taking me on in 2008. You encourage everyone to bring their game and be the greatest person they can be, and I truly believe that I've learned and evolved as a researcher and a person under your leadership (throughout these years).
Per Marits[3] my second co-supervisor, and second main supervisor, for reassuring talks during project doubts (telling me the era of "just finding a new single disease antigen" is over) and for immense help during writing, throughout all stages of my PhD studies. Also, for being a likeminded spirit in public discussions of politics (consensus wouldn't make a good discussion).
Louise Sjöholm[4], my third co-supervisor, for taking me on in times of great need, opening your lab and guiding me to all the necessities in the field of epigenetics. Also, for reminding me that moms can do anything and everything. This thesis would have looked very different and poorer without you.
My first supervisor in practice, as well as my first co-supervisor, **Peter Janson**[5]. I can't beginner to thank you for teaching me both all the laboratory skills, and also how to be "street smart" in the lab (a mind-set I didn't start practicing until years after you left).

My mentor, **Kai Ödeen**[6], professor (emeritus) at Kungliga Tekniska Högskolan, for being the greatest mentor ever, advising and supporting in stormy weathers. For not mentioning that I didn't attend *the greatest school*, and for being present and supportive from day 1 of my life.

Amir Sherif, although not formally my supervisor, you have been of indispensable help during the last years of my studies; in knowledge, in writing, and in cheering on when I'm feeling rueful. Your dedication to the field of urology is incomparable, in practice and research, and I can't thank you enough for all the work you've put down for me and others.

All my **co-authors**, for the most diverse types of help during lab work, collection of materials, writing and cheering me on.

The members of Ola W group, **Adi**[7], for filling the vacuum from all the once that left, with friendship, intellectual and laboratory collaborations, parties, work out, food and wine; Always! **Evelina**[8], for long-lasting friendship, sharing everything from Maggio to salty butter. Prince **Ali**[9], for bringing that well needed smile, reminding me that by worry I make it

double. When in doubt, you can always go downtown. **Sofia B**, for your immense contribution to the group, the work within this thesis and for being my IBS sister in arms. **Ludvig**[10], for not getting arrested that time when running around royal Haga, for letting "America" soften your heart to First aid kit, and for merely letting me be the second most tipsy at AW. **Emma L**[11], for taking all the harsh epithets, so that I was spared, and for being an adult gateway to Harry Potter, red wine and anchovy olives. **Johan K**, for tagging along above mentioned. **Malin**, you are truly a superwomen. You're my go-to person with all life matters. **Lu**, for being my supplier of coconut candy. **Kurt**, for bringing me stories from the bright side of my origins. **David**, for sharing the journey from Basåret all the way to defence, and for keeping the bisexual discussion going. **Mona**, for reminding me that a functional brain is above all other priorities, and for letting me shoulder the mini-Mona mantel of personal integrity. **Jin**, for your endless patience with me learning 5 different mao. **Max**, for all your patience with me, regarding everything from psychological conditions to factor analysis. I believe everything you say. **Michael M**, for believing in my abilities and not judging me for not liking violent sports. **Christian L**[12], for holding the line during endless late night lab work featuring E&A's playlist and for never turning down a party. **Robert W**, for your authority skepticism (although I don't always share it). **Augusta**[13], for great Disney duets, HP love and restaurant tips in Lund. **Philipp**[14], for embracing the *Pompeii* feeling during lab late nights.
Victor Blenow, whiteout you I would probably not have finished this book by now.

All my **students**[15] throughout the years; **Deepika**, **Pia** and **Anna-Chiara**, **Axel**, **Sofie Solmaz** and **Maxi**, thanks for letting me be the Queen Bee for a while. A special thanks to **Sofie Önnestam**[16] who illustrated the picture in this thesis. I have a lot to thank you for.

The laboratory environment at **L2:04**[1] is widely known for being something extra. Everyone passing through, long term or briefly, has contributed to this, and I am forever grateful for the helpfulness and warmth that has permeated all my years here. I would like to acknowledge a few present and former members of the epic and ending era of L2:04.

Annika Scheynius and **Marianne Van Hage**, your leadership is inspiring. You are role models for every woman in the scientific world that is still dominated by men.
The other group leaders, for sharing this openness to students that didn't officially belong to you; **Susanne**, for being an inspiring scientist as well the person to rely on for sauna. **John**, for being my unofficial mentor. **Eduardo**, for inviting me to your group the moment you started, for pushing me to meet with invited speakers and for giving me lifelong access to your fruit basket. **Gunnar**, for helping out with lab books from ancient days (Mats'), **Karin** and **Anna**, for bringing new fuel into the scientific fire.

New and long gone lab friends, in no particular order; **Rosanne**[17]**,** for never missing a HP reference, and thanks for the recent "nature vs nurture" comment. **Martina**[4]**,** I'm still dreaming of your risotto. Keep up the fierce scientific spirit. You'll go far! **Muksheed**, for

being my “knight in shining armour” when it comes to PCR machine booking. **Oscar**[18], for being my new dance partner. **Stefanie**[4], for pushing me to my first midnattsloppet, and for being on the same page regarding life, priorities and late night snacks. **Liz**, for joining me in carrying the party queen crown. **Konrad**[19], for being a true friend from the right side of town. **Anna A**[20]**,** from Baltimore to managing the biohazard group. Absint is da shit! **Patricia**[21], for the dance battles at “ett ställe”. **Catharina J**, the lab was never the same since you left. **Jeanette**, for keeping up the police force throughout the years in cell lab. **Hans G**, for true party spirit, all the way home to Lidingö. **Klas**, for teaching me all the tricks with agarose gels and “lefty Lucy”. **Ola N**[22], since you left there’s no queen in the lab. **Emilie**[21], no one made movies like you! Thanks for teaching me some tricks. **Anna-Maria**, for being my first lab mate at biomed program. **Karen**, for an unexpected friendship and a great hen party! **Cindy**[23] for loving Danny Saucedo and for introducing me to the dark room. **Annika J**, for all the help in the final stages of my studies, and for being the saviour when me and my family needed last minute bank help for moving.

Biomed buddies **Susanna** and **Elisabet**. I still can’t believe I passed it all up to here. I’m sure your great spirits and our ”hydrogen-jon”-sång had a great deal to do with that. I miss our board game nights.

DSA (inkl. Ola N, Arash H and **John Steen)** and the members of “kurs- och programmkommittén” for teaching me how KI *really* works. And for keeping me for so long.

During my past education, there are two people I’d like to thank especially;
Claes Abrahamsson and **Anna Högberg**. During my time at Gångsätra , you two contributed greatly to my continuous education and career in the field of natural sciences, and for that I’m forever grateful.

Mina vänner utanför lab och utbildning, **Blanka**[24], från dag ett har du varit min allra bästa vän. Tack för att du stått ut med mig i vått och torrt. Du är en bättre vän än jag förtjänar.
Malin[25], från galna spanskalektioner till F*cking Åmål och hem igen. Kanske är det dags att slå slag i saken med ”kiss me”? **Emma och Anna-Carin**[26], Tänk när vi pratade om att starta ett radioprogram med Gurra! Och förlåt för att jag lånade eran Magnus Uggla-CD så länge. Jag hoppas att i fått den tillbaka… **Talar**, för att vänskap kan födas och frodas helt utan näring! **Fofo**, för att du ser till att hålla ihop gänget, och för att du var den första killen jag känt som dansade (back in Hastings).
Dagisvänner, **Lotta och Karin**[27], tänk att vi känt varandra hela livet. Oavsett var i världen ni är så finns ni alltid med i tanken. Tack för att ni fortfarande finns i mitt liv.
André[28], tack för att du varit min bästa killkompis någonsin. Du har varit med när allting hänt. Viva forever!

Everyone else, who I forgot to mention in stressful time. I’m sorry for not being a more present friend.

My “in-laws”, min ingifta familj. Tack **Görel**, för den välbehövliga småstadssemester ditt hem ger oss, och för att du har förståelse för kämpigt småbarnsliv. **Johan**[29], för att du står ut med alla mina, sent påkomna, musikprojekt. Du är en fantastisk musiker, producent, it-tekniker, konsult etc. **Bengt**, för att du är ett levande bevis på att *uppfinnare* fortfarande är ett yrke. Det är väldigt inspirerande.
Tommy, du är den bästa lekledaren och tillför även lite mer (behövlig) händighet till familjen Ahlén.

Min familj, **Mamma Barbro**[30], för att det inte gått en dag då du inte trott på mig. För att du pushat mig och bevisat för mig att jag kan åstadkomma allt. **Pappa Bertil**[31], bara du och jag vet det riktiga värdet av *egentid*. Ni två har gett mig allt, och jag kan stå för att jag är ett äkta curlingbarn! **Min syster Victoria**[32], ingen lyssnar som du. Tack för att du alltid finns där.
Mattheo, du är mosters solstråle!

Till sist vill jag tacka de mina, för att ni vet att denna bok inte definierar mig som människa/fru/mamma. **Felicia**[33] och **Simon**[34], ni är mitt allt! Tack för att ni förstår att en bok inte skrivs på en dag. Min äkta man, **Kristian**[35], för att du står ut med mig när jag knappt står ut med mig själv. *Det finns inte ord nog.*

PLAYLIST REFERENCES

	Song	Artist
1	Changing	Sigma, Paloma Faith
2	(I used to want to be a) Cowboy	Chris LeDoux
3	Guld och döda skogar	Imperiet
4	Runt the world (girls)	Beyoncé
5	Human	The Killers
6	Sommar, sommar, sommar	Radions underhållningskorkester
7	When we were young	Adele
8	Dancing on my own	Robyn
9	Downtown	Petula Clark
10	Fjäriln vingad syns på Haga	*Carl Michael Bellman*
11	Hey ya!	Outcast
12	Son of a preacher man	Aretha Franklin
13	Belle	Sofia Källgren
14	Pompeii	Bastille
15	Royals	Lorde
16	Firework	Katy Perry
17	Do the Hippogriff	Weird Sisters
18	Séra Séra	Shakira
19	Från Djursholm till Danvikstull	Orup
20	Good morning Baltimore	Nikki Blonsky
21	I gotta feeling	The Black Eyed Peas
22	Don’t stop me now	Queen
23	In the club	Danny Saucedo
24	Sanna Vänner	Carola
25	Underground	Broder Daniel
26	Pom pom	Magnus Uggla
27	More than words (Mer än snor)	Extreme
28	Graduation (Friends forever)	Vitamin C
29	Music	Madonna
30	Because you loved me	Celine Dione
31	Easy Silence	Dixie Chicks
32	Håll om mig hårt	Panetoz
33	Vart jag än går	Stiftelsen
34	Vart du än går	Lisa Nilsson
35	Pure shores	All Saints

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