Clinical Implications of DNA Methylation for Kidney Transplantation

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Colofon

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Listen with curiosity
Speak with honesty
Act with integrity

Roy T. Bennett,
The Light in the Heart

Contents

Part I – Introduction

Chapter 1 General introduction and objectives 11

Part II – DNA methylation and the *in vitro* environment

Chapter 2 Interferon-gamma DNA methylation is affected by mycophenolic acid but not by tacrolimus after T-cell activation – *Frontiers in Immunology, 2017* 25

Chapter 3 Epigenetic changes in umbilical cord mesenchymal stromal cells upon stimulation and culture expansion – *Cytotherapy, 2018* 47

Part III – DNA methylation in organ transplantation

Chapter 4 Clinical potential of DNA methylation in organ transplantation – *Journal of Heart and Lung Transplantation, 2016* 77

Chapter 5 Variations in DNA methylation of interferon gamma and programmed death 1 in allograft rejection – *Clinical Epigenetics, 2016* 97

Chapter 6 Differentially methylated regions in T cells identify kidney transplant patients at risk for *de novo* skin cancer – *Clinical Epigenetics, 2018* 119

Chapter 7 Disrupted regulation of serpinB9 in circulating T cells is associated with an increased risk for post-transplant skin cancer – *Accepted at Clinical and Experimental Immunology* 147

Part IV – Summary and discussion

Chapter 8 Summary and general discussion 177

Chapter 9 Nederlandse samenvatting 191

Part V – Appendices

List of abbreviations 199

List of publications 201

PhD portfolio 202

Curriculum Vitae auctoris 204

Acknowledgements (Dankwoord) 205

Part I

Introduction

The epigenetic mechanism DNA methylation

Deoxyribonucleic acid (DNA) stores the information necessary for all forms of life, including humans. DNA is a complex molecule composed of two DNA strands that coil around each other, known as the double helix. The building blocks of the DNA (nucleotides) are cytosine (C), guanine (G), adenine (A) and thymine (T), where the C is always coupled to the G and the A is always coupled to the T in the double helix structure. The specific order of the four different nucleotides is referred to as the genomic sequence and determines whether an individual has blue or brown eyes for example. DNA regions that code for a functional molecule (protein) are what we call genes and the average length of a human gene is 67,000 nucleotides[1]. Humans have approximately 19,000 protein-coding genes and these comprise 1-2% of the complete human genomic sequence[2].

Inside the nucleus of the cell, the DNA sequence of a gene is transcribed into messenger RNA (mRNA), a molecule that functions as an information-carrier between DNA and protein. This mRNA is then translated into protein and the different proteins that are produced within a cell largely determine the function of that cell. However, not all genes are translated into protein, genes can be active, producing a lot of protein, or silenced, producing little to no protein, this is referred to as gene expression levels.

Tight regulation of gene expression is essential in maintaining proper cell function and this regulation is done by epigenetic mechanisms. These epigenetic mechanisms influence gene expression without changing the underlying genomic sequence of the DNA and therefore represent the interface between the genomic information and the environment. Three main categories of epigenetic mechanisms can be identified[3] (Figure 1). The first is DNA methylation, which is the covalent addition of a methyl-group (CH_3) to the cytosine in the DNA and, to this day, the most studied and best-understood epigenetic mechanism. The second category is post-translational modifications of histones, these are the proteins around which the DNA is wrapped. Modifications of histone proteins involve acetylation, phosphorylation, methylation and more. The third category is the higher-order 3-dimensional structure of the DNA such as loop formation and positioning of the DNA inside the nucleus. All these epigenetic mechanisms together determine whether a specific gene is accessible for gene transcription. In this thesis we will focus on DNA methylation as the epigenetic mechanism of interest.

DNA methylation in mammals occurs almost exclusively on cytosines (C) that are followed by a guanine (G) in the DNA, referred to as a CpG dinucleotide or CpG site. The methyl-group is present on both strands of the DNA and is copied onto the daughter-strand during DNA replication by the enzyme DNA methyltransferase 1 (DNMT1). DNA methylation

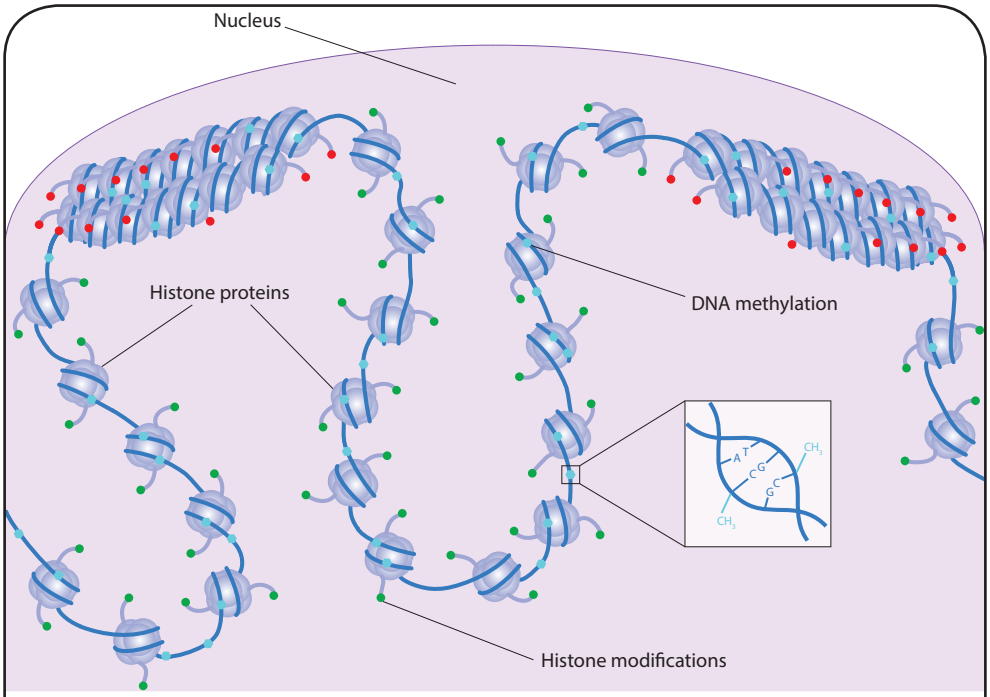

Figure 1. The three main categories of epigenetic mechanisms. *The light blue hexagons represent DNA methylation, which is depicted in more detail within the square. The DNA double helix is wrapped around 8 histone proteins, the histone tails can be modified to repress gene expression (red dots) or to activate gene expression (green dots). In genomic regions where the DNA is tightly packed genes are silenced and in open genomic areas genes can be expressed.*

can also be introduced to previously unmethylated sites by *de novo* methyltransferases (DNMT3a, DNMT3b). Removal of the methyl-group occurs either passively during cell division or actively by ten-eleven translocating enzymes (TET)[4]. In most cases, high DNA methylation in the promoter of a gene is associated with gene silencing. The methylation complicates binding of transcription factors to initiate transcription and may recruit other gene repressing epigenetic marks[5]. Whilst promoter DNA methylation regulates gene expression at close proximity in the genome, the effect of DNA methylation outside promoter regions is less clear[6]. Recently, more research is focused on DNA methylation within enhancer regions, which are regulatory regions typically located far away from the genes they regulate[7].

Cellular identity and differentiation

As explained previously, DNA methylation changes gene expression without changing the underlying DNA sequence. This is a crucial concept in cellular identity, since essentially all

cells in the body have the same DNA sequence whilst different cell types have very different functions. Changes in DNA methylation profiles play a critical role in the differentiation of stem cells and progenitor cells towards differentiated cell types[8]. For example T cells, which are derived from hematopoietic stem cells and play a central role in adaptive immunity, experience demethylation of lineage-specific genes during hematopoietic differentiation[9,10]. Once the T cells are matured and the CD4 (T helper cells) and CD8 (cytotoxic T cells) phenotypes are established, they leave the thymus as naive T cells. Naive T cells are characterized by high DNA methylation of T cell effector genes such as *interferon gamma* (*IFNγ*)[8] and *programmed death 1* (*PD1*)[11]. Upon recognition of antigen via the T-cell receptor, naive T cells will differentiate into effector cells and eventually memory cells. During differentiation, demethylation of effector genes ensures that the appropriate gene expression profile is established[12,13].

DNA methylation as biomarker

Even though cell identity is largely determined by the DNA methylation profile, there is a degree of plasticity in DNA methylation. Environmental conditions such as diet[5], psychological stress[14] and exposure to chemical components[15] have shown to affect DNA methylation, leading to long-term phenotypic effects. An excellent model to study environmental effects are identical twins[16,17]; they have exactly the same DNA sequence whilst different environmental conditions can lead to different DNA methylation profiles[18]. Disease-discordant twin studies have been used to identify DNA methylation differences associated with autoimmune disorders such as systemic lupus erythematosus[19] and psychiatric disorders such as bipolar disease[20]. In addition, epigenome-wide association studies (EWAS) are increasingly identifying DNA methylation differences associated to disease[21], highlighting the potential of DNA methylation as biomarker. In oncology there are several well-established DNA methylation biomarkers such as *VIM* methylation for colorectal cancer[22], *SHOX2* for lung cancer[23,24] and *MGMT* for glioblastoma[25]. The current challenge in the field of epigenetics is to move from demonstrating an association with disease to elucidating the etiological role of DNA methylation changes in human disease[26,27].

Measuring DNA methylation

Methylated cytosines are not detectable by regular DNA sequencing methods and if the DNA needs amplification by polymerase chain reaction (PCR), the methyl-group disappears. To circumvent this problem, the DNA can be treated with sodium bisulfite to induce methylation dependent changes to the DNA. With this chemical treatment, unmethylated cytosines are converted to uracil (U), which is usually found in RNA, whilst methylated cytosines are protected from this conversion[28] (Figure 2A). During subsequent PCR the uracil is then copied as a thymine (T) (Figure 2B).

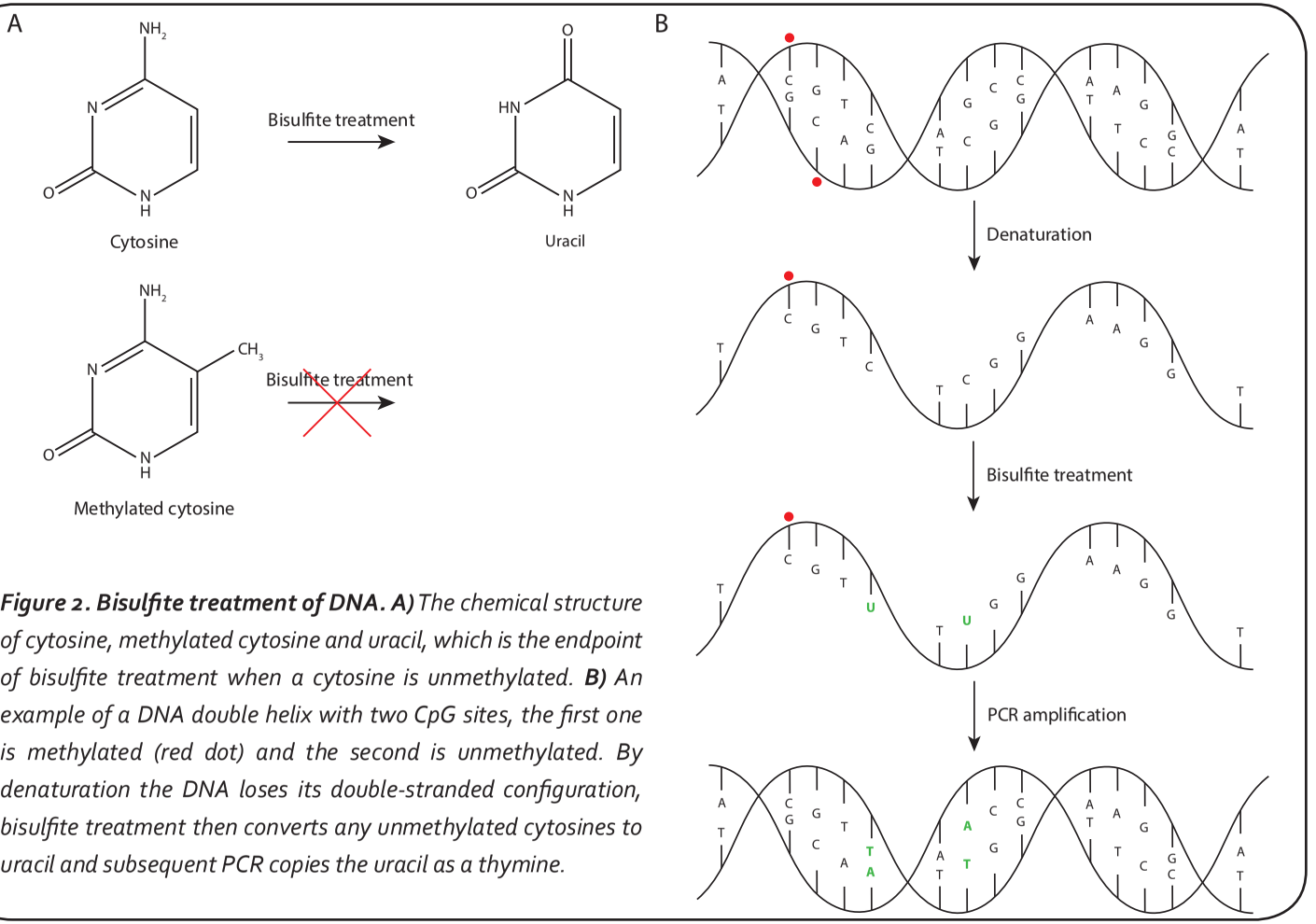

Figure 2. Bisulfite treatment of DNA. A) *The chemical structure of cytosine, methylated cytosine and uracil, which is the endpoint of bisulfite treatment when a cytosine is unmethylated.* ***B)*** *An example of a DNA double helix with two CpG sites, the first one is methylated (red dot) and the second is unmethylated. By denaturation the DNA loses its double-stranded configuration, bisulfite treatment then converts any unmethylated cytosines to uracil and subsequent PCR copies the uracil as a thymine.*

After bisulfite treatment, several methods are available to measure DNA methylation at a single site resolution. An example of a targeted method to measure DNA methylation is pyrosequencing, which can quantitatively measure DNA methylation of a region of up to 200 base pair (bp) per sequence reaction[29]. After bisulfite treatment and PCR of the target sequence, the real-time incorporation of nucleotides is detected by an enzyme-mediated light flash whenever a specific nucleotide is built in. The percentage methylation for a single CpG site is then calculated from the ratio of the thymidine and cytosine peak intensities at the site of interest. Within a single cell two chromosomes, thus two copies of each CpG site, are present and the percentage methylation can be 0%, 50% or 100%. Most often a sample contains multiple cells and the percentage therefore represents the average methylation for all the DNA molecules within the sample.

There are also methods that measure DNA methylation at a genome-wide scale such as the 450k (>450.000 CpG sites) or EPIC (>850.000 CpG sites) methylation arrays by Illumina[30]. These arrays consist of a glass slide with small pieces of DNA (probes) attached that specifically bind sequences of the bisulfite treated DNA, the probes are specific for a methylated or an unmethylated site. The array covers not only 99% of known human genes but also intergenic regions, microRNA promoters and regions that were previously identified as differentially methylated in a wide range of tumor types. The EPIC array additionally covers many recently identified enhancers[31]. The methylation values are expressed as a beta-value between 0-1, where 0 represents unmethylated and 1 represents fully methylated.

Organ transplantation

Organ transplantation is the best treatment option for patients experiencing end-stage organ failure[32]. Heart, lung, liver and kidney are among the majority of transplanted organs, whereby liver and kidney transplantation occur most frequently. In the Netherlands, 950 to 1000 kidney transplantations are performed each year[33] of which around 200 in our center, Erasmus MC. To prevent an immune response by the recipient towards the donor organ, transplant recipients require lifelong immunosuppressive treatment. Nowadays, maintenance immunosuppressive treatment after kidney transplantation consist of a proliferation inhibitor such as mycophenolate mofetil (MMF), and a calcineurin inhibitor (CNI) such as tacrolimus[34]. These immunosuppressive drugs suppress immune cells, including T cells since these cells play a key role in the recipients' immune response towards the allograft.

Complications after kidney transplantation

Even though quality of life improves significantly after transplantation, there are several

complications that transplant recipients can experience. Despite immunosuppressive treatment, acute rejection of the graft still occurs in up to 20% of the kidney transplant recipients[35]. Acute rejection is defined as a rejection episode that develops within a short time-frame and is associated with a sharp decrease in kidney function. Acute rejection is, in most cases, treated successfully with high dosages of steroids[36]. Chronic rejection, a process that develops on the long-term, is more difficult to treat and may lead to graft failure and even death. The current gold standard to diagnose a rejection is a biopsy, in which tissue damage and infiltrating immune cells can be assessed. This is an invasive method with sub-optimal sensitivity[37], specific and sensitive prediction tools for rejection that can be analyzed non-invasively are still lacking[38].

T cells play a key role in the rejection process. Before encountering any antigen, T cells are in a naive cell state. After recognizing the donor antigen, presented to the T cells by antigen presenting cells (APC), T cells will differentiate towards the effector cell state and produce immune signaling molecules called cytokines to alert and recruit other immune cells to the organ. These cytokines induce proliferation and differentiation of the T cells and, once recruited to the allograft, the CD8-compartment of the T cells (cytotoxic T cells) will induce cell death by apoptosis of the target (donor) cells. As a result of encountering an antigen, some T cells will differentiate into a memory state that, upon re-encountering the same antigen, can more rapidly respond than naive T cells. In addition to the cellular immune response, T cells may also activate B cells to produce donor specific antibodies, thereby contributing to a humoral immune response. These immune processes can lead to tissue damage and thereby compromise the function of the allograft. For these reasons, immunosuppressive treatment to suppress T-cell activity is an essential part of post-transplant care.

Complications other than rejection are often related to the systemic suppression of the immune system in transplant recipients which affects all immune responses, not only those directed at the graft. Increased incidences of infections and malignancies are very common in transplant recipients[39,40], associated with high morbidity and mortality in these patients[41]. Skin cancer is the most common malignancy in transplant recipients[42], specifically cutaneous squamous cell carcinoma (cSCC). Studies have shown a 65 to 200 times increased incidence of cSCC in transplant recipients compared to the general population[43,44] and a 30-year cumulative incidence of over 60%[45]. Risk factors include human papilloma virus (HPV) infection, history of sunburn, fair skin color, exposure to ultraviolet (UV) radiation, but most importantly a previous cSCC[42]; indicating that cSCC is often a recurring disease in these patients.

cSCC represents a high burden for transplant recipients and can significantly decrease

their quality of life. Treatment requires frequent hospital visits where surgical excision of the cSCC is often the treatment of choice for non-metastatic disease[46,47]. Early recognition and treatment of a pre-cancerous lesions such as warts or actinic keratosis reduces the burden for patients and may prevent development of an invasive malignancy. Preventing the development of cSCC is difficult, reducing sun exposure and applying adequate sun protection in combination with frequent screening to facilitate early detection is currently the recommended approach[47].

The immune system plays a conflicting role in post-transplant skin cancer patients: it needs to be suppressed to prevent rejection but at the same time it must be activated to provide anti-tumor immune surveillance. With this in mind, several studies have been conducted towards immune phenotypes associated to post-transplant cSCC. High number of T regulatory cells (Treg) and senescent T cells ($CD8^+CD57^+$) have been associated to post-transplant cSCC[48-50], but only to a recurrence of the cSCC. Tools to predict the development of a first post-transplant cSCC are currently unavailable.

Objectives of this thesis

Despite advances in surgical procedures and the development of better and more specific immunosuppressive drugs in kidney transplantation, complications such as rejection and malignancy remain problematic for transplant recipients. There is a need to explore novel and innovative methods to identify transplant recipients at increased risk for complications and thereby improve and personalize treatment for these patients. Since epigenetic mechanisms such as DNA methylation underlie changes in functional behavior, studying changes in DNA methylation may improve risk assessment for post-transplant complications.

The main objective of this thesis is to explore the role of DNA methylation changes in complications after kidney transplantation. To answer this two complementary approaches were employed.

- First, we aim to unravel if environmental conditions relevant in transplantation affect DNA methylation; by investigating the stability of DNA methylation in experimental, *in vitro* systems in the presence of immunosuppressive drugs and cytokines.
- Second, we explore whether DNA methylation profiles can identify kidney transplant recipients who are at increased risk for rejection or skin cancer after kidney transplantation.

In chapter 2, we describe the effect of the immunosuppressive drugs tacrolimus and MMF (active ingredient MPA), on DNA methylation of T cells. We investigated the changes in *IFNγ* DNA methylation after stimulation of the T cells in the presence of these drugs, both in total T cells and in naive and memory T cells. Chapter 3 focuses on the effect of cytokines added to the in vitro culture system as well as culture expansion alone, on the DNA methylation profiles of mesenchymal stromal cells (MSCs) as a model system. MSCs are an interesting cell type to study in transplantation since they have immunomodulatory and regenerative capacities. Here we applied a genome-wide analysis of DNA methylation instead of a targeted analysis.

In chapter 4, the potential of DNA methylation in organ transplantation is introduced. We reviewed the literature and provide an overview of the clinical potential of DNA methylation as a biomarker for complications after transplantation and for monitoring the immune system. Chapter 5 describes DNA methylation of *IFNγ* and *PD1* in patients who developed a rejection after kidney transplantation. We focused on DNA methylation within the naive and memory subsets of the CD8+ T cell compartment. In chapter 6 we describe a different complication after transplantation: skin cancer. Genome-wide DNA methylation profiles of T cells were studied before transplantation, to identify patients at increased risk for skin cancer after transplantation. Chapter 7 then describes a disrupted regulation of serpinB9 as risk factor for post-transplant skin cancer. Here we studied DNA methylation profiles, RNA and protein expression of serpinB9 in circulating T cells after transplantation.

Chapter 8 summarizes and discusses the results described in this thesis and provides a perspective on the future implications of our findings.

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Part II

DNA methylation and the *in vitro* environment

Chapter 2

Interferon-gamma DNA Methylation is Affected by MPA but not by Tacrolimus after T-cell Activation

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Abstract

Immunosuppressive drug therapy is required to treat patients with autoimmune disease and patients who have undergone organ transplantation. The main targets of the immunosuppressive drugs tacrolimus and mycophenolic acid (MPA; the active metabolite of mycophenolate mofetil) are T cells. It is currently unknown whether these immunosuppressive drugs have an effect on DNA methylation - an epigenetic regulator of cellular function. Here, we determined the effect of tacrolimus and MPA on DNA methylation of the gene promoter region of *interferon gamma* (*IFNγ*), a pro-inflammatory cytokine. Total T cells, naive T cells ($CCR7^{+}CD45RO^{-}$) and memory T cells ($CD45RO^{+}$ and $CCR7^{-}CD45RO^{-}$) were isolated from CMV seropositive healthy controls and stimulated with α-CD3/CD28 in the presence or absence of tacrolimus or MPA. DNA methylation of the *IFNγ* promoter region was quantified by pyrosequencing at 4 hours, day 1, 3 and 4 after stimulation. In parallel, T-cell differentiation, and IFNγ protein production were analyzed by flow cytometry at day 1 and 3 after stimulation. Our results show that MPA induced changes in *IFNγ* DNA methylation of naive T cells; MPA counteracted the decrease in methylation after stimulation. Tacrolimus did not affect *IFNγ* DNA methylation of naive T cells. In the memory T cells, both immunosuppressive drugs did not affect *IFNγ* DNA methylation. Differentiation of naive T cells into a central-memory-like phenotype (CD45RO+) was inhibited by both immunosuppressive drugs, while differentiation of memory T cells remained unaffected by both MPA and tacrolimus. IFNγ protein production was suppressed by tacrolimus. Our results demonstrate that MPA influenced *IFNγ* DNA methylation of naive T cells after stimulation of T cells, while tacrolimus had no effect. Both tacrolimus and MPA did not affect *IFNγ* DNA methylation of memory T cells.

Introduction

Patients who have undergone organ transplantation as well as patients with autoimmune disease require lifelong immunosuppression to inhibit the immune response towards alloantigen or autoantigen. This immune response involves interaction between different immune cells including dendritic cells, macrophages, T and B cells. T cells proliferate, differentiate and produce effector cytokines in response to antigen[1,2] and therefore immunosuppressive drugs are often designed to suppress T-cell activity.

After activation, the differentiation of T cells is regulated to great extent by DNA methylation – an essential epigenetic regulator of several cellular functions[3-5]. DNA methylation is the addition of a methyl group on a cytosine (C) that is followed by a guanine (G) in the DNA, also known as a CpG dinucleotide. High methylation in the promoter region of a gene is related to a closed chromatin structure and transcriptional silencing of the gene[6,7]. When T cells differentiate during an immune response, the promoter regions of various effector genes become demethylated, thereby allowing the cells to upregulate these genes and produce effector cytokines[8,9]. Naive T cells are therefore characterized by methylated promoter regions of effector genes, whereas effector and memory T cells are demethylated at those regions.

Epigenetic regulators such as DNA methylation are dynamic and susceptible to cues from the environment[10,11]. These cues include internal factors such as cytokines and hormones as well as external factors such as food, toxins and drugs. Several common-used pharmaceutical drugs, not designed as epigenetic drugs, have an effect on epigenetic mechanisms in the cell[12,13]. These findings suggest that immunosuppressive drugs could affect DNA methylation in T cells and thereby modulate T-cell function.

Today, the immunosuppressive drugs that are most often prescribed to organ transplant recipients include tacrolimus and mycophenolate mofetil[14,15]. Tacrolimus represses the calcineurin pathway downstream of the T-cell receptor (TCR). It inhibits calcineurin phosphatase activity, thereby reducing levels of dephosphorylated nuclear factor of activated T lymphocytes (NFAT), which ultimately inhibits T-cell activation[16,17]. Mycophenolate mofetil's active ingredient is mycophenolic acid (MPA). MPA is an inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key enzyme in *de novo* purine synthesis[18]. Inhibition of IMPDH reduces synthesis of guanosine nucleotides, which are essential for DNA synthesis in T cells, resulting in reduced proliferation of T cells[19,20]. Despite the fact that the mechanism of action is largely known for these two drugs, it is not known whether their effect on cellular function involves epigenetic regulation, nor whether they affect the epigenetic regulation of cytokine expression. A further understanding of

the effect of different immunosuppressive drugs on epigenetic regulators of T-cell function will contribute to optimization of the immunosuppressive regimen.

We hypothesized that tacrolimus and MPA induce changes in DNA methylation of T cells. We focus on promoter DNA methylation of the pro-inflammatory cytokine IFNγ which plays a prominent role in immune responses. Not only have high expression levels of IFNγ been linked to acute rejection after organ transplantation[21-23], it is also highly expressed during the inflammation seen in autoimmunity[24,25]. IFNγ expression – along with that of many other cytokines – is known to be regulated by DNA methylation[26-28]. To study the effect of immunosuppressive drugs on *IFNγ* DNA methylation after activation of T cells, we stimulated T cells *in vitro* in the absence or presence of tacrolimus or MPA. After stimulation, DNA methylation was measured at two sites within the *IFNγ* promoter. Since DNA methylation is cell-type specific[29], the experiments were performed on total T cells as well as on isolated naive and memory T cells.

Materials and methods

Study subjects

Our study population consisted of 19 healthy individuals aged between 26-75 (68% female). Peripheral blood of these subjects was collected after informed consent and according to biobank protocol with approval of the local ethics committee (MEC-2010-022). We chose to study healthy individuals to eliminate confounding effects of disease on DNA methylation[30]. It is also known that *IFNγ* DNA methylation is significantly lower in CMV seropositive individuals than in CMV seronegative individuals[31]. To compose a homogeneous group and eliminate CMV effects on inter-individual differences in methylation levels, only CMV seropositive individuals were included in the study.

Isolation of total T cells, naive T cells and memory T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Chicago, IL, US). Isolated PBMCs were stored at -140°C until further use. Total T cells were isolated from the PBMCs by magnetic cell separation on the autoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the pan T cell protocol using the deplete S settings. Purities were >90% $CD3^+$ cells after isolation.

The naive and memory T-cell populations were isolated from the PBMCs using fluorescence-activated cell sorting (FACS) by the BD FACSAria™ II (BD Biosciences, San Jose, CA, US). The PBMCs were stained with CD3 Brilliant Violet 510 (Biolegend, San Diego, CA, US), CD4 Pacific Blue (BD Biosciences), CD8 APC-cy7 (BD Biosciences), CD45RO APC (Biolegend),

CCR7 PE-cy7 (BD Biosciences) and to exclude nonviable cells the cells were also stained with 7AAD PerCP (BD Biosciences). Naive cells were defined as CCR7+CD45RO-, central memory (CM) cells as CCR7+CD45RO+, effector memory (EM) as CCR7-CD45RO+ and the highly differentiated EMRA cells as CCR7-CD45RO-[32]. After cell sorting, the purities were >95% for each sorted fraction.

T-cell stimulation

The T cells were stimulated for 4 days with α-CD3/CD28 coated Dynabeads® (Gibco, Waltham, MA, US) in a bead to cell ratio of 1:1 at day 0. 50,000 cells were cultured per well in a 96-well plate. The cells were cultured in the absence or presence of tacrolimus, MPA or 5-aza-2'deoxycytidine (decitabine). Tacrolimus (Prograf®, Astellas Pharma, Tokyo, Japan) was added to the cells in a concentration of 10 ng/mL which is a clinically relevant concentration that is reached in transplant recipients[33]. MPA (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells in a concentration of 0.2 µg/mL, a concentration at which the cells are still able to proliferate. Our positive control, the demethylating agent decitabine (Sigma-Aldrich)[34], was added to the cells in a concentration of 10^{-6} M, a concentration at which the cells are still able to proliferate. Each drug-treated sample has a matched negative control (stimulation alone).

The cells were incubated at 37°C in 5% CO_2 and harvested at 4 hours, day 1, 3, and 4 for DNA methylation analysis and at day 1 and 3 for flow cytometry analysis. To assess viability and proliferation, the cells were counted before and after stimulation using conventional light microscopy and Trypan Blue staining (Thermo Fisher Scientific, Waltham, MA, US).

Flow cytometry

Flow cytometry was used to determine the phenotype of T cells immediately after isolation and at day 1 and 3 after stimulation. We also measured the percentage of IFNγ producing cells at these time points. The samples were treated with Brefeldin A (GolgiPlug™, BD Biosciences) for 16 hours prior to flow cytometry analysis. The monoclonal antibodies used for cell surface staining were the same as previously described for the FACS cell sorting. In addition, the cells were permeabilized using permeabilize solution 2 (BD Biosciences), and stained for intracellular IFNγ with FITC labelled IFNγ (BD Biosciences). The cells were then analyzed on the FACSCanto II (BD Biosciences) with FACSDiva software. All flow cytometry data were analyzed using Kaluza software 1.3 (Beckman Coulter, Brea, CA, US).

DNA isolation, bisulfite conversion and PCR

After harvesting, the cells they were pelleted, frozen in liquid nitrogen and stored at -80°C until bisulfite conversion. The T-cell pellets were digested with proteinase K and bisulfite

treatment was performed using the EZ DNA Methylation-Direct kit (Zymo Research, Irvine, CA, US) according to the manufacturer's protocol. Bisulfite treatment introduces methylation-dependent changes in the DNA, demethylated cytosines are converted into uracil whereas methylated cytosines remain unchanged. The bisulfite treated DNA was amplified by PCR. A 230 base pair (bp) region of the *IFNγ* promoter was amplified using the Pyromark PCR kit (Qiagen, Venlo, The Netherlands). A forward primer with the sequence 5'-ATGGTATAGGTGGGTATAATGG-3' and a biotin-labelled reverse primer with the sequence 5'-CAATATACTACACCTCCTCTAACTAC-3' (Sigma-Aldrich) were used, both at a concentration of 10 pmol/μL[31]. The PCR conditions were 15 minutes at 95°C, 45 cycles of 30 seconds 94°C, 30 s 58°C, 30 s 72°C followed by 10 min at 72°C and final storage at room temperature (21°C). Prior to pyrosequencing, the PCR product was visualized on a 1% agarose gel to verify the size of the amplicon. Two important CpG sites are inside this amplicon, CpG -186 and CpG -54. These sites are within binding domains of transcription factors[26,31].

Pyrosequencing

Pyrosequencing is an excellent technique to quantitatively measure DNA methylation at single CpG-site resolution, yielding accurate and reproducible results[35,36]. The *IFNγ* PCR product was sequenced using a PyroMark Q24 pyrosequencer (Qiagen). Minor adjustments were made to the manufacturer's protocol: to immobilize the PCR product 1 μL Streptadivin Sepharose High Performance Beads (GE Healthcare) was used per sequence reaction and annealing of the sequence primers was done for 3 minutes at 80°C. The CpG -186 sequence primer was 5'- GGTGGGTATAATGGG-3' and the CpG -54 sequence primer was 5'- ATTATTTTATTTTAAAAAATTTGTG-3', both at a concentration of 10 μM[31]. Two DNA methylation standards were used as control, human high and low methylated DNA (EpigenDx, Hopkinton, MA, US). Research shows that methylation at adjacent sites is correlated[37] therefore the methylation percentages of the two CpG sites, site -54 and -186, were pooled per individual and the mean DNA methylation percentage is presented in the results.

Statistical analysis

Statistical analyses were performed with SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, US). The Mann-Whitney U test was used for unpaired analysis to identify differences between the conditions at a certain time point. The Wilcoxon signed rank test was used for paired analysis when comparing different time points within a condition. A p value < 0.05 was considered statistically significant.

Results

Effect of tacrolimus and MPA on *IFNγ* DNA methylation of total T cells

To exclude complete cell cycle arrest as a cause for methylation differences, we compared cell numbers under the different conditions after stimulation. Cell numbers were lower if cells were cultured with either tacrolimus, MPA or decitabine than if the cells were cultured without those factors, but due to overlapping ranges this difference was not statistically significant (Supplementary Figure S1). Our results suggest that the cells were still able to proliferate under the chosen concentrations of the different drugs.

To determine the changes in DNA methylation after T-cell stimulation, we analyzed *IFNγ* promoter methylation at several time points after stimulation. *IFNγ* DNA methylation of total T cells increased significantly after stimulation with α-CD3/CD28 (p=0.002; Figure 1B). Stimulated T cells showed a median DNA methylation percentage of 47% (range: 35%-59%) at day 0 and this was significantly increased at day 4 (59%; 46%-66%).

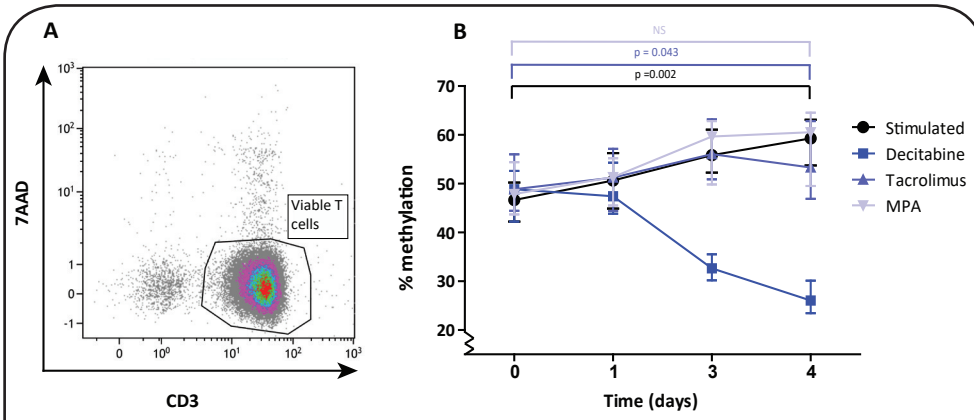


Figure 1. A) *A representative example of the CD3+ purity and viability after MACS isolation.* ***B)*** *Median and interquartile range of IFNγ DNA methylation at day 0, 1, 3 and 4 after α-CD3/CD28 stimulation of total T cells under the different culture conditions: stimulated (n=15), decitabine (n=7), tacrolimus (n=5), MPA (n=4). P values were calculated with a Wilcoxon matched pairs test.*

DNA methylation of T cells cultured in the presence of tacrolimus increased significantly from 49% (42%-59%) to 53% (44%-67%) (p=0.043) and did not differ significantly from the stimulated condition at any of the given time points (Figure 1B). DNA methylation of T cells cultured in the presence of MPA increased from 48% (43%-56%) to 61% (46%-66%) and also did not differ significantly from the stimulated condition (Figure 1B). Our positive

control, T cells cultured in the presence of decitabine, significantly decreased in DNA methylation between day 0 and day 4 (p=0.028; Figure 1B).

Since our total T-cell population was a heterogeneous mixture of naive and memory T cells with different methylation profiles[29], we continued to study isolated cell populations to infer whether tacrolimus or MPA did influence these cell types individually.

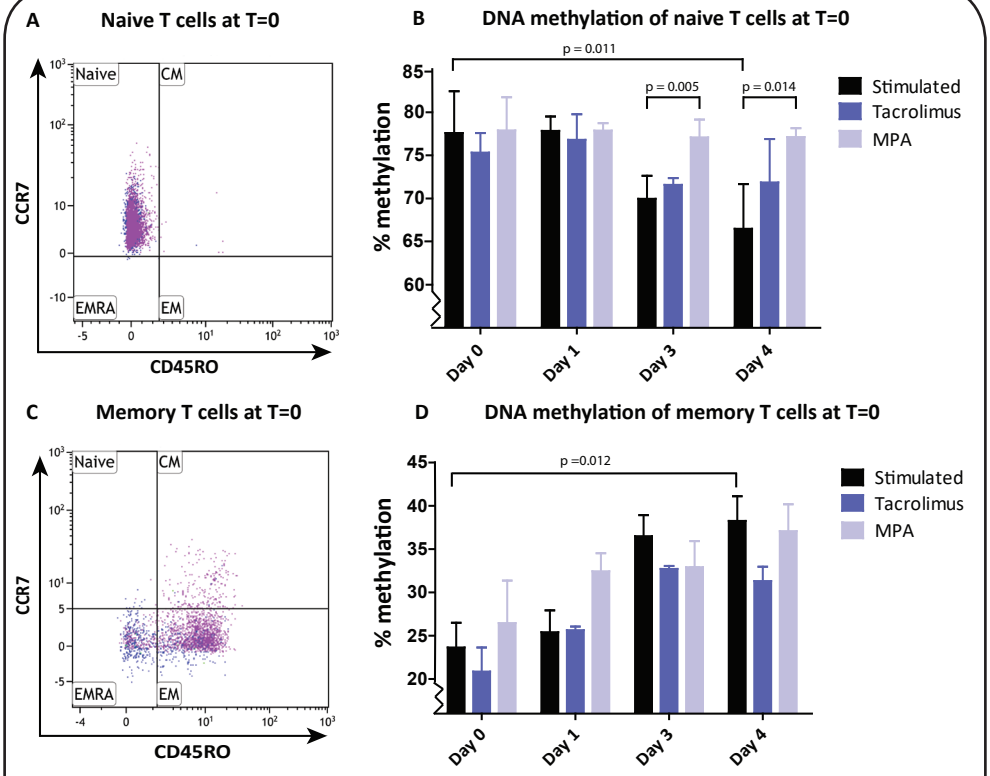


Figure 2. A) *A representative example of the naive CCR7+CD45RO- T cells after sorting.* ***B)*** *Median and interquartile range of IFNγ DNA methylation of sorted naive T cells stimulated in the absence (n=9) or presence of tacrolimus (n=3) or MPA (n=4).* ***C)*** *A representative example of the memory CD45RO+ and CCR7-CD45RO- T cells after sorting.* ***D)*** *Median and interquartile range of IFNγ DNA methylation of the sorted memory T cells stimulated in the absence (n=9) or presence of tacrolimus (n=3) or MPA (n=3). The pink dots in the FACS plots (***A,C***) represent the CD4+ cells and the blue dots the CD8+ cells. P values were calculated with a Wilcoxon matched pairs test (T=0 vs T=3 within one condition) or Mann-Whitney U test (between conditions).*

Effect of tacrolimus and MPA on *IFNγ* DNA methylation of naive and memory T cells

Pure naive (CCR7+CD45RO-) (Figure 2A) and memory (CD45RO+ and CCR7-CD45RO-) (Figure 2C) T-cell subsets were stimulated separately. *IFNγ* DNA methylation significantly decreased in the naive start population in the absence of tacrolimus or MPA, from 78% (75%-83%) at day 0 to 67% (61%-77%) at day 4 (p=0.011; Figure 2B). The two immunosuppressive drugs had differential effects on this reduction in DNA methylation. While tacrolimus had no effect, MPA neutralized the effect of stimulation significantly and DNA methylation did not decrease (78%;76%-82% at day 0 and 77%;75%-78% at day 4). This differential effect resulted in a significant difference between stimulation only and the addition of MPA on day 3 (p=0.005) and day 4 (p=0.014; Figure 2B).

In the total memory start population, *IFNγ* DNA methylation significantly increased in the absence of tacrolimus or MPA, from 24% (19%-31%) at day 0 to 38% (30%-46%) at day 4 (p=0.012; Figure 2D). This increase was not affected by tacrolimus nor MPA, both these conditions were not significantly different from stimulation alone.

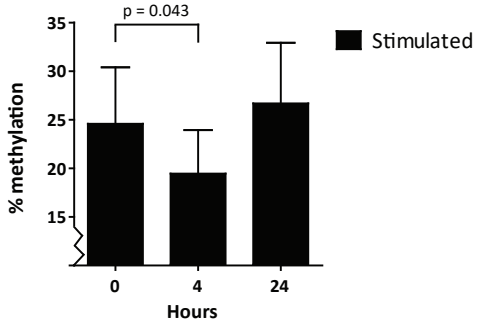


Figure 3. Median and interquartile range of IFNγ DNA methylation *of the sorted memory T cells at 0, 4 and 24 hours after α-CD3/CD28 stimulation (n=5). P value was calculated with a Wilcoxon matched pairs test.*

As explained in the introduction, we expected effector-gene promoters to demethylate after activation to allow transcription of the corresponding effector gene. We observed this in the naive T cells, demethylation of the *IFNγ* promoter took place after 3 days of stimulation (Figure 2B). However, the *IFNγ* promoter of the memory T cells did not demethylate after 1, 3 or 4 days after stimulation (Figure 2D). Therefore we speculated that demethylation occurred in a shorter timeframe than 24 hours, to allow memory T cells to produce IFNγ protein. To address this question we harvested memory T cells at 4 hours after stimulation and indeed we observed a significant decrease (3-12%; p=0.043) in methylation followed by remethylation to base levels after 24 hours (Figure 3).

Phenotypic changes after α-CD3/CD28 stimulation of the naive T cells

The isolated naive T cells, which were CCR7+CD45RO- at day 0, were analyzed for the expression of CD45RO and CCR7 after 1 and 3 days of stimulation in the absence and

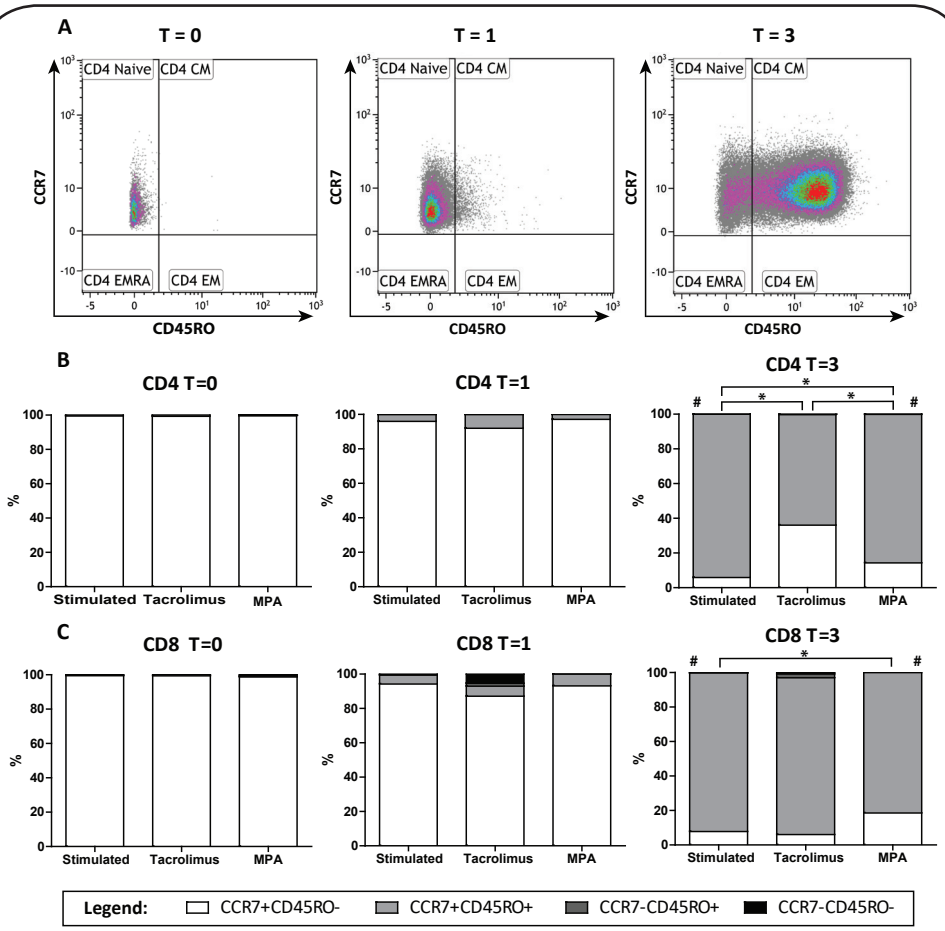


Figure 4. Phenotypic changes of the naive T cells in the absence or presence of tacrolimus or MPA*: stimulated (n=9), tacrolimus (n=3) and MPA (n=4).* ***A)*** *A representative gating example of the CD4+ T cells directly after isolation (T=0) and at day 1 (T=1) and day 3 (T=3) after stimulation.* ***B)*** *Median percentages of CD4+ subsets in the absence or presence of tacrolimus or MPA at day 0, 1 and 3.* ***C)*** *Median percentages of CD8+ subsets in the absence or presence of tacrolimus or MPA at day 0, 1 and 3. *p<0.05 (Mann-Whitney U test to compare two conditions) #p<0.05 (Wilcoxon matched pairs test to compare T=0 with T=3 within one condition).*

presence of tacrolimus or MPA. CD4+ and CD8+ T cells were gated separately (Figure 4), the percentages CD4+/CD8+ do not differ significantly between the conditions (Supplementary Figure S2). After one day of stimulation the phenotype did not differ significantly from day 0 in both CD4+ and CD8+ T cells. On day 3 there was a significant shift towards CD45RO+ cells in the stimulated condition (p=0.008). The shift was observed in all three conditions

and in both the CD4+ and CD8+ T cells (Figure 4B,C). These cells, which were CD45RO- at day 0, upregulated their CD45RO expression showing a central-memory-like phenotype at day 3. When we compared the different conditions with stimulation only at day 3, tacrolimus (p=0.013) and MPA (p=0.039) significantly repressed CD4+ differentiation and MPA also significantly repressed CD8+ differentiation (p=0.014; Figure 4B,C).

Phenotypic changes after α-CD3/CD28 stimulation of the memory T cells

The isolated memory T cells, which were CD45RO+ and CCR7-CD45RO- at day 0, were also analyzed by flow cytometry after 1 and 3 days of stimulation in the absence or presence of tacrolimus or MPA. CD4+ and CD8+ T cells were gated separately (Figure 5). The percentage of CD8+CD45RO+ cells increased significantly after 3 days of stimulation, both in the CCR7+ (p=0.008) and CCR7- (p=0.021) population (Figure 5C). In the CD4+ population we observed an increase in the CCR7+CD45RO+ population (p=0.011) and a decrease in the CCR7- population (p=0.021) (Figure 5B). When we compared the different conditions with stimulation only at day 3, no significant differences were found.

IFNγ protein production of the memory population

IFNγ protein production was measured using intracellular staining in both the sorted naive T cells and the sorted memory T cells (Figure 6). The sorted naive T cells did not produce IFNγ protein at day 1 after stimulation (data not shown) while 10% (3%-19%) of the sorted memory T cells did produce IFNγ. Tacrolimus significantly inhibited IFNγ production, hardly any cells produced IFNγ in the presence of tacrolimus (Figure 6B). MPA did not have a significant effect on IFNγ production and the percentage IFNγ producing cells did not differ from stimulation only. Three days after stimulation of the sorted memory T cells, few cells still produce IFNγ both in the presence and absence of tacrolimus or MPA.

Discussion

To our knowledge, this is the first study to investigate the effect of immunosuppressive medication on DNA methylation of primary T cells[38,39]. The study design allowed us to track changes over time after activation. Also, by combining the results of our analyses of DNA methylation, phenotype and protein production, we were able to determine the effects of immunosuppressive drugs on cellular dynamics after T-cell activation. Our results show that after T-cell activation, MPA affected *IFNγ* DNA methylation of naive T cells but notthat of memory T cells, while tacrolimus had no effect on *IFNγ* DNA methylation of T cells (Figure 1,2).

The mechanism by which MPA counteracts the effect of T-cell stimulation on *IFNγ* DNA methylation is unknown. We can however suggest a possible mechanism by looking at the

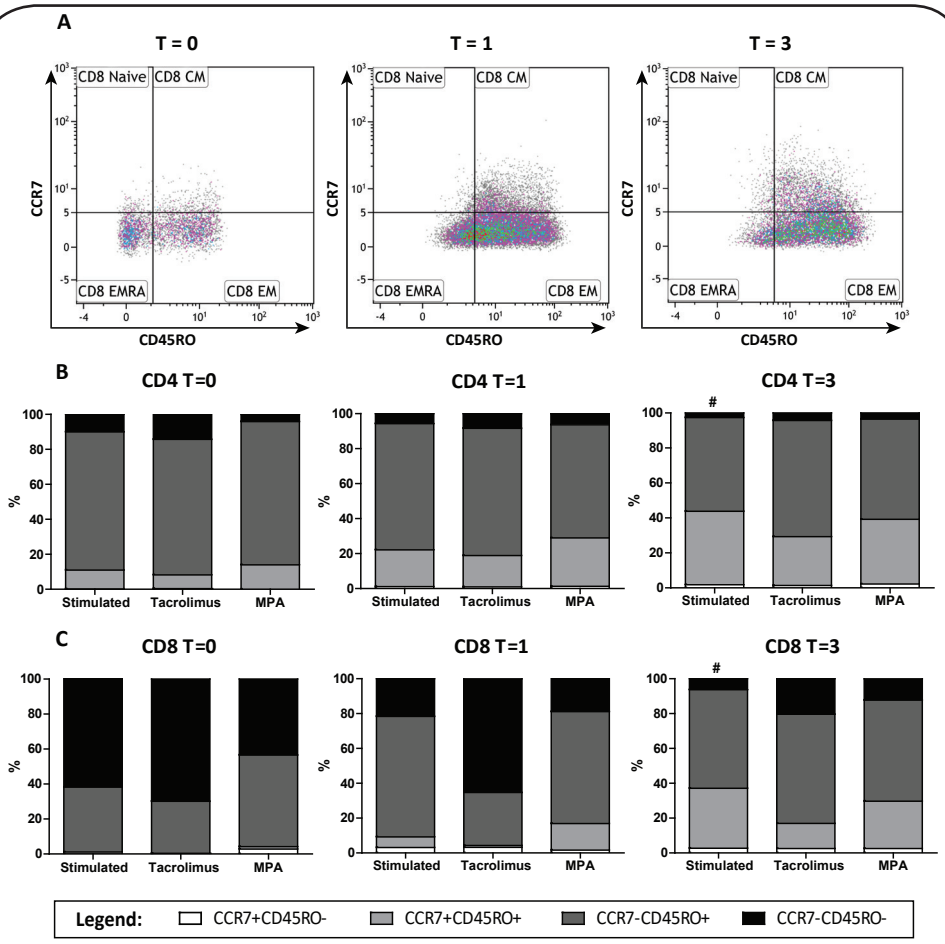


Figure 5. Phenotypic changes of the memory T cells in the absence or presence of tacrolimus or MPA: *stimulated (n=9), tacrolimus (n=3) and MPA (n=3).* ***A)*** *A representative gating example of the CD8+ subsets of the stimulated cells directly after isolation (T=0) at day 1 (T=1) and day 3 (T=3) after stimulation.* ***B)*** *Median percentages of CD4+ subsets in the absence or presence of tacrolimus or MPA at day 0, 1 and 3.* ***C)*** *Median percentages of CD8+ in the absence or presence of tacrolimus or MPA at day 0, 1 and 3. #p<0.05 (Wilcoxon matched pairs test to compare T=0 with T=3 within one condition).*

different enzymes that regulate DNA methylation in general. DNA methyl transferases (DNMTs) are a family of enzymes that maintain DNA methylation during cell division (DNMT1) and cause *de novo* DNA methylation (DNMT3a,b)[4]. Lower activity of DNMT1 leads to passive demethylation, the methylation "dilutes" during cell division[5,40]. Possibly, MPA has a direct or indirect effect on DNMT1 activity during differentiation of naive T cells. A similar suggestion was made by He et al.[41] in relation to an increased CD70 expression

induced by MPA.

While the two drugs' effects on DNA methylation were different, their effects on T-cell differentiation were similar (Figure 4,5). Tacrolimus and MPA both suppressed the differentiation of naive T cells (CD45RO-) towards CD45RO+ cells. This phenotypic marker is a characteristic marker for memory T cells[32] but it has been described as an activation marker as well[42,43]. Since tacrolimus inhibited differentiation of the naive T cells significantly but did not influence *IFNγ* DNA methylation of those cells, we believe that the differentiation can occur independently from changes in *IFNγ* DNA methylation. On the other hand, the changes in T-cell phenotype and *IFNγ* DNA methylation after stimulation alone both occur after three days, indicating a relation between these two parameters. Taken together, the exact relationship between phenotypic changes and changes in *IFNγ* DNA methylation after stimulation remains unclear.

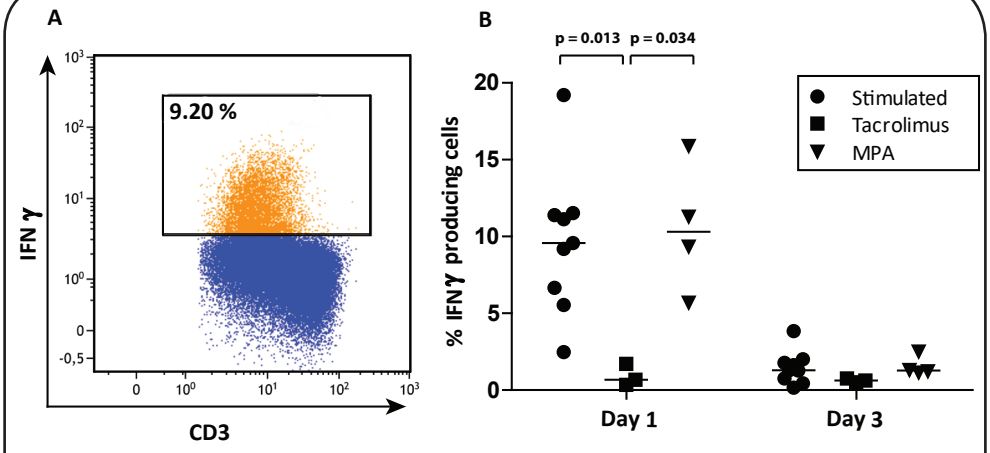


Figure 6. A) *A representative gating example of IFNγ production by the sorted memory T-cell population on day 1 after stimulation.* ***B)*** *Percentages and median of IFNγ producing memory T cells on day 1 and 3 of all three conditions measured by intracellular staining and flow cytometry. P-values were obtained with the Mann-Whitney U test.*

While we had expected T cells to become demethylated on their *IFNγ* promoter upon stimulation, we were surprised to note that, in both total T cells and memory T cells, *IFNγ* promoter methylation actually increased (Figure 1B,2D). In line with the results of previous studies[44,45], *IFNγ* DNA methylation decreased shortly after stimulation of the memory T cells (Figure 3). After the demethylation phase of these cells, *IFNγ* DNA methylation returned to base-level and from day 1 onwards DNA methylation steadily increased. Since the phenotype of the cells changed after stimulation, each time point reflected

a heterogeneous cell population. This makes it difficult to assign the increasing *IFNγ* DNA methylation to a specific cell type. The ideal situation would be to isolate pure cell populations at each time point using surface markers before analyzing their methylation profile – this is practically challenging however.

We are currently uncertain what the biological reason is behind the increase in *IFNγ* DNA methylation (remethylation) that we observed. Similar remethylation of gene promoters after stimulation has thus far been reported for PD1 and IL2. Youngblood et al.[46] studied the *PD1* locus in antigen-specific CD8+ T cells in mice and found that after 8 days of LCMV infection, the *PD1* locus in effector cells had been partially remethylated. This finding was only seen in an acute infection model however: when the mice were chronically infected, the locus remained demethylated and the CD8+ cells became exhausted[46]. A study on *IL2* promoter DNA methylation in HIV-infected patients showed that *IL2* DNA methylation was higher in all CD_4+ effector memory subsets of HIV-infected patients than in those of healthy controls, indicating that chronic HIV infection increased methylation levels in these cell types[47]. The remethylation of the *IFNγ* promoter that we observed may be similar to that of the *PD1* and *IL2* promoters described in the above-mentioned papers.

Although DNA methylation of *IFNγ* was not affected by the presence of tacrolimus, IFNγ protein production by the memory cells was suppressed in the presence of tacrolimus (Figure 6). As mentioned in the introduction, the mechanism of action of tacrolimus is known. Tacrolimus-induced inhibition of the calcineurin pathway inhibits the activity of NFAT, a transcription factor that regulates *IFNγ* gene expression[48,49]. Our results demonstrate that this tacrolimus-induced suppression of IFNγ protein production is independent of changes in DNA methylation of *IFN*γ.

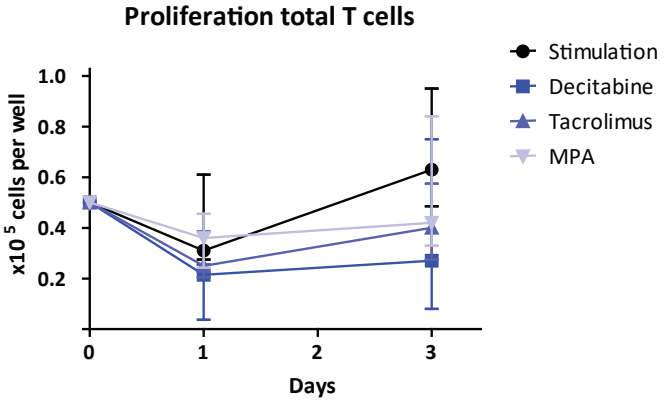
MPA did not affect the percentage of IFNγ producing memory cells in our experiments but the results reported in literature vary. He et al.[41] reported that MPA inhibited IFNγ production in CD_4+ T cells after α-CD_3/CD28 stimulation. Whereas Egli et al.[50] did not find a strong decrease in IFNγ production after adding MPA to CMV-stimulated PBMCs. In both studies, IFNγ concentration was measured in the culture supernatant, and such concentration is strongly related to the number of cells present. Since proliferation decreases under the influence of MPA[18,51], cytokine production should be corrected for cell numbers as we did by measuring intracellular IFNγ. In addition, Egli et al.[50] did not measure T-cell specific IFNγ production and since NK cells are also capable of producing IFNγ this may have influenced their results. These experimental differences could explain the difference between our findings and the results reported in literature.

Here we focused on the *IFNγ* gene promoter to study differences in DNA methylation. Possibly, immunosuppressive drugs have much stronger effects on DNA methylation of

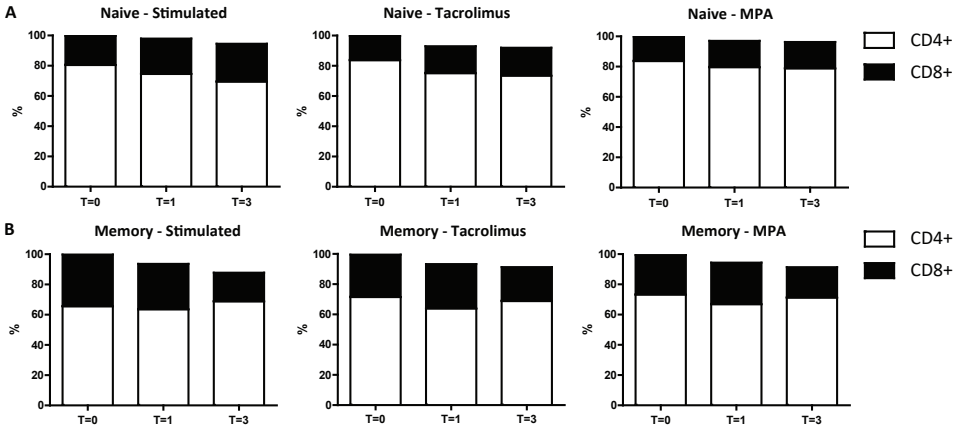
other genes or even at intergenic regions[12]. To find the most affected regions, a genome-wide methylation study could be performed. Due to the explorative nature of this study a genome-wide approach was outside the scope of this paper.

The findings presented here demonstrate that *IFNγ* DNA methylation in T cells was not affected in the same manner by tacrolimus and MPA and therefore we conclude that these immunosuppressive drugs differentially affect *IFNγ* DNA methylation in CMV seropositive individuals. Our study also shows that naive and memory T cells did not only have distinct DNA methylation profiles, but also that they were not affected equally by the immunosuppressive drugs studied. These findings may be of significance for future research into the efficacy of immunosuppressive drugs. Knowledge on the effect of immunosuppressive drugs on DNA methylation of T-cell effector genes and thereby T-cell function could optimize the treatment regimen. When developing and testing immunosuppressive drugs, we recommend to include DNA methylation studies thereby improving our understanding of their effect on the function of patients' immune cells.

Supplementary figures



Supplementary Figure S1. Proliferation of total T cells presented as the median of cells per well in time. *Stimulation (n=9), decitabine (n=7), tacrolimus (n=5) and MPA (n=4). 50,000 cells were stimulated at day 0 and the cells were counted at day 1 and 3 after stimulation with conventional light microscopy after staining the cells with Trypan Blue.*



Supplementary Figure S2. Median percentages of CD4+ and CD8+ populations *within the CD3+ cells of* ***A)*** *the naive start population (CCR7+CD45RO-) in the presence and absence of tacrolimus or MPA and* ***B)*** *the memory start population CD45RO+ and CCR7-CD45RO-) in the presence and absence of tacrolimus or MPA.*

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Chapter 3

Epigenetic changes in umbilical cord mesenchymal stromal cells upon stimulation and culture expansion

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Abstract

Background

Mesenchymal stromal cells (MSC) are studied for their immunotherapeutic potential. Prior to therapeutic use MSC are culture expanded to obtain the required cell numbers and to improve their efficacy MSC may be primed *in vitro*. Culture expansion and priming induce phenotypical and functional changes in MSC and thus standardisation and quality control measurements come in need. We investigated the impact of priming and culturing on MSC DNA methylation and examined the use of epigenetic profiling as a quality control tool.

Methods

Human umbilical cord-derived MSC (ucMSC) were cultured for three days with IFNγ, TGFβ or a multi-factor combination (MC; IFNγ, TGFβ and retinoic acid). In addition, ucMSC were culture expanded for 14 days. Phenotypical changes and T-cell proliferation inhibition capacity were examined. Genome-wide DNA methylation was measured with Infinium MethylationEPIC Beadchip.

Results

Upon priming, ucMSC exhibited a different immunophenotype and ucMSC(IFNγ) and ucMSC(MC) had an increased capacity to inhibit T-cell proliferation. DNA methylation patterns were minimally affected by priming, with only one significantly differentially methylated site (DMS) in IFNγ and MC-primed ucMSC associated with autophagy activity. In contrast, 14 days after culture expansion ucMSC displayed minor phenotypical and functional changes but showed more than 4000 significantly DMS, mostly concerning genes involved in membrane composition, cell adhesion and transmembrane signalling.

Discussion

These data show that DNA methylation of MSC is only marginally affected by priming, whereas culture expansion and subsequent increased cellular interactions have a large impact on methylation. On account of this study we suggest that DNA methylation analysis is a useful quality control tool for culture expanded therapeutic MSC.

Introduction

Mesenchymal stromal cells (MSC) have been extensively examined in clinical trials regarding their immunotherapeutic potential[1-4]. Prior to their application in the clinic, MSC are commonly expanded to obtain clinically relevant numbers. However, during long-term *in vitro* culture expansion the phenotype and function of MSC is affected[5-7]. Previous studies have shown that during long-term expansion their proliferative capacity decreases[7,8]. In addition, long-term culture expansion affects the immunomodulatory properties of MSC, for instance their capacity to inhibit of T-cell proliferation[8]. Recently, there has been a growing interest in the optimization of the immunomodulatory properties of MSC *in vitro*. MSC can be primed with stimuli to enhance their immunomodulatory properties with the aim to improve their therapeutic efficacy[9-17]. Prior to their clinical application, MSC are routinely tested for multiple parameters to assess their safety and functionality, such as karyotype, morphology (spindle-shape) and viability as well as their cell surface protein expression and differentiation capacity according to the recommendations of the International Society for Cellular Therapy[1-4,18-21]. These tests give a global indication of the state of MSC. However, MSC have a great ability to adapt and culture expansion and priming may therefore modify MSC on a different level. Therefore, we endeavored to perform a more in depth analysis of the effects of culture expansion and stimulation on MSC.

Epigenetic modifications of the genome can be both hereditary as well as environmentally influenced. These epigenetic modifications affect gene expression without altering the genomic sequence and are important regulators of cellular function[22-25]. Methylation of cytosines at cytosine-phosphate-guanine (CpG) sites in the DNA, is one of the main mechanisms of epigenetic modifications. Methylation at a CpG site may block the start of transcription and in particular methylation of CpG islands at transcriptional start sites (TSSs) is associated with long-term gene silencing[22]. *In vitro* procedures may affect DNA methylation; potentially resulting in changes in their gene expression and subsequently their phenotype and function.

Previously, it was demonstrated that there is an association between osteogenic differentiation of MSC and their DNA methylation pattern[26-29]. In addition, other studies demonstrated that during long-term culture expansion, where MSC were cultured over 10 passages, MSC became senescent and their DNA methylation patterns changed[30,31]. However, no study to date has addressed the effect of priming MSC *in vitro* with various stimuli to optimize their immunomodulatory properties on the DNA methylation. Furthermore, it remains unclear whether during culture expansion DNA methylation patterns of MSC are affected. Elucidation of the effects of MSC expansion and priming on

DNA methylation may result in additional quality control tools that help in development and application of MSC therapy in clinical setting. This will ensure the use of better standardized MSC therapeutic products. Therefore in this study we investigated the changes in methylation in the epigenome of MSC during priming by various stimuli and also after two weeks culture expansion.

Materials and Methods

Isolation and culture of MSCs

Human umbilical cord tissue was collected by Tissue Solutions Ltd. (Glasgow, UK) from Caesarean section deliveries from virally screened healthy donors. Whole cord tissue of the neonatal side was used for MSC isolation. All cord tissues provided by Tissue Solutions were obtained according to the legal and ethical requirements of the country of collection, with the approval of an ethics committee (or similar) and with anonymous consent from the donor. Isolation of the $CD362^+$ subset of ucMSC was performed according to previous manuscripts by de Witte et al.[8,10]. After isolation, each cell fraction was counted, seeded for expansion and cryopreserved at passage 2 for shipment to Erasmus Medical Center. Here ucMSC were cultured in minimum essential medium Eagle alpha modification (MEM-α; Sigma-Aldrich, St Louis, MO, USA) containing 2 mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100IU/ml penicillin, 100IU/ml streptomycin; Lonza) and supplemented with 15% batch tested fetal bovine serum (FBS; Lonza) and 1 ng/ml basic fibroblast growth factor (bFGF) (Sigma) and kept at 37°C, 5% CO_2 and 20% O_2. Once a week medium was refreshed and ucMSC were passaged using 0.05% trypsin-EDTA (Life technologies, Paisley, UK) at ~80-90% confluence. All ucMSC used in experiments were between passage 3-6.

Characterization of ucMSC was performed by flow cytometric analysis of the cell surface markers: CD31 (PB, BD Biosciences), CD45 (APC-Cy, BD Biosciences), CD13 (PE-Cy7, BD Biosciences), CD73 (PE, BD Pharmingen), CD90 (APC, R&D systems) and CD105 (FITC, R&D systems). After labeling the cells were washed and measured on the FACSCanto II flow cytometer (BD Biosciences) (Supplementary Figure S1).

Experimental design

The experimental design consists of two parts: 'Priming of MSC' and 'Culture expansion of MSC', see also Figure 1.

Priming of ucMSC

ucMSC of 4 different umbilical cord donors were stimulated with factors were known to

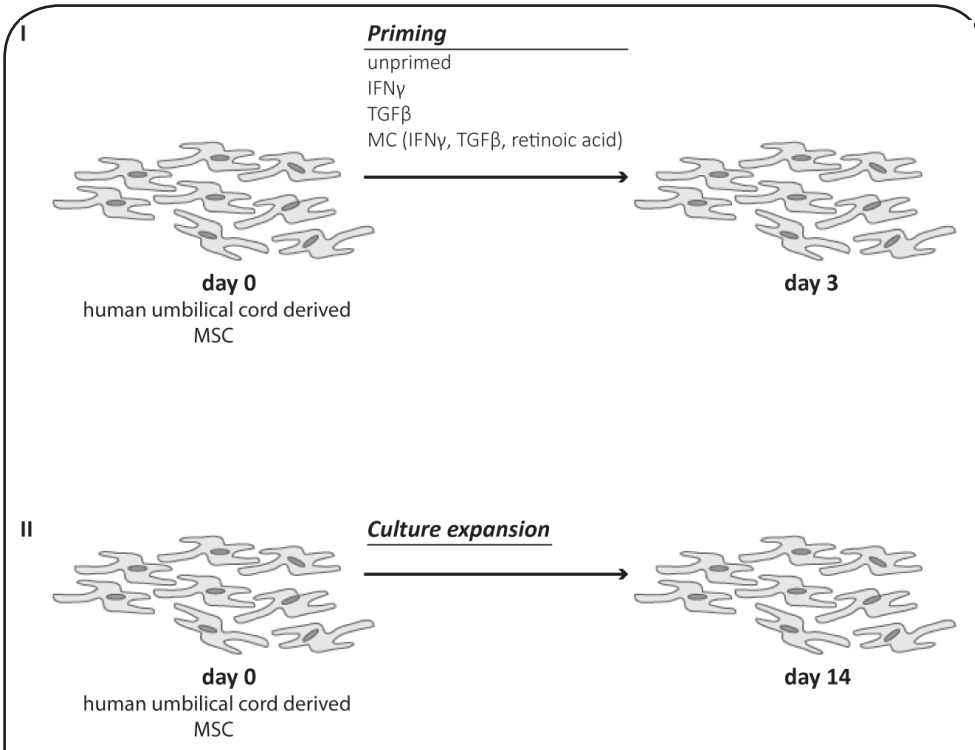


Figure 1. Study design. (Top) *In the first part of the experimental design, ucMSCs were left unprimed or primed with IFNγ, TGFβ, or a multifactor combination of IFNγ + TGFβ + retinoic acid (MC) for 3 days. Thereafter, whole-genome DNA methylation analysis was performed on unprimed and primed ucMSCs.* ***(Bottom)*** *In the second part, ucMSCs were culture expanded for 14 days. Whole-genome DNA methylation analysis was performed on ucMSCs before and after culture expansion.*

modify MSC function or phenotype, as demonstrated in previous studies[10,32]. At day 0, MSC (confluent culture) were stimulated for three days with interferon gamma (IFNγ, 50 ng/ml; Life technologies, transforming growth factor beta 1 (TGFβ, 10ng/ml; R&D systems, MN, USA) or a multi-factor combination (MC) of IFNγ, TGFβ and retinoic acid (RA, 100 μM; Sigma). At day 3, cells were trypsinised and either used for experiment or snap frozen in pellets containing 300,000 cells and stored at -80°C.

Culture expansion of MSC

At day 0, MSC of 4 different umbilical cord donors were seeded (250,000 cells/T175 flask). Medium was partly refreshed (50%) every 5/6 days. At day 14, cells were used for experiment or snap frozen as pellets for future use.

DNA extraction procedure

DNA was isolated from ucMSC with the QIAamp DNA Micro kit (Qiagen; Germany) according to the manufacturer's instructions. DNA concentration was measured with spectrophotometry (Nanodrop spectrometer, Thermo Scientific, USA). DNA quality of the samples was estimated by measuring the ratio of absorbance at 260/280nm (between 1.7-2) and with agarose gel electrophoresis.

Bisulfite treatment and DNA methylation measurement

To determine DNA methylation profiles, samples underwent bisulfite conversion. During this conversion unmethylated cytosines were converted into uracil (Supplementary Figure S2). The bisulfite conversion was performed using 500 ng genomic DNA per sample and using the EZ-96 DNA Methylation Kit (Shallow; Zymo Research, CA, USA). Bisulfite converted samples were then hybridized to the Illumina 850k DNA methylation array (Infinium MethylationEPIC Beadchip; Illumina; USA) according to the manufacturer's instructions.

In short, the Infinium MethylationEPIC BeadChip applies both Infinium I and II assay chemistry technologies. The infinium I assay uses two bead types: methylated (M) and unmethylated (U). Whereas Infinium II assay uses a single bead type, with the methylated state determined at the single base extension step after hybridization. This array provides methylation data of over 850,000 CpG sites in the genome. These CpG sites are located in CpG islands, shores and shelves, the 5'UTR, 3'UTR and bodies of RefSeq genes, FANTOM5 enhancers, ENCODE open chromatin and ENCODE transcription factor binding sites.

The raw data of the DNA methylation arrays is deposited at the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) under accession number GSE113527 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113527).

Analysis

Analysis of the methylation data was performed using Rstudio (RStudio Desktop 1.1.3.83) and comb-p software. R Bioconductor packages DMRcate[33,34], limma[35,36], minfi[37] and missMethyl[38] were used. Firstly, the raw methylation data was imported in R. Subsequently the data were normalized (Subset quantile normalization: SQN (within and between array)). Probes on the sex chromosomes were removed from the dataset, resulting in a remaining 810,005 sites. Subsequently M-value (equation 1) and β-value (equation 2) were calculated.

$$M = log2(\frac{methylated}{unmethylated}) \quad [1]$$

$$\beta = \frac{methylated}{methylated + unmethylated} \quad [2]$$

The M-value is the log2 ratio of the intensities of methylated probe versus unmethylated probe. When M=0 there are equal amounts of methylated and unmethylated sites, when M>1 there are more methylated than unmethylated molecules and when M<1 there are more unmethylated than methylated molecules. β-value is the ratio of the methylated probe intensity and the overall intensity (with β=0: completely unmethylated and β =1: fully methylated). According to Du et al. the use of M-values is more appropriate when doing differential methylation analysis[39]. Therefore, M-values of 810,005 CpG sites were used for further analysis.

To determine differences in DNA methylation after culture expansion or priming, paired analyses were performed. Firstly, differences in methylation between the different conditions for each ucMSC donor were identified, followed by joining the differences across ucMSC donors to determine (significant) differences in the mean methylation level of each CpG site (paired testing). Sites with a p_{adj}<0.05 were considered significantly differentially methylated.

The lists of significantly differentially methylated CpG sites were subsequently used to perform gene ontology testing, using the gometh function in R. Furthermore, the list was used to find differentially methylated regions (DMRs) in a command line tool and python library: Comb-P[40]. By calculating auto-correlation, combining adjacent p-values Stouffer-Liptak-Kechris correction, performing false discovery adjustment, finding regions of enrichment (i.e. series of adjacent low P-values) and assigning significance to regions with irregularly spaced p-values, Comb-P enables identification of significant DMRs. The size of the regions analyzed was set to 500 basepairs (bp) with the seed at p<0.01. Multiple testing was taken into account by correcting using a Šidák correction.

T cell proliferation assay

Primed and culture expanded ucMSC were seeded into 96-wells plates and left overnight to adhere in the incubator. The next day PBMC were labeled with Cell Trace CFSE (Life Technologies) according to the instructions of the manufacturer and seeded on top of the ucMSC, at different [MSC:PBMC] ratios: [1:10], [1:5] and [1:2.5] in RPMI supplemented with 2 mM L-glutamine, 100IU/ml penicillin, 100IU/ml streptomycin and 15% FBS. αCD3/CD28 stimulation was added (0.5 µg / ml αCD3 antibody, 0.5 µg/ml αCD28 antibody and 1 µg/ml goat-α-mouse antibody; Life Technologies). The co-cultures were left for 3 days and

PBMC were collected. PBMC were stained for CD4 (APC; eBioscience) and CD8 (Pe-cy7; eBioscience). With the use of the FACSCanto II flow cytometer the proliferation of PBMCs was measured.

RT-PCR

mRNA was isolated from ucMSC, from the same samples as the DNA extraction, using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 500ng mRNA with random primers (Promega Benelux B.V., The Netherlands). Quantitative gene expression was determined using TaqMan Gene Expression Master Mix (Life Technologies) and Assays-on-demand for HS1BP3 (Hs00916454_m1).

Results

Priming alters ucMSC immunophenotype and functionality

ucMSC were cultured for three days in the presence of IFNγ, TGFβ or a combination of IFNγ, TGFβ and RA (MC). We observed that whilst ucMSC maintained their spindle shaped morphology (Figure 2A) and expression of MSC markers CD13, CD73, CD90 and CD105 (Supplementary Figure S1) upon priming, expression of HLA type I, II and PD-L1 was increased (percentage expressing cells as well as the MFI) (Figure 2B). The immunomodulatory capacity of ucMSC, determined by their potential to inhibit CD4 and CD8 T-cell proliferation, significantly increased after priming ucMSC with IFNγ and MC (Figure 2C and Supplementary Figure S3).

Impact of priming of ucMSC on DNA methylation

To examine whether priming of ucMSC leaves an epigenetic imprint that can be used to identify MSC potency or as a inclusion or exclusion criterion of MSC for clinical use, genome-wide DNA methylation profiles were generated of ucMSC after 3 days priming with IFNγ, TGFβ or MC. We compared DNA methylation profiles of unprimed ucMSC to those of primed ucMSC and demonstrated that priming of ucMSC with IFNγ and MC but not TGFβ led to differential methylation at a single site located on chromosome 2 (Figure 3A-D). This site, Cg00221794, was hypomethylated in ucMSC primed with IFNγ and MC compared to unprimed ucMSC (Figure 3E). No differentially methylated regions were detected in any of the primed ucMSC (Figure 3D).

Modified expression near the IFNγ and MC induced hypomethylated site Cg00221794

The CpG site Cg00221794 is located near an area annotated as the Hematopoietic Cell-Specific Lyn Substrate 1 binding protein 3 (HS1BP3) gene. To investigate whether hypomethylation of this site affects HS1BP3 gene expression levels, mRNA levels of

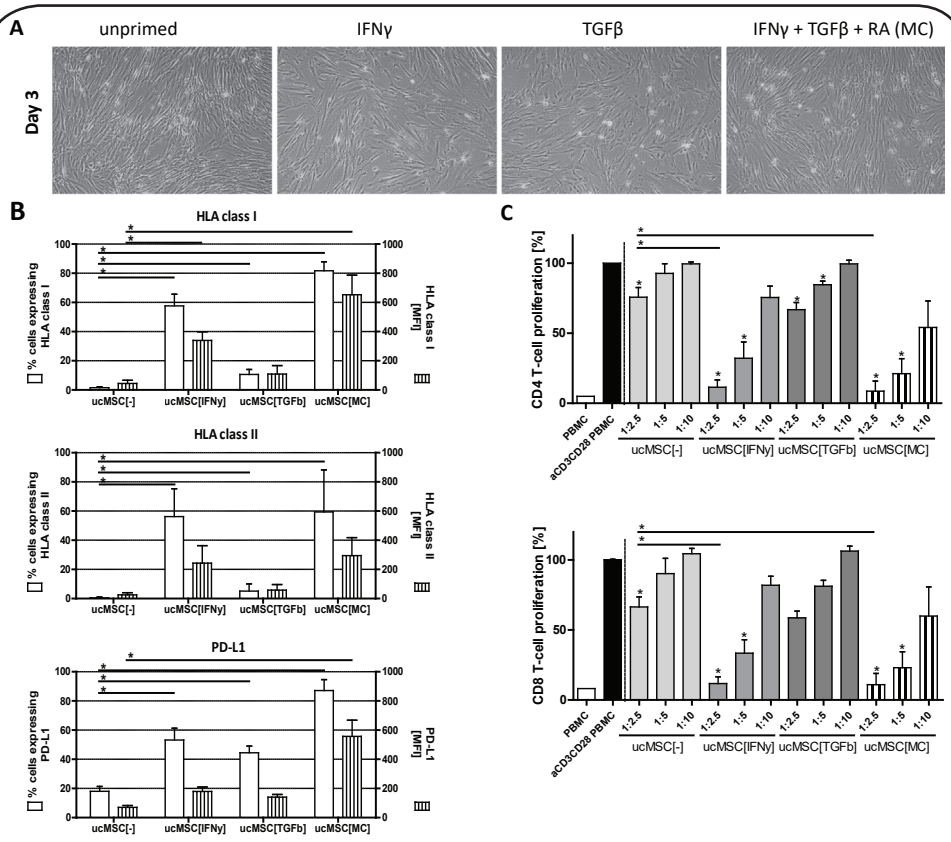
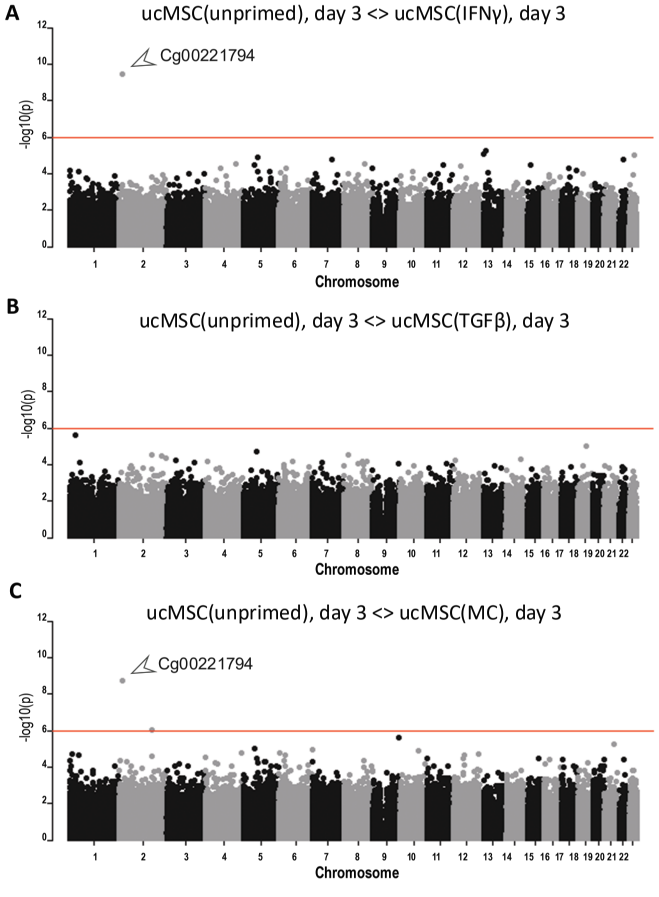


***Figure 2. Morphology, surface marker expression and immunosuppressive capacity of primed ucMSCs**. **A)** Representative bright field photos of unprimed ucMSCs and after priming with IFNγ, TGFβ or the MC. **B)** Expression of HLA class I (top), HLA class II (middle) and PD-L1 (bottom) in percentage of positive cells (clear bars, on left axis of graph) or in MFI (striped bars, on right axis of graph). **C)** Inhibition of CD4 T-cell proliferation (top) and CD8 T-cells (bottom) by unprimed and primed ucMSCs, in different ratios. *P<0.05*

HS1BP3 were analyzed in unprimed and IFNγ, TGFβ or MC primed ucMSC. Gene expression levels of HS1BP3 were significantly upregulated upon priming of ucMSC with IFNγ or MC compared to unprimed ucMSC (Figure 4).

Culture expansion alters the immunophenotype of ucMSC

To assess the effects of culture expansion the morphology, immunophenotype and capacity to suppress T cell proliferation of ucMSC were assessed before and after 14 days of culture expansion. After 14 days of culture expansion, the cultures were >90% confluent (Figure 5A). There was no change in expression of the MSC markers CD13, CD73, CD90 and



D

Comparison between		# Differentially methylated site (DMS)	# Differentially methylated region (DMR)
ucMSC[-] *day 3*	ucMSC[**IFNγ**] *day 3*	1	0
ucMSC[-] *day 3*	ucMSC[**TGFβ**] *day 3*	0	0
ucMSC[-] *day 3*	ucMSC[**MC**] *day 3*	1	0

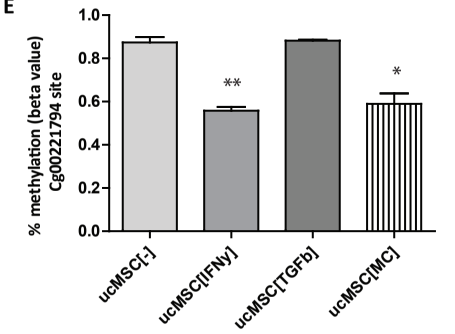


Figure 3. Analysis of differentially methylated sites (DMSs) and regions of primed ucMSCs*. Manhattan plots of DMSs of ucMSCs 3 days after priming with* ***A)*** *IFNγ,* ***B)*** *TGFβ or* ***C)*** *MC compared with unprimed ucMSCs. Genome-wide significance levels of 1 x 10^{-6} from the Bonferroni correction were used. Horizontal lines mark significance cut-offs. Arrow indicated significantly methylated site.* ***D)*** *Summarizing table of the number of DMSs and DMRs after priming of ucMSCs.* ***E)*** *Percentage methylation (beta-values) of cg00221794 site at day 3 after priming. N=4 per condition. *P<0.05, **P<0.005 compared with ucMSC[-]*

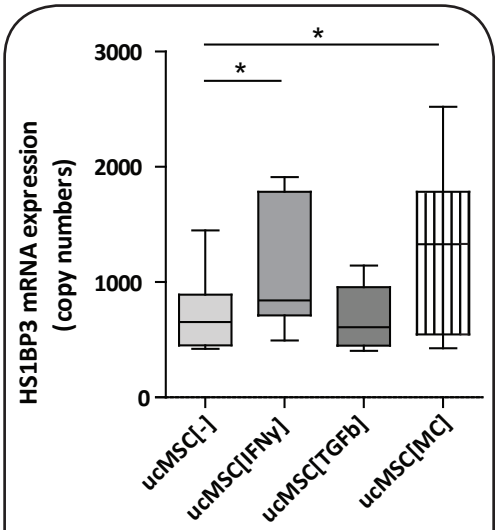


***Figure 4. mRNA expression levels** of HS1BP3 in ucMSCs after 3 days of priming. *P<0.05*

CD105 (Supplementary Figure S1) while there was significant increased protein expression of HLA type II and PD-L1 on ucMSC at day 14 compared to prior to culture expansion (day 0) (Figure 5B). Furthermore, there was no significant difference in the ability of day 0 versus day 14 ucMSC to suppress T cell proliferation (Figure 5C).

ucMSC undergo major epigenetic changes during culture expansion

DNA methylation patterns were determined in MSC 14 days after culture expansion. In contrast to the minor effects of priming of ucMSC with IFNγ, TGFβ or MC on DNA methylation, 14 days of culture expansion led to 4831 significantly differentially methylated sites (DMS) (Figure 6A). Gene ontology analyses revealed these differences were located in genes involved in plasma membrane composition, cell adhesion and transmembrane signaling. We furthermore observed 545 differentially methylated regions (DMR) (Figure 6B), of which 47 were hypermethylated and 498 hypomethylated (Figure 6C). This suggests in general elevated expression of cell membrane associated proteins upon increase confluency following 14 days of culture of ucMSC.

The top 10 most significantly hyper or hypo-methylated regions are shown in Supplementary Tables S1 and S2. These results demonstrate that in contrast to priming of ucMSC with IFNγ, TGFβ or MC, culturing ucMSC for 14 days has a major impact on DNA methylation profiles of ucMSC. The significant changes in DNA methylation between newly seeded MSC and MSC cultured for 14 days suggest that methylation profiling can be used to differentiate between MSC cultures of different culture phase and potentially as an inclusion/exclusion assay for MSC for clinical therapy.

Discussion

Our data demonstrate that priming of ucMSC, despite inducing immunophenotypical and functional changes, does not induce major epigenetic changes. In contrast, culture expansion over 14 days (one passage) has lesser effects on ucMSC phenotype and function, but has a major impact on the epigenetic profile of the cells. This suggests that ucMSC that

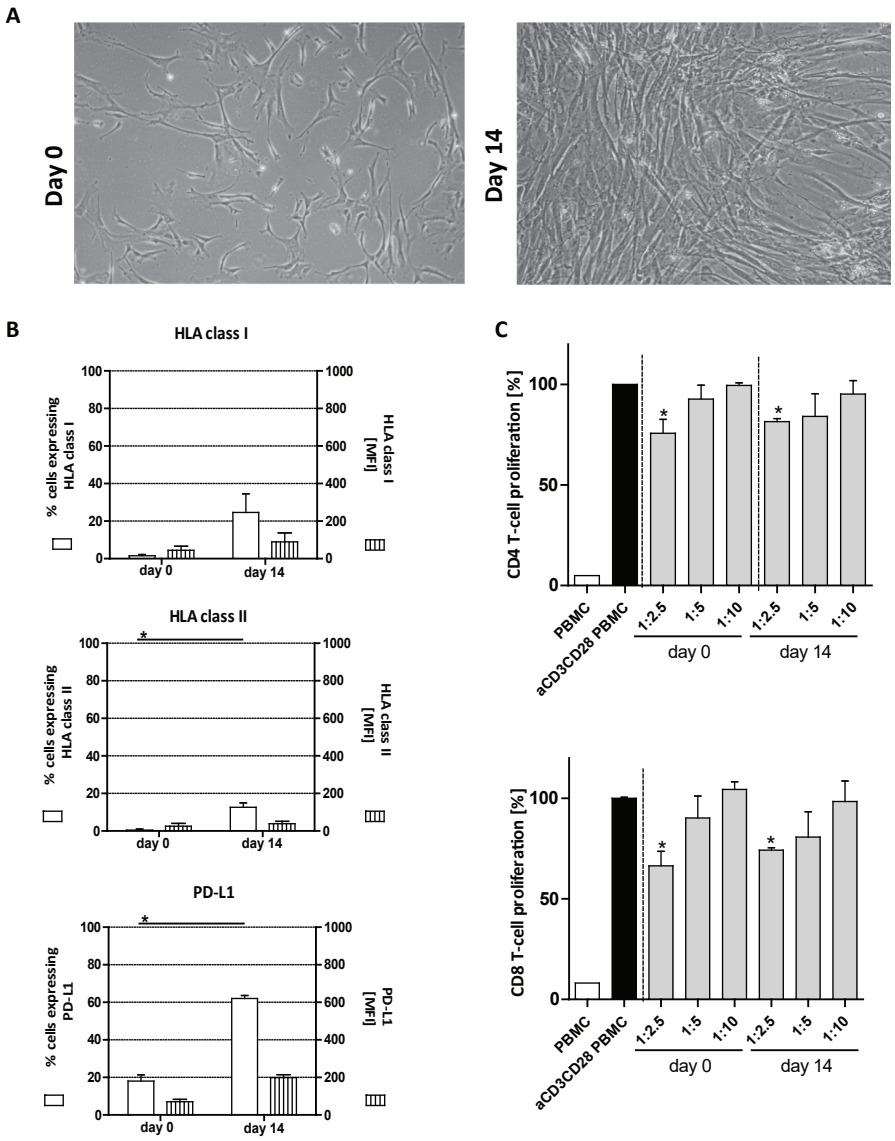


Figure 5. Morphology, surface marker expression and immunosuppressive capacity of culture expanded ucMSCs. ***A)*** *Representative bright field photos of ucMSCs after 14 days of culture expansion. Original magnification 50x.* ***B)*** *Expression of HLA class I (top), HLA class II (middle) and PD-L1 (bottom) in percentage of positive cells (clear bars, on left axis of graph) or in MFI (striped bars, on right axis of graph).* ***C)*** *Inhibition of CD4 T-cell proliferation (top) and CD8 T cells (bottom) by ucMSCs prior to culture expansion (day 0) or post-culture expansion (day 14) in different ratios. *P<0.05 compared with aCD3CD28 PBMC.*

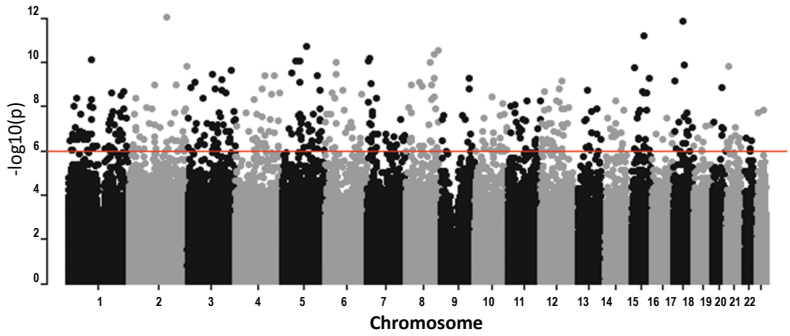
are immunophenotypically identical may represent cells of a different standard. Epigenetic analysis of MSC may therefore represent a useful tool to validate MSC according to set epigenetic profile standards.

Culture expansion is a necessity when working with MSC to generate sufficient numbers of cells, although preferably MSC are used at a low passage for research and for clinical trials to minimize risks associated with their stability, safety and functionality. Long-term *in vitro* culture expansion of MSC increases the probability of genetic instabilities, and studies have reported increasing aneuploidy of MSC cultures during long-term expansion[41-43]. Recently, we showed that during long-term culture expansion, ucMSC remain genetically and phenotypical stable, but their immunosuppressive capacity decreases[5]. This is not observed for short-term culture expansion. In the present study, despite minor effects on immunophenotypical parameters, we identified major differences in the DNA methylation pattern of ucMSC after 14 days of culture expansion.

We postulate that clonal expansion, aging of the cells or confluency of the culture may contribute to the major changes observed in DNA methylation. Firstly, ucMSC are a heterogeneous population, such that across MSC cultures there are differences in their secretome, surface marker expression, gene expression and also in their epigenome. Throughout the experiment, ucMSC were seeded and cultured in the same flask for 14 days, during which period extensive proliferation took place. It is plausible that certain ucMSC have higher proliferation rates, which would lead to an enrichment of this population, representing clonal expansion[44]. Secondly, the amount of proliferation and duration of the expansion may have led to ageing of the cells. DNA methylation levels have been demonstrated to change during cellular senescence of MSC[45,46], with Dahl et al reporting a shift towards more DNA methylation over time in culture of MSC[47]. In contrast with these findings, we detected more hypomethylation after 14 days of culture expansion. During 14 days of culture expansion a high cell density (up to >90% confluency) was reached. Under these conditions the cells are forced to increase intercellular interactions, which is likely to affect matrix and membrane composition and intercellular signaling. This is supported by our findings that genes related to membrane composition, cellular adhesion and transmembrane signaling were hypomethylated, suggesting increased expression. Confluency upon harvest will therefore affect MSC and DNA methylation analysis is a tool to monitor this in a quantitative manner.

DNA methylation affects gene expression, but this is not necessarily a direct consequence of methylation changes of the gene of interest itself[22,23]. When priming ucMSC with IFNγ or MC a single hypomethylated site, namely Cg00221794 was identified, which is located near the HS1BP3 gene. Hypomethylation in a promotor region is suggested to lead to increased

A



B

Comparison between		# Differentially methylated site (DMS)	# Differentially methylated region (DMR)
ucMSC ***day 0***	ucMSC ***day 14***	4831	545

C **Differentially methylated regions after 14 days of culture expansion**

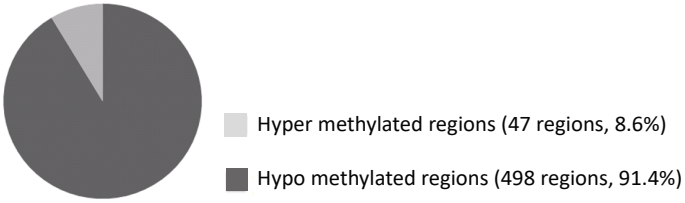


Figure 6. Analysis of DMSs and DMRs *of ucMSCs at day 0 and after 14 days of culture. Manhattan plot of DMSs of* ***A)*** *ucMSCs after 14 days of culture expansion compared with ucMSCs before 14 days of culture expansion. Genome-wide significance levels of 1 x 10^{-6} from the Bonferroni correction were used.* ***B)*** *Summarizing table of the number of DMSs and DMRs after culture expansion of the ucMSCs.* ***C)*** *Pie chart revealing the distribution of observed hypermethylated and hypomethylated regions. N=4 per condition.*

gene expression[22]. Although site Cg00221794 is not located in the promotor region of the HS1BP3 gene but in close proximity, HS1BP3 gene expression was increased in ucMSC after priming with IFNγ or MC, which suggests a role for site Cg00221794 in the regulation of HS1BP3. HS1BP3 has recently been identified as a regulator of autophagy[48]. Its depletion inhibits autophagosome formation by interacting with phosphatidic acid on endosomes thereby preventing endosomal development into autophagosomes[49]. MSC display a high level of autophagy under homeostatic conditions, which is up or downregulated under stress or during differentiation[50]. The role of HS1BP3 in MSC is unknown, but it may well be

implicated in the regulation of MSC autophagy.

Whereas priming of ucMSC with IFNγ or MC had only minor effects on DNA methylation, it had a significant effect on the T cell inhibition capacity of MSC and their expression of PD-L1 and HLA class I and II. On the contrary, 14 days of culture induced significant changes in DNA methylation, but had no effect on T cell inhibition capacity of MSC and small effects on PD-L1 and HLA expression. It is however possible that other functions of MSC that were not investigated, such as their capacity to modulate monocyte function or secrete trophic factors, are affected by the DNA methylation changes induced after 14 days of culture. Depending on the functional requirements of MSC for different types of applications, it becomes important to test the behavior of primed or prolonged culture expanded MSC in relevant assays.

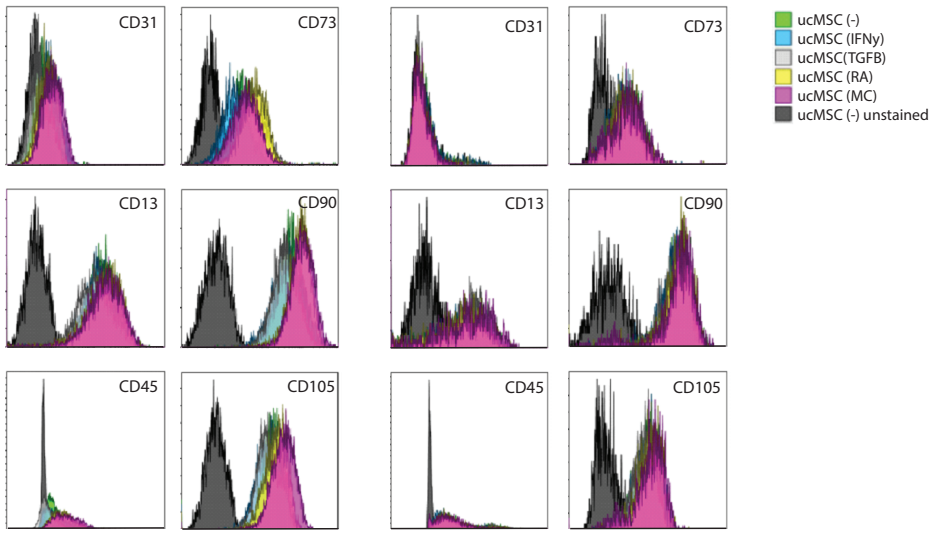
The NIH Roadmap Epigenomics Consortium generated reference epigenetic profiles of various human cell types, which supplies data concerning annotation and functional information of genomic sites and regions[51], which we used to annotate functions to differentially methylated sites in ucMSC (Supplementary Tables S1, S2). However, the Roadmap relates to adipose tissue derived MSC and bone marrow derived MSC and there is no data available for ucMSC. Although MSC from various tissue sites resemble each other, it is also clear that there are tissue specific differences. Therefore it is possible that particular methylation sites have a different function in ucMSC than described in the Roadmap.

The use of a standardized therapeutic MSC product, is crucial to guarantee safety and predictable functionality. Although, it is unclear what the full impact of changes in DNA methylation of ucMSC on safety and functionality is, methylation status offers a global view on the state of a cell culture. In our hands, T cell inhibitory capacity of ucMSC did not change in response to changes in methylation induced by prolonged culture, but it is likely that other properties of the cells were affected. The mechanisms of MSC therapy have not been fully elucidated and therefore at this moment it is not possible to test relevant functional properties of the cells in relation to methylation status. When more is known about the mechanisms of action of MSC therapy, the effect of epigenetic changes on these particular mechanisms can be determined. Therefore, DNA methylation profiling could be used as a part of the characterization of therapeutic MSC to ensure the use of MSC of a fixed state, for standardization properties.

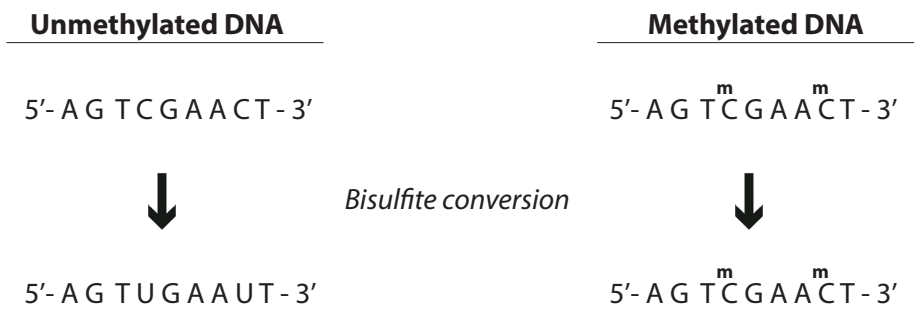
To conclude, MSC can be subjected to *in vitro* manipulations that lead to various phenotypical and functional changes. Our data showed that priming MSC with various stimuli has a minor impact on their DNA methylation, whereas during *in vitro* culture expansion MSC exhibit more extensive changes in DNA methylation profiles. These major

changes in DNA methylation may influence the safety and efficacy of MSC therapy, which needs to be further investigated. Our results reveal that epigenetic profiles may be used as a quality control measure for MSC for experimental and in particular clinical use. Additional assessment of their DNA methylation pattern prior to their (pre)-clinical use, next to testing e.g. their karyotype, viability and phenotype will give a more in-depth analysis of their state. Moreover, assessment of their DNA methylation pattern as a quality control will contribute to the standardization of therapeutic MSC.

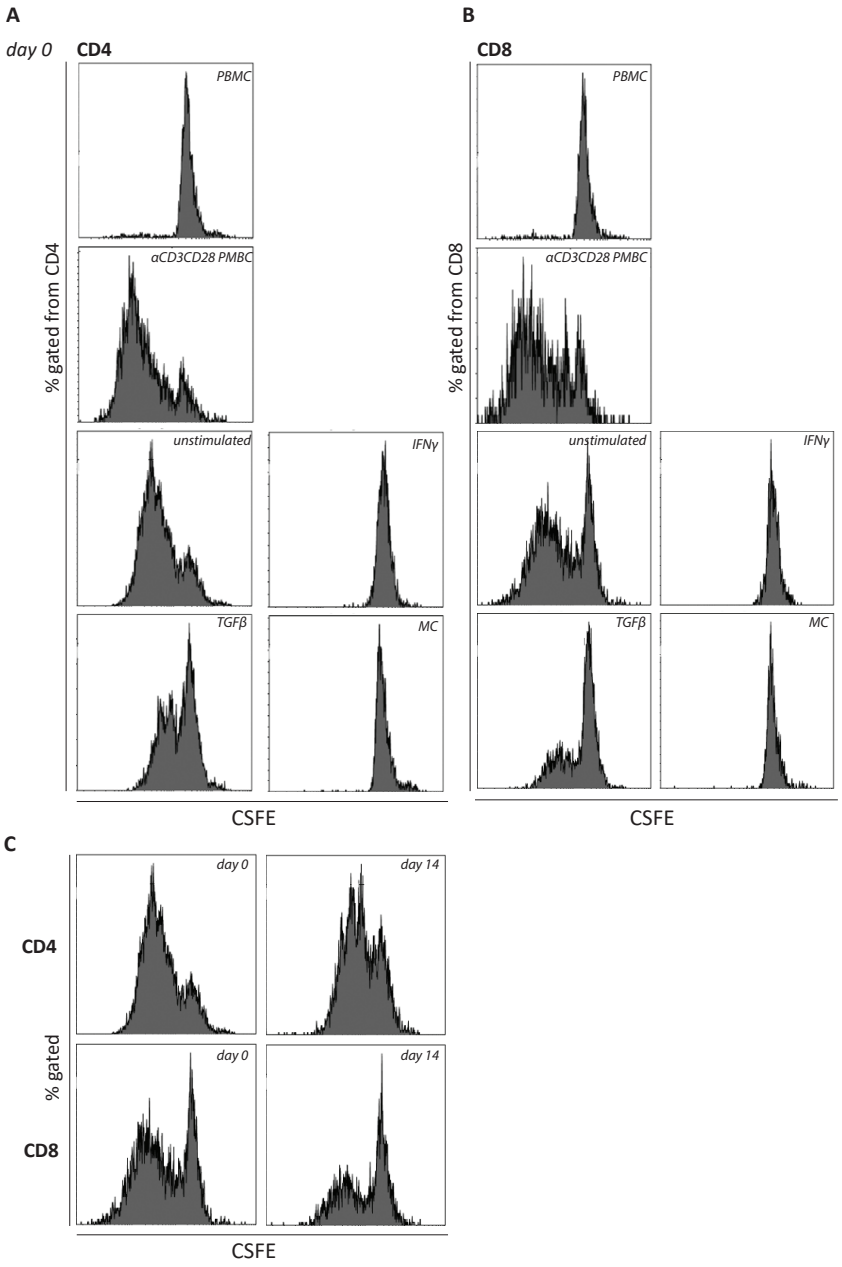
Supplementary Figures



Supplementary Figure S1. Characterization of ucMSC. *Immunophenotyping of ucMSC by flow cytometry. Representative histograms (green: ucMSC(-), blue: ucMSC(IFNy), light grey: ucMSC(TGFb), yellow: ucMSC(RA), pink: ucMSC(MC) and dark grey: unstained ucMSC(-)) of expression of characteristic MSC markers CD13, CD73, CD90, CD105 and negative expression of endothelial marker CD31 and hematopoietic marker and CD45 by ucMSC post 3 day priming* ***(top)*** *and after 14 days of culture expansion* ***(bottom)****.*



Supplementary Figure S2. Example of bisulfite conversion*. All samples underwent bisulfite conversion: unmethylated cytosines are converted into uracil.*



Supplementary Figure S3. T cell suppression by ucMSC. *UcMSC and αCD3CD28 stimulated CSFE labeled PBMCs were co-cultured at a 1:2.5 ratio for 3 days. Representative histograms of CSFE in gated CD4* ***(A)*** *and CD8 T-cells* ***(B)*** *co-cultured with primed ucMSC and with 14 days culture expanded ucMSC* ***(C)****.*

Supplementary Tables

Supplementary Table S1. *Top 10 hypomethylated regions in ucMSC after 14 days of culture expansion. Annotated to ROADMAP reference data for adipose derived MSC and bone marrow derived MSC. The p-values of the regions and the number of CpG sites within the significantly hypomethylated regions are indicated.*

	#	Chr	Start	In region	P_{region}	#CpG
day 0 <> day 14 ucMSC	**1**	7	23387365	This DMR is located in an area annotated to the Insulin like growth factor 2 mRNA binding protein 3 (IGF2BP3) gene and is present in a region known to be a strong enhancer region and an active TSS.	6.01×10^{-18}	5
	2	5	159894868	This DMR is located in an area annotated to the MIR3142 Host Gene (MIR3142HG) gene and is present in a region known to be (near) an active TSS.	2.39×10^{-17}	5
	3	5	73928997	This DMR is located in an area annotated to the Ectodermal-Neural Cortex 1 (CCL28, ENC1) gene and is present in a region known to be a strong enhancer and transcription region.	9.4×10^{-18}	3
	4	9	118135710	This DMR is located in an area annotated to the Deleted In Esophageal Cancer 1 (DEC1) gene and is present in a region known to be a strong enhancer region and an active TSS.	3.34×10^{-17}	2
	5	4	160319500	This DMR is located in an area annotated to the RP11-138A23.2 gene and is present in a region known to be an enhancer and transcription region	4.34×10^{-15}	3
	6	4	123693559	This region was not annotated to a gene by ROADMAP reference data	8.26×10^{-15}	2
	7	7	18548468	This DMR is located in an area annotated to the Histone deacetylase 9 (HDAC9) gene and is present in a region known to be an active TSS.	5.8 x 10-14	4

#	Chr	Start	In region	P_{region}	#CpG
8	6	29454623	This DMR is located in an area annotated to the MAS1 Proto-Oncogene Like (MAS1L) gene and is present in a region known to be quiescent.	1.68×10^{-13}	12
9	4	74606107	This DMR is located in an area annotated to the Interleukin-8 (IL-8, CXCL8) gene and is present in a region known to be a enhancer region and an active TSS.	7.83×10^{-13}	4
10	6	29429909	This DMR is located in an area annotated to the Olfactory receptor 2H1 (OR2H1) gene and is present in a region known to be quiescent.	8.34×10^{-13}	6

Supplementary Table S2. *Top 10 hypermethylated regions in ucMSC after 14 days of culture expansion. Annotated to ROADMAP reference data for adipose derived MSC and bone marrow derived MSC. The p-values of the regions and the number of CpG sites within the significantly hypermethylated regions are indicated.*

	#	Chr	Start	In region	P_{region}	#CpG
day 0 <> day 14 ucMSC	**1**	12	14996143	This DMR is located in an area annotated to the ADP-Ribosyltransferase 4 (ART4) gene and is present in a region known to be an enhancer and transcription region, quiescent and near a TSS.	5.74×10^{-12}	11
	2	17	77018501	This DMR is located in an area annotated to the C1QTNF1 Antisense RNA 1 (C1QTNF1-AS1) gene and is present in a region known to be a strong enhancer region, quiescent and a repressed polycomb.	1.51×10^{-11}	8
	3	8	72757787	This DMR is located in an area annotated to the MSC Antisense RNA 1 (MSC-AS1) gene and is present in a region known to be an enhancer region, near a TSS, bivalent promotor and a repressed polycomb.	4.67×10^{-9}	5
	4	15	74466337	This DMR is located in an area annotated to the Immunoglobulin superfamily containing leucine rich repeat (ISLR) gene and is present in a region known to be an active TSS.	4.67×10^{-9}	6
	5	12	16760040	This DMR is located in an area annotated to the LIM Domain Only 3; Microsomal Glutathione S-Transferase 1 (LMO3) gene and is present in a region known to bea repressed polycomb, bivalent promotor and near a TSS.	1.5×10^{-8}	12

#	Chr	Start	In region	P_{region}	#CpG
6	2	239799314	This DMR is located in an area annotated to the Twist Family BHLH Transcription Factor 2 (TWIST2) gene and is present in a region known to be a transcription region and quiescent.	1.53×10^{-8}	4
7	4	111561070	This DMR is located in an area annotated to the Paired Like Homeodomain 2 (PITX2) gene and is present in a region known to be repressed polycomb and quiescent.	1.55×10^{-8}	3
8	12	3259078	This DMR is located in an area annotated to the Tetraspanin 9 (TSPAN9-IT1/TSPAN9) gene and is present in a region known to be a enhancer region.	5.27×10^{-8}	2
9	8	27468684	This DMR is located in an area annotated to the Clusterin (CLU) gene and is present in a region known to be a strong enhancer region and near a TSS.	1.83×10^{-7}	9
10	4	99417260	This DMR is located in an area annotated to the Tetraspanin 5 (TSPAN5) gene and is present in a region known to be a enhancer and transcription region.	1.96x10-7	4

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Part III

DNA methylation in organ transplantation

Chapter 4

Clinical potential of DNA methylation in organ transplantation

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Abstract

Identification of patients at risk for post-transplant complications is a major challenge and will improve clinical care and patient health after organ transplantation. The poor predictive values of current biomarkers strengthens the need to explore novel and innovative methods such as epigenetics for the discovery of biomarkers. Cell differentiation and function of immune cells is dependent on epigenetic mechanisms, which regulate gene expression without altering the original DNA sequence. These epigenetic mechanisms are dynamic, potentially heritable, change with age and can be regulated and influenced by environmental conditions. One of the most well-known epigenetic mechanism is DNA methylation, which comprises the methylation of a cytosine (C) next to a guanine (G; CpG dinucleotides). Aberrant DNA methylation is increasingly associated with diseases, including immune-mediated diseases, and these alterations precede the clinical phenotype. The impact of DNA methylation profiles on transplant acceptance and rejection as well as on other post-transplant complications is unknown. Here we will discuss the current evidence of the functional role of recipient and donor DNA methylation on outcome after organ transplantation. Changes in DNA methylation may predict the risk of developing post-transplant complications including infections, malignancies and allograft rejection. We speculate that identification of these changes in DNA methylation contributes to the earlier diagnosis and prevention of post-transplant complications leading to improved patient care.

Introduction

A major challenge in organ transplantation today is the identification of patients at risk for post-transplant complications. The current method for diagnosing rejection in heart transplantation is invasive and large variability is observed between pathologists in biopsy interpretation[1,2]. Crespo-Leiro et al.[3] evaluated the ISHLT 2004 acute cellular rejection (ACR) grading scheme within the CARGO II pathology panel and found low all-grade agreement, strengthening the need to discover and develop novel non-invasive methods to monitor the allograft. Ideally, rejection and other complications can be diagnosed and possibly predicted non-invasively from markers present in the peripheral blood or urine.

Several methods have been investigated to assess their potential to identify patients at risk for post-transplant complications. Immune-related chemokines and cytokines in the peripheral blood have been studied as non-invasive markers for acute and chronic rejection[4,5]. The Clinical Trials in Organ Transplantation (CTOT)-05 is a recently published multicenter cohort study which analyzes the potential of several biomarkers previously studied in both heart and kidney transplant recipients[6]. Their results show no significant associations between the majority of tested biomarkers and biopsy-proven acute rejection (BPAR) or CAV, including reactive T cell panels (interferon-gamma (IFNγ) Enzyme-Linked Immuno Spot (ELISPOT)), anti-Human Leukocyte Antigen (HLA) class II or anti-Donor-Specific Antibodies (DSA), and gene expression of 6 specific genes in blood. The results of this study do not support routine use of the studied assays to predict BPAR or CAV, however the authors point out the heterogeneity due to different clinical practice among centers as a weakness of the study. Even though Starling et al.[6] found no associations, other studies show promising results when analyzing gene expression in heart transplant patients, possibly due to a wider range of genes studied. Allomap® is a commercially available blood-based test to diagnose cardiac allograft rejection, based on gene expression levels of 11 genes[7]. It is developed based on results from the IMAGE (Invasive Monitoring Attenuation through Gene Expression)[8] and CARGO (Cardiac Allograft Rejection Gene Expression Observational Study) trials[9]. In both studies gene expression analysis resulted in significantly fewer biopsies taken to monitor the allograft. However, its results are also critically discussed[10] and we should be careful when extrapolating these results to high-risk patients. Another promising approach is the analysis of cell-free donor derived DNA (cfdDNA) in the serum of transplant patients, which is based on the assumption that the concentration of the cfdDNA correlates with the severity of the cardiomyocyte and endothelial damage[11]. Levels of cfdDNA were significantly higher in patients during acute rejection compared to stable transplant patients[12,13]. The predictive value of these assays is now explored and additional validation is necessary before clinical implementation[4,14].

Biomarkers for the diagnosis and/or prediction of post-transplant complications should be accurate with high specificity, high sensitivity and its results should be reproducible. The goal is to find biomarkers that display changes at the molecular level as early indicator for these post-transplant complications, before the clinical signs appear, as this may prevent irreversible tissue damage[15]. In addition to the gene expression studies, mechanisms that regulate gene expression, the so-called epigenetic mechanisms, can be studied as well. Genome-wide analysis of epigenetic features in transplant recipients may lead to the discovery of new biomarkers for the identification of patients at risk for post-transplant complications. This will provide tools to improve diagnostics and current treatment strategies. The first indication that epigenetics can be used for the diagnosis of rejection comes from studies by Mehta et al.[16]. The authors propose hypermethylation of the *CALCA* gene in urine of kidney transplant recipients as a biomarker for acute kidney injury. Nevertheless, more research should be performed to explore the potential of epigenetics in transplantation[17]. Integration of molecular techniques to provide biomarkers for complications after organ transplantation holds potential to improve the currently used diagnostics[7,11,15].

Epigenetic mechanism: DNA methylation

There are several epigenetic mechanisms which influence the condensation of the chromatin to make specific genes accessible or inaccessible to transcription factors, and thereby determine which genes are transcribed[18]. Histone modifications such as methylation or acetylation, non-coding RNA molecules which bind the DNA (siRNAs, lncRNAs, microRNAs) and methylation of DNA are the main epigenetic mechanisms[19] which are nicely illustrated in an extensive review by Portela et al.[20]. In the current review, we focus on DNA methylation which is the methylation of a cytosine (C) followed by a guanine (G), a CpG dinucleotide (Figure 1A). A methyl group is added to the DNA by DNA methyltransferases (DNMT) (Figure 1B), these are specific enzymes. CpG dinucleotides are unequally distributed across the genome and regions with a high number of CpG sites are called CpG islands which are mostly located in promoter regions[21]. High methylation of promoter CpG sites is associated with a tight, closed chromatin structure and transcriptional silencing of the associated gene (Figure 1C)[22]. DNA methylation is thereby responsible for the fine control of different cellular functions, including T cell differentiation during an immune response[23,24]. It is a dynamic feature, susceptible to cues from the environment[25], infections, chemical agents and drugs are examples of external factors that can influence methylation. Also internal stimuli like cytokines and hormones influence DNA methylation profiles[26,27].

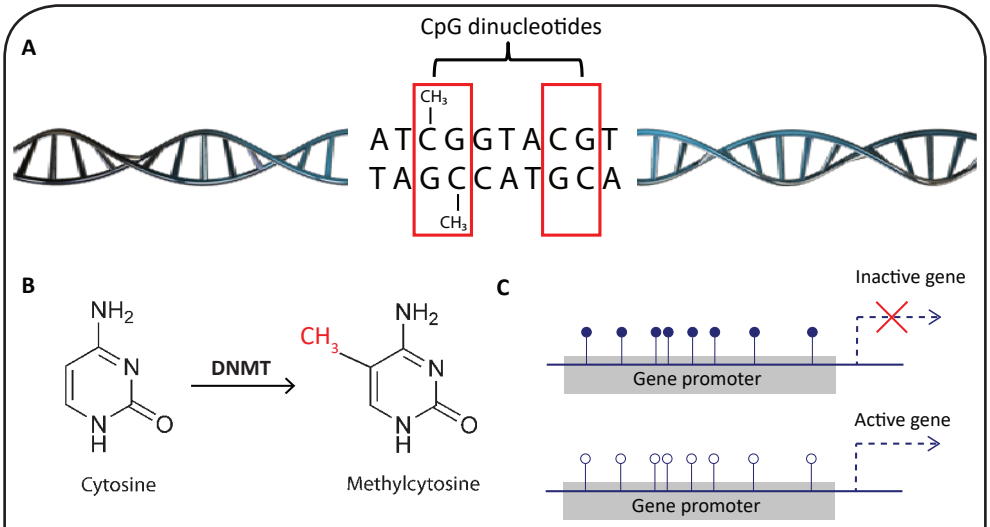

Figure 1. DNA methylation A) *A DNA strand with the nucleotides Adenosine (A), Thymidine (T), Cytosine (C) and Guanine (G). The red boxes highlight the CpG dinucleotides where the left is methylated and the right is demethylated.* ***B)*** *The addition of a methyl group to a cytosine is catalyzed by the enzyme DNA methyltransferase (DNMT).* ***C)*** *Two promoter regions where filled spots represent CpG sites which are methylated and empty spots represent CpG sites which are demethylated.*

Recent evidence indicates that functional DNA methylation also occurs outside of promoters in intra- and intergenic regions[28,29], affecting the three-dimensional organization of the DNA[30]. This is especially interesting because changes in methylation on CpG sites outside promoters might be associated with a specific disease, highlighting their potential as biomarker[21,31]. Smyth et al.[27] found intragenic CpG sites of which the DNA methylation was associated with chronic kidney disease without a significant change in the gene expression, suggesting a more distant function of the DNA methylation.

DNA methylation is one of the most studied epigenetic mechanism since it can be quantified relatively easy[32]. Bisulfite sequencing is currently the golden standard and a costly but more comprehensive approach is provided by Illumina for genome-wide DNA methylation analysis[33]. The advantage of studying DNA methylation over the whole epigenome is that not only DNA methylation on CpG islands and promoter regions is quantified but also intra- and intergenic regions are represented on the microarray. Due to cell-specific DNA methylation profiles it is recommended to focus DNA methylation studies on a specific cell type and not on a heterogeneous cell population as for example peripheral blood mononuclear cells (PBMCs)[34-36].

DNA methylation in disease

Aberrant DNA methylation profiles have been associated with different types of complex diseases[27,37,38]. This was first discovered in oncology where they found global loss of DNA methylation and regional increase in methylation[39]. To date the use of epigenetic biomarkers is well established for detection and diagnosis of several cancers[40]. Some of these markers can be detected not only by analyzing the affected tissue but also by other, less invasive methods, e.g. detection in urine or peripheral blood[41,42]. Already in 1999 Esteller et al. discovered hypermethylation of tumor suppressor genes in serum DNA as diagnostic marker for small-cell lung cancer[43].

In patients with immune disease such as systemic lupus erythematosus (SLE), diabetes, rheumatoid arthritis (RA) abnormal DNA methylation of immune cells is observed[44-46]. Heart failure is also associated with epigenetic changes and DNA methylation[47,48]. Differential DNA methylation of peripheral blood leukocytes (PBL) in repetitive elements *ALU*, *Satellite 2* and *LINE-1*, which is a measure for global DNA methylation, showed to be associated with ischemic heart disease and its risk factors[49,50]. However, exact cell composition was unknown in these PBL samples and the association was only significant in male subjects.

Discovery of a comprehensive set of biomarkers for diagnostic purposes improves (early) detection of the disease but also opens up the possibility to use them as therapeutic targets[39,51]. In oncology, epigenetic drugs such as demethylating agents 5'-azacitidine and decitabine, have proven to be an effective treatment and are both FDA approved[52]. Costa et al.[53] showed that 5'-azacitidine is effective in chronic myelomonocytic leukemia by decreasing activity of DNA methyltransferase, however its function is global and unspecific. For patients with cancer an epigenetic drug might be the last possible treatment, though the potential of these drugs for modulating DNA methylation in a more specific way should be carefully studied in a wider range of diseases.

DNA methylation in organ transplantation

Successful organ transplantation is the net-result of the overall cumulative injury caused by several events in the donor e.g. age, life-style, ischemia/reperfusion injury (IRI) and the immune response in the recipient[54-57]. Research shows that IRI causes epigenetic changes in the donor organ. Specifically the promoter region of the *C3* gene becomes demethylated in the kidney, which is associated with chronic nephropathy post-transplantation[58,59], a similar change in DNA methylation might occur during heart transplantation. The initial immune response by the recipient towards the transplanted organ largely depends on the IRI induced changes in the donor organ[60]. DNA methylation is a large contributor to the balanced immune response towards the graft as it regulates the function of cells of

the immune system[26]. B cells, NK cells, T cells and other immune cells are established during differentiation of hematopoietic stem cells resulting in distinct DNA methylation profiles for each cell type[61,62]. As in organ transplantation T cells play prominent roles in alloreactivity and are the key target for immunosuppressive drugs, we focus in this review on DNA methylation in T cells.

A direct link between DNA methylation profiles and organ rejection is not yet established, however several genes involved in alloreactivity are under regulation of DNA methylation. A summary of relevant genes, which can potentially serve as biomarkers for acute cellular rejection and immune activation in organ transplant recipients, is provided in Table 1. During an immune response the T cell differentiation from naïve to memory cells is controlled by DNA methylation. When genes encoding effector molecules become transcriptionally upregulated, repressive epigenetic marks as DNA methylation are lost[24,26,63]. The presence of memory T cells is both associated with acute and chronic rejection after organ transplantation[63-65]. Memory T cells express chemokine receptor type 6 (CCR6) at a higher level compared to naïve T cells, which is the consequence of hypomethylation of *CCR6*[66]. In CD8+ cytotoxic T cells the differentiation from naïve to effector cells is established by demethylation of the promoter region of specific effector genes as granzyme B and *IFN*γ[67]. The ability of memory CD8+ cells to rapidly demethylate effector genes on antigen re-exposure is most likely an interplay between DNA methylation and histone modifications[67]. The CD_4+ T cell differentiation is more complex due to the T helper cell lineage diversity within the effector population[23,24,68]. For example, the promoter region of *IFN*γ is methylated in naïve CD_4+ T cells and upon antigen stimulation, in the presence of Thelper1 (Th1) polarizing cytokines (e.g. Interleukin (IL)-12), the promoter region of *IFN*γ becomes demethylated enabling expression of *IFN*γ in CD_4+ Th1 cells. Consequently, cellular instability and plasticity[69] are both under control of DNA methylation. Cellular instability is a major concern in organ transplantation while considering regulatory T cell therapy. Under inflammatory conditions regulatory T cells can earn effector functions as IFNγ production[70] and IL-17 production through demethylation[71,72].

Important regulators of the immune response are regulatory T cells (Treg; CD_4+$FOXP_3$+)[100]. The role of these Treg in the induction and maintenance of tolerance in organ transplantation has been demonstrated in several experimental models of transplantation[101]. In humans high urinary mRNA levels of *FOXP3* have been associated with rejection reversal[102] though on the contrary high *FOXP3* expression in the allograft is found during acute rejection in transplanted organs[94,103,104]. Treg develop in the thymus, natural Treg (nTreg), and in addition Treg develop in the periphery (induced Treg; iTreg) under a variety of conditions including antigen stimulation in the presence of IL-2 and TGFβ[105,106]. The transcription factor $FOXP_3$ is essential for the maintenance and immune suppressive function of Treg

Table 1. *Genes involved in T cell alloreactivity under control of DNA methylation.*

Gene product	Gene	T cell source	Reference
Cytokines	*IFNγ*	CD4 (Th1)/CD8	73-77
	IL2	T cells	78, 79
	IL4	CD4 (Th2)	73, 80, 81
	IL10	Treg	73, 82
	IL17	CD4 (Th17)/CD8	83
Costimulatory molecules	*PD1*	CD8	84
	CD40L	CD4	85, 86
Cytotoxic proteins	*Granzyme B*	CD4/CD8	87 in mice
	Perforin 1	CD8	88, 89
Transcription factors	*FOXP3*	Treg	90-95
	RORC (RORγt)	CD4 (Th17)	72, 77
	TBX21 (T-bet)	CD4 (Th1)	96, 97
	GATA3	CD4 (Th2)	98, 99
Receptor	*CCR6*	Memory T cells	66

and its expression is controlled by DNA methylation[90,91]. The *FOXP3* gene contains one region, the Treg Specific Demethylated Region (TSDR), which is demethylated in nTreg and methylated in other peripheral blood leukocyte subtypes, including iTreg and recently activated T cells. Demethylation of this region results in a stable, constitutive expression of *FOXP3* and is used as a marker to identify nTreg[92,93].

Recently, we studied demethylation of the *FOXP3* gene in endomyocardial biopsies (EMB) after heart transplantation[95]. According to the International Society for Heart and Lung Transplantation (ISHLT) definition of rejection, only patients with EMB scored ≥ 2R are considered to experience a clinical relevant rejection requiring therapy[107]. Multiple EMB were analyzed of both patients who remained free from rejection (non-rejectors; 1R EMB) and patients who developed a histologically proven acute rejection (rejectors; 2R EMB and 1R EMB sampled 8 days before the 2R EMB). The percentage of demethylated *FOXP3* was significantly higher in the 1R EMB collected before rejection compared to the 1R EMB of the non-rejectors (Figure 2A). The question is whether this difference can be used to predict rejection which enables timely intensifying of the immunosuppressive therapy and possibly prevention of tissue damage. ROC analysis demonstrated a relatively good discrimination between the rejectors and non-rejectors (Figure 2B, area: 0.79, 95% confidence interval: 0.65-0.93). Nevertheless, the overlap between both groups is that large that at this moment a true cut-off point of % demethylated *FOXP3* to predict rejection is impossible to identify. By studying larger cohorts of 1R EMB or in combination with other,

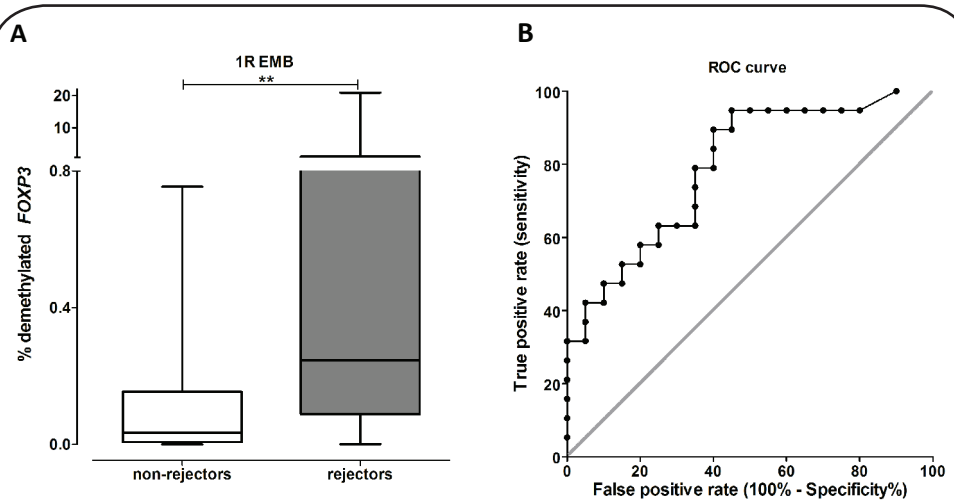

Figure 2. FOXP3 demethylation in endomyocardial biopsies A) *The percentage of demethylated FOXP3 is significantly higher in 1R EMB of rejectors collected before rejection (n=19) compared to 1R EMB of non-rejectors (n=20)* ***B)*** *Receiver operating characteristic (ROC) curve of the ability to predict rejection. Area under the ROC curve: 0.79 and the optimal threshold for predicting rejection is 0.088% of demethylated FOXP3 with 78,9% sensitivity and 65% specificity. ** indicates p<0.005*

to be identified, predictive markers, the % of demethylated *FOXP3* might be proven to be predictive for a clinical relevant rejection in the future.

Currently prescribed immunosuppressive drugs prevent the occurrence of rejection, however long-term complications of life-long use of these immunosuppressive drugs has become a major problem[108]. Demethylation of *FOXP3* is not only studied for predicting rejection but also for predicting long-term complications. Organ transplant recipients are 200 times more likely to develop a cutaneous squamous cell carcinoma (cSCC)[109], a specific type of skin cancer, for which immunosuppressive medication seems to be a large risk factor. Sherston et al.[110] studied demethylation of the TSDR as a marker for cSCC in kidney transplant recipients. They followed a cohort of 58 kidney transplant recipients in time and found a significant increase in the proportion of demthylated $CD4+$ $FOXP3+$ cells in patients who had previously developed an cSCC. The immune phenotype was stable in time, emphasizing its potential as biomarker for cSCC post-transplantation[110].

After heart transplantation cardiac allograft vasculopathy (CAV) is a common reason for retransplantation[111] and a common cause of death after 3 years, together with malignancy and renal failure[112]. Once CAV has been initiated in a patient it cannot be reversed, therefore early detection or prediction is important[113]. To date no reliable biomarkers have been found

to assess the risk for developing CAV[6], possibly the study of epigenetics in patients with CAV could complement or improve current diagnostics and hopefully provide therapeutic targets[114].

Conclusion

Epigenetics is part of the molecular mechanisms underlying the functional processes we have been studying in the past. Studying epigenetics over the whole epigenome will unravel the clinical potential of DNA methylation in organ transplant recipients. Most likely these studies will lead to the discovery of novel biomarkers for the identification of patients at risk for post-transplant complications. The advantage of studying DNA methylation over the whole epigenome is that not only DNA methylation in promoter regions is quantified but also intra- and intergenic regions are included, the exact function of DNA methylation in these regions is not entirely known yet. CpG sites outside of the promoter, which do not directly have an influence on gene expression, might be a discriminating biomarker for certain diseases. We speculate that DNA methylation analysis in organ transplant recipients will contribute to improved diagnostics of post-transplant complications with earlier detection, prediction and possibly prevention.

In Figure 3 we illustrate three hypothetical situations where several risk factors are assessed by quantifying DNA methylation on specific places in the genome. When risk factors can be estimated for a patient their treatment strategy can be adjusted, not only by altering medication but also by providing more specific lifestyle advices. Hopefully treatment of transplant patients can be customized in the future, moving more towards personalized medicine. The discovery of new epigenetic biomarkers also opens the possibility to use them as therapeutic targets, epigenetic drugs are becoming available in oncology and these should be studied to explore their application in transplantation.

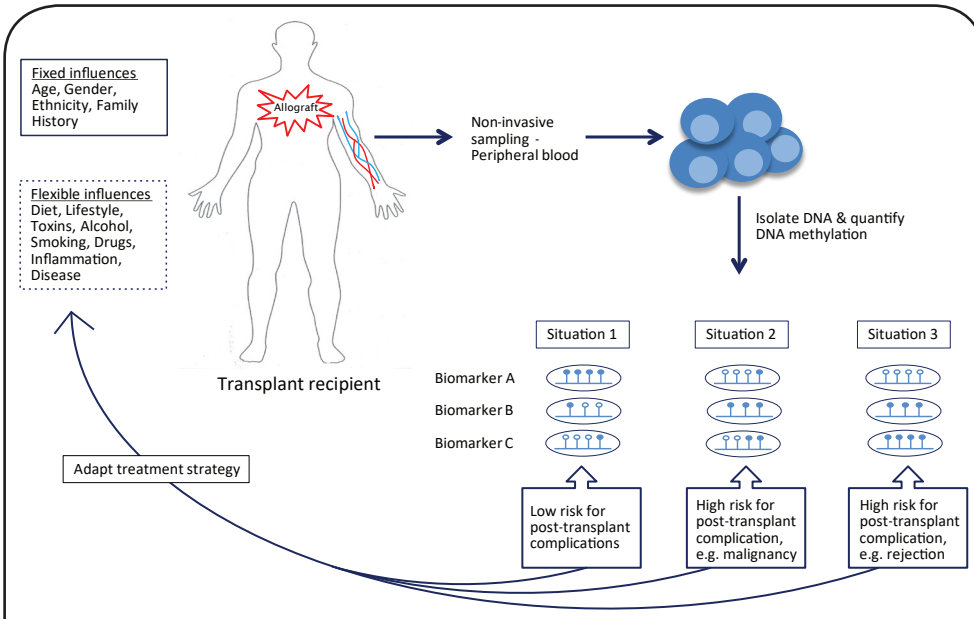

Figure 3. DNA methylation as biomarker in transplant patients. *Illustration of the workflow when biomarkers would be used to assess risk factors in transplant patients. The use of a non-invasive sampling method and a specific cell population to infer DNA methylation on specific locations in the genome. When risk factors are assessed the treatment strategy could be adjusted.*

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Chapter 5

Variations in DNA methylation of interferon gamma and programmed death 1 in allograft rejection after kidney transplantation

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Abstract

Background

The role of DNA methylation in the regulation of the anti-donor directed immune response after organ transplantation is unknown. Here, we studied the methylation of two mediators of the immune response: the pro-inflammatory cytokine *interferon γ* (*IFN*γ) and the inhibitory receptor *programmed death 1* (*PD1*) in naïve and memory CD8+ T-cell subsets in kidney transplant recipients receiving immunosuppressive medication. Both recipients experiencing an episode of acute allograft rejection (rejectors) as well as recipients without rejection (non-rejectors) were included.

Results

CpGs in the promoter regions of both *IFNγ* and *PD1* were significantly (p<0.001) higher methylated in the naïve CD8+ T cells compared to the memory T-cell subsets. The methylation status of both *IFNγ* and *PD1* inversely correlated with the % of IFNγ or PD1 producing cells. Before transplantation the methylation status of both *IFNγ* and *PD1* was not significantly different from healthy donors. At 3 months after transplantation, irrespective of rejection and subsequent anti-rejection therapy, the *IFNγ* methylation was significantly higher in the differentiated effector memory CD45RA+ (EMRA) CD8+ T cells (p=0.01) whereas the PD1 methylation was significantly higher in all memory CD8+ T-cell subsets (CD27+ memory; p=0.02: CD27- memory; p=0.02: EMRA; p=0.002). Comparing the increase in methylation in the first 3 months after transplantation between rejectors and non-rejectors demonstrated a significantly more prominent increase in the *PD1* methylation in the CD27- memory CD8+ T cells in rejectors (increase in rejectors: 14%, increase in non-rejectors: 1.9%, p=0.04). The increase in DNA methylation in the other memory CD8+ T cells was not significantly different between rejectors and non-rejectors. At 12 months after transplantation the methylation of both *IFNγ* and *PD1* returned to baseline levels.

Conclusions

The DNA methylation of both *IFNγ* and *PD1* increases the first 3 months after transplantation in memory CD8+ T cells in kidney transplant recipients. This increase was irrespective of a rejection episode indicating that general factors of the kidney transplantation procedure, including the use of immunosuppressive medication, contribute to these variations in DNA methylation.

Background

Kidney transplantation is currently the best treatment option for patients with irreversible, end-stage kidney disease[1]. Successful kidney transplantation is hampered by different complications including immune-mediated complications such as acute rejection[2]. Several non-invasive biomarkers for acute rejection have been studied, including proteins involved in cytotoxic lymphocyte function (e.g. perforin and granzyme B), cytokines (e.g. interferon (IFN) γ) and immune related chemokines (e.g. CXCL9 and CXCL10)[3,4]. Nevertheless it remains difficult to predict and regulate the host immune response after transplantation. The host immune response is orchestrated by a tightly regulated cascade of gene expression changes which are regulated by epigenetic mechanisms like histone modifications, DNA methylation, microRNA interactions and chromatin remodeling complexes[5-8]. Variations in these epigenetic mechanisms might serve as an additional marker to monitor the host immune response after organ transplantation.

An important player of the host immune response is the pro-inflammatory cytokine IFNγ and high expression of IFNγ is associated with both acute and chronic allograft rejection[9-11]. The expression of *IFNγ* is regulated by DNA methylation with the addition of methyl groups on cytosine phosphate guanine sites (CpGs) in the *IFNγ* promoter region silencing its expression. The CpG methylation pattern of *IFNγ* discriminates different T-cell subsets. First, naïve (antigen unexperienced) T cells versus memory (antigen experienced) T cells (both CD4+ and CD8+ T cells) with memory T cells having a lower methylation profile[12-14]. Second, the different T helper cell (Th) subsets with Th1 cells being hypomethylated compared to the Th2 and Th17 subsets[15-17]. Another important molecule involved in the regulation of the anti-donor immune response is the inhibitory receptor programmed cell death (PD) 1. Aggressive recipient T cells that attack the transplanted organ, the so-called alloreactive T cells, are inhibited by PD1 signaling. In addition, PD1 signaling promotes the generation of induced regulatory T cells[18,19]. The expression of *PD1* is also dependent on DNA methylation and while mainly methylated in naïve T cells, *PD1* is demethylated during differentiation into memory T cells[20].

Regulation of gene expression by DNA methylation is a well-known epigenetic mechanism with a critical role in physiological development and normal cell function by coordinating the lineage- and tissue-specific expression of genes[21]. DNA methylation is dynamic and susceptible to stimuli from the environment including internal stimuli like cytokines and hormones and external stimuli like chemical agents, pollutants, dietary components and chronic viral infections[16,22-24]. Aberrant DNA methylation profiles are associated with the pathogenesis of disease. Initially, DNA methylation was associated with tumor formation and progression[25], but later on variations in DNA methylation have been associated with

other diseases[26,27] including chronic kidney disease (CKD)[28,29] and immune-mediated diseases such as rheumatoid arthritis[30] and allergy[31,32]. In addition, variations in DNA methylation of immune related genes orchestrate the host immune response after organ transplantation[5-8].

Graft infiltrating cytotoxic CD8+ T cells play a major role in the rejection process and elevated numbers of effector and memory CD8+ T-cell subsets are associated with an increased risk for acute rejection[33-35]. Here we examined the influence of variations in DNA methylation of *IFNγ* and *PD1* in different CD8+ T-cell subsets on allograft rejection. The DNA methylation of *IFNγ* and *PD1* was determined in kidney transplant recipients before and 3 and 12 months after transplantation and both kidney transplant recipients who experienced a rejection episode within the first 3 months after transplantation and recipients who remained free from rejection were included. To exclude gender-[32] or chronic viral infection-[24] related differences we first analyzed whether the DNA methylation of either *IFNγ* or *PD1* was different in males versus females or in cytomegalovirus (CMV) seropositive healthy donors versus CMV seronegative healthy donors.

Results

IFNγ methylation is significantly decreased in CMV seropositive individuals

In PBMCs of CMV seronegative healthy donors, the DNA methylation of *IFNγ* was 51.2 ± 4.4 % (mean ± SD). The *IFNγ* methylation was significantly lower in PBMCs of age-matched CMV seropositive healthy kidney donors (45.1 ± 7.2%, p = 0.009; Figure 1A). In both males and females, the methylation of *IFNγ* was lower in the CMV seropositive individuals (Figure 1A) and there was no significant difference between males and females. The DNA methylation of *PD1* in PBMCs of CMV seronegative healthy donors was comparable to the *PD1* methylation in CMV seropositive healthy donors (40.5 ± 5.3% *versus* 38.9 ± 6.3%; Figure 1B). Subdividing the PBMCs into the different CD8+ T-cell subsets (Figure 1C) demonstrated significantly lower methylation of *IFNγ* in naïve, CD27+ memory and CD27- memory CD8+ T cells in CMV seropositive individuals compared to CMV seronegative individuals (Figure 1D). The methylation of *PD1* was not significantly different between the CMV seropositive individuals and CMV seronegative individuals in all the studied CD8+ T-cell subsets (Figure 1E).

DNA methylation inversely correlates with protein expression

To determine whether variations in DNA methylation at the described CpGs[20,36] are associated with changes in protein expression, we measured the expression of IFNγ and PD1 in the different CD8+ T-cell subsets (Figure 2A). A clear-cut difference was observed

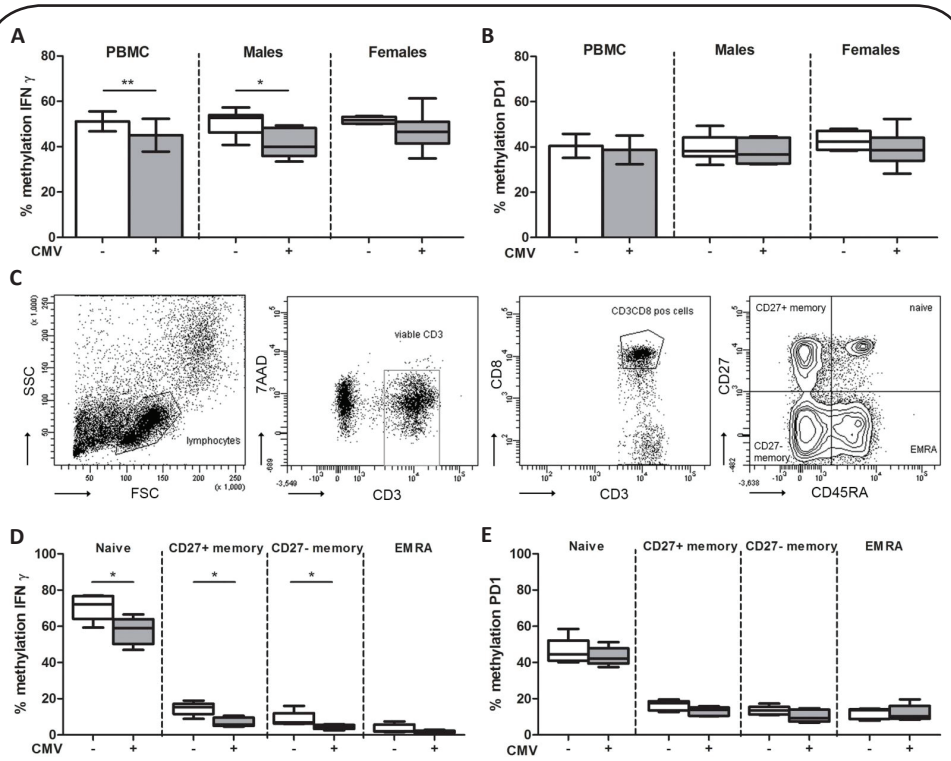

Figure 1. IFNγ and PD1 methylation in CMV seropositive and CMV seronegative healthy kidney donors. *The percentage of DNA methylation of IFNγ* ***A)*** *and of PD1* ***B)*** *in CMV seronegative (n=15; open bars) and CMV seropositive healthy donors (n=15; grey bars) in PBMCs (mean ± SD) and stratified by gender (box and whiskers min to max).* ***C)*** *Gating strategy of the different CD8+ memory T-cell subsets. The percentage of DNA methylation of IFNγ* ***D)*** *and of PD1* ***E)*** *in CMV seropositive (n=5; open bars) and CMV seronegative healthy donors (n=5; grey bars) in cell sorted CD8+ T-cell subsets; naïve, CD27+ memory, CD27- memory and differentiated effector memory CD45RA+ (EMRA). Box and whiskers (min to max); * p < 0.05 and ** p < 0.01*

between the naïve CD8+ T cells compared to the memory CD8+ T cells where 14.6 ± 16.4% (mean ± SD) of naïve CD8+ T cells expressed IFNγ *versus* 50.3 ± 18.9% of the CD27+ memory, 52.6 ± 20.6% of the CD27- memory and 66.1 ± 19.8% of the EMRA CD8+ T cells expressed IFNγ (p<0.0001; Figure 2B). In parallel, a significantly lower percentage of naïve CD8+ T cells expressed PD1 compared to the memory CD8+ T-cell subsets (naïve: 27.3 ± 16.5%, CD27+ memory: 67.9 ± 5.1%, CD27- memory: 68.4 ± 12.2% and EMRA: 51.4 ± 20.1; p<0.0001; Figure 2E). The highest percentage of IFNγ expressing cells was found within the EMRA CD8+ T cells while the CD27+ and CD27- memory CD8+ T-cell subsets contained the

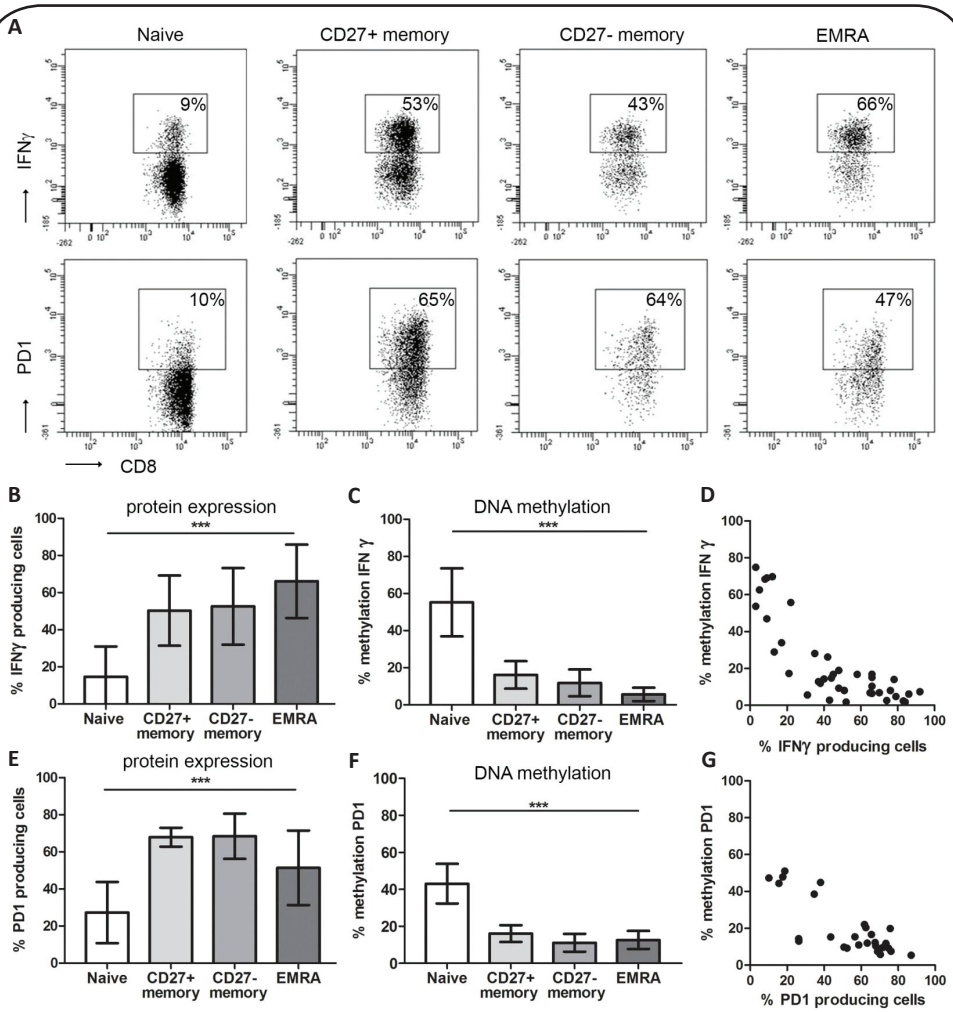

Figure 2. IFNγ and PD1 protein expression and IFNγ and PD1 DNA methylation. *FACS plots of IFNγ and PD1 expression in naïve, CD27+ memory, CD27- memory, and differentiated effector memory CD45RA+ (EMRA) CD8+ T cells in (**A**; representative example). Mean protein expression and percentage of DNA methylation in the different CD8+ T-cell subsets in kidney transplant recipients before transplantation (n=10; IFNγ in **B-D** and PD1 in **E-G**; mean ± SD). *** p < 0.001.*

highest percentages of PD1 expressing cells. The DNA methylation of both *IFNγ* and *PD1* demonstrated the opposite pattern with the highest percentage of methylation in naïve CD8+ T cells. Naïve CD8+ T cells were methylated for 55.2 ± 18.3% at the *IFNγ* locus and for 43.1 ± 10.7% at the *PD1* locus. This methylation was significantly higher (p<0.0001 for both *IFNγ* and *PD1*) compared to the different memory CD8+ T-cell subsets (Figure 2C and F).

This inverse relation between the DNA methylation and protein expression confirms the regulatory capacity of the studied CpGs (Figure 2D and G).

Variations in DNA methylation in kidney transplant recipients before transplantation

Before kidney transplantation, the methylation of *IFNγ* in CMV seronegative kidney recipients was comparable to the methylation levels in CMV seronegative healthy donors for naïve, CD27+ memory, CD27- memory and EMRA CD8+ T cells (Figure 3A). The same pattern was seen for the methylation of *PD1* (Figure 3B). Subdividing the transplant recipients into the ones that went on to experience a rejection after transplantation, the rejectors, and the non-rejectors, did not reveal any significant differences in methylation of *IFNγ* nor *PD1*, either between the two recipient groups nor in comparison to the healthy donors (data not shown).

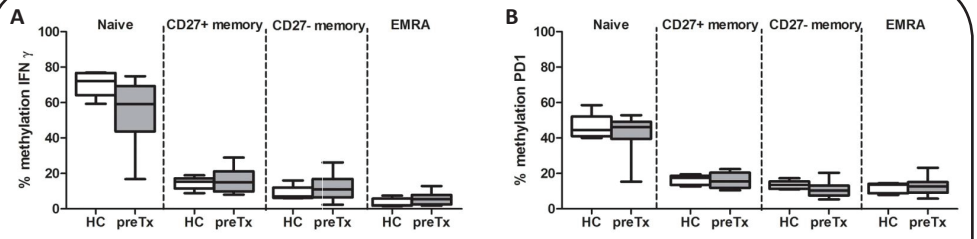

Figure 3. IFNγ and PD1 methylation in healthy donors and kidney transplant recipients before transplantation. *The percentage of DNA methylation of IFNγ* ***A)*** *and PD1* ***B)*** *in healthy controls (HC, n=5; open bars) and kidney transplant recipients before transplantation (preTx, n=10; grey bars) in cell sorted CD8+ T-cell subsets; naïve, CD27+ memory, CD27- memory and differentiated effector memory CD45RA+ (EMRA). Box and whiskers (min to max).*

Variations in DNA methylation in kidney transplant recipients after transplantation

After kidney transplantation the percentage of methylation of *IFNγ* did not change significantly in the naïve, CD27+ memory and CD27- memory CD8+ T cells during the first year after transplantation (Figure 4A-C). In the EMRA CD8+ T cells, the methylation of *IFNγ* was significantly higher at 3 months after transplantation compared to the methylation before transplantation irrespective of rejection and the subsequent anti-rejection therapy (p=0.01; Figure 4D). Focusing on rejection demonstrated that the methylation of *IFNγ* was significantly higher at 3 months after transplantation in the rejectors (14.3% *versus* 6.3% before transplantation; p=0.01) while the non-rejectors increased from 4.9% to 8.6% (not significant). Both rejectors and non-rejectors demonstrated elevated *IFNγ* methylation

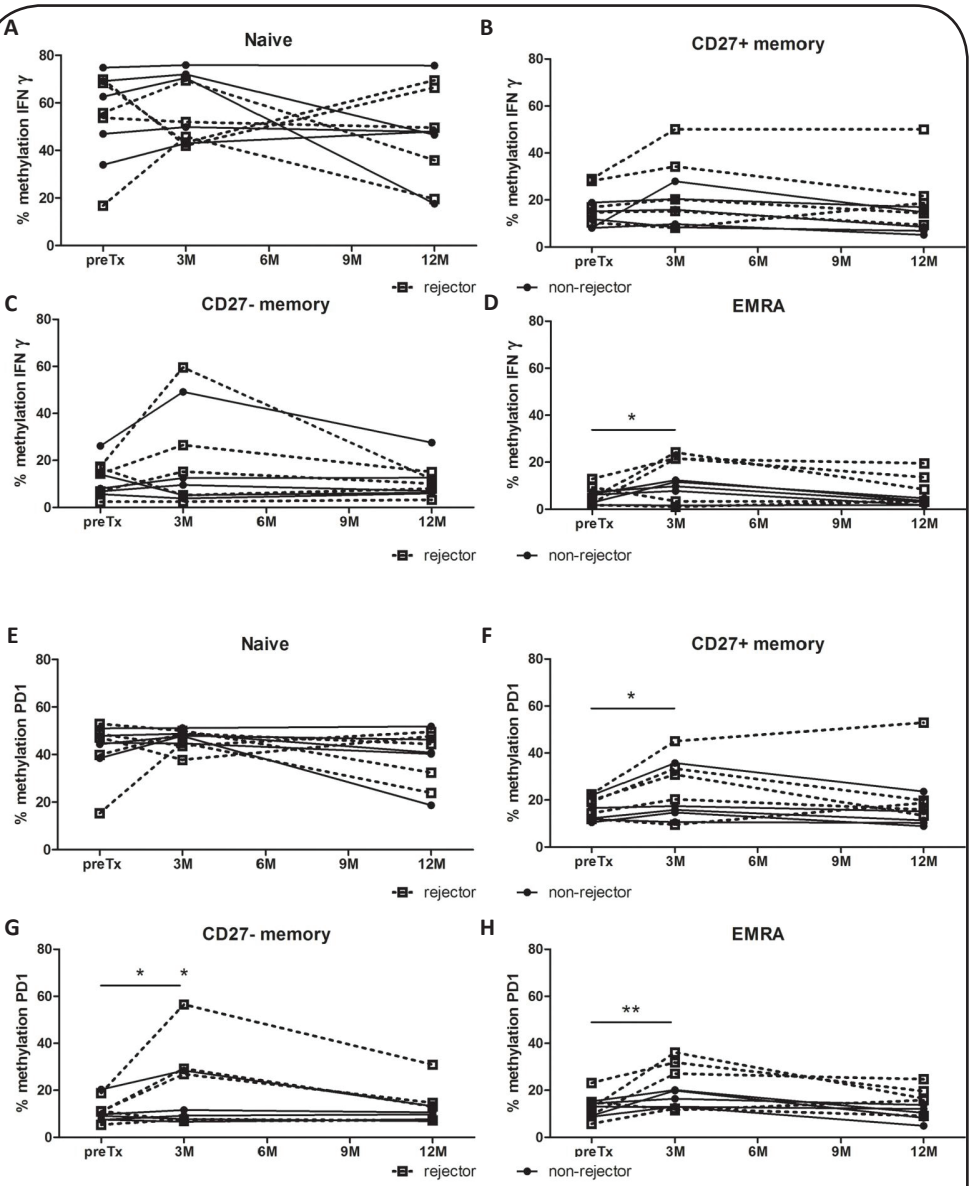

Figure 4. IFNγ and PD1 methylation in kidney transplant recipients during the first year after transplantation. *The percentage of DNA methylation of IFNγ (A-D) and of PD1* ***(E-H)*** *in kidney transplant recipients before and 3 and 12 months after transplantation in cell sorted CD8+ T-cell subsets; naïve* ***(A and E),*** *CD27+ memory* ***(B and F)****, CD27- memory* ***(C and G)*** *and differentiated effector memory CD45RA+ (EMRA;* ***D and H).*** ** p < 0.05 and ** p < 0.01.*

levels in the EMRA CD8+ T cells at 3 months after transplantation but this increase in methylation was not significant different between rejectors and non-rejectors (p=0.3). At 1 year after transplantation the methylation of *IFNγ* was comparable to the levels measured before transplantation.

The methylation of *PD1* did not change significantly in the naïve CD8+ T cells during the first year after transplantation (Figure 4E). Irrespective of rejection, the methylation of *PD1* significantly increased during the first 3 months after transplantation in CD27+ memory CD8+ T cells with 7.2% (p=0.02), in CD27- memory CD8+ T cells with 7.9% (p=0.02) and in EMRA CD8+ T cells with 7.5% (p=0.002; Figure 4F-H)). Focusing on rejection demonstrated a more prominent increase in DNA methylation in the rejectors compared to the non-rejectors in all memory CD8+ T-cell subsets (CD27+ memory: rejectors: 27.8% *versus* 17.6%, p=0.02 and non-rejectors: 18.9% *versus* 14.6% p=0.3; CD27- memory: rejectors: 25.4% *versus* 11.4%, p=0.002 and non-rejectors: 12.7% *versus* 10.9%, p=0.6; EMRA: rejectors: 23.8% *versus* 13.2%, p=0.002 and non-rejectors: 16.5% *versus* 12.1%, p=0.2; methylation at 3 months *versus* before transplantation respectively). The increase in *PD1* methylation in rejectors during the first three months after transplantation was not significantly different from the increase in *PD1* methylation in non-rejectors in both the CD27+ memory CD8+ T cells (p=0.3) and EMRA CD8+ T cells (p=0.2). In the CD27- memory CD8+ T cells the increase in *PD1* methylation was significantly higher in the rejectors (14%) compared to the non-rejectors (1.9%, p=0.04). In parallel with the methylation of *IFNγ*, the methylation of *PD1* returned to normal levels at 1 year after transplantation.

Discussion

The clinical potential of DNA methylation in organ transplantation, either as diagnostic or prognostic biomarker or as therapeutic target has been proposed by many[5-8,37,38]. Nevertheless, this is the first study where DNA methylation of two selected genes, *IFNγ* and *PD1*, was actually studied in CD8+ T cells in a small cohort of human kidney transplant recipients over time in relation to acute allograft rejection. Irrespective of rejection, we observed at 3 months after transplantation significant elevated DNA methylation levels of *IFNγ* in the differentiated EMRA CD8+ T cells, while the DNA methylation of PD1 was significantly higher in all CD8+ memory T-cell subsets. This increase in *IFNγ* methylation was not significantly different between rejectors and non-rejectors, while the increase in *PD1* methylation was significantly higher in the rejectors in the CD27- memory CD8+ T cells. In the other CD8+ memory T cells subsets (CD27+ memory and EMRA) the increase in DNA methylation of *PD1* was not significantly different between rejectors and non-rejectors.

Kidney transplantation will activate the recipient's immune system accompanied by an increase in cytokine production, including production of the pro-inflammatory IFNγ[35,39,40],

and upregulation of PD1 expression[41]. As protein expression inversely correlates with DNA methylation levels at gene promoter sites, kidney transplantation induces demethylation of genes involved in immune activation. However, for both *IFNγ* and *PD1* an increase in DNA methylation was observed in rejectors and non-rejectors in the first 3 months after transplantation, indicative for lower expression levels of IFNγ and PD1. Likely, the expected demethylation is only detectable in the donor-antigen specific T cells. The low percentage of these cells within the selected CD8+ T cells explains why the expected decrease in methylation was not observed. The observed increase in *IFNγ* and *PD1* DNA methylation most likely does not reflect the immune response against the foreign donor antigen but demonstrates a down regulation of the immune system achieved by the given immunosuppressive medication which non-specifically block all T cell subsets. For example the usage of prednisolone. In this study, prednisolone was tapered to 5 mg at month 3 and thereafter completely withdrawn. At 1 year after transplantation the DNA methylation levels returned to baseline.

In a clinical transplantation setting it is impossible to measure the DNA methylation of either *IFNγ* or *PD1* just before rejection. Currently rejection cannot be predicted as the moment of rejection strongly varies between individuals and therefore those samples are not available. Although material was only available of a small number of patients we had the unique opportunity the follow the same patients over time. Variations in DNA methylation are more profoundly found in the period after withdrawal of stress exposure (e.g. drugs) compared to the period during exposure[42,43]. Translation to the field of organ transplantation implies that after a rejection episode including anti-rejection therapy, rejectors would have more variations in DNA methylation compared to non-rejectors. However this was not true for the methylation of either *IFNγ* or *PD1* at 12 months after transplantation, indicating that allograft rejection has no imprinted effect on the DNA methylation of those immune genes.

Despite differences in immune activity of the distinct memory CD8+ T-cell subsets, the variations in DNA methylation in either memory subset were comparable. The EMRA CD8+ T cells are potentially the most aggressive subtype with a strong cytolytic activity, while the CD27+ memory cells display weak cytolytic activity producing effector cytokines such as interleukin (IL) 2, IFNγ, tumor necrosis factor (TNF) α and IL4[44,45]. The CD27- memory CD8+ T cells, which are functionally in between the CD27+ memory CD8+ T cells and the EMRA CD8+ T cells, represents the smallest subpopulation and it is unclear why specifically these cells demonstrated a significant difference in increase in methylation of *PD1* between rejectors and non-rejectors.

DNA methylation is adjustable by cues from the environment, *e.g.* viral infections[20,24,46],

though the exact cues and mechanisms remain largely unknown[16,22,23]. The uremic condition during chronic kidney disease (CKD) modifies DNA methylation profiles[47-49]. Although, before transplantation we did not observe significant changes in the methylation of either *PD1* or *IFNγ* compared to age-matched healthy donors. Either the previously observed effect on DNA methylation is gene specific and not applicable to *IFNγ* and *PD1* or the included transplant recipients here had less severe kidney disease compared to the CKD patients studied previously.

In contrast to previous observations where males demonstrated a significantly higher DNA methylation of *IFNγ* compared to females[32], significant differences in DNA methylation between males and females were not observed. However, we observed a significantly lower % of *IFNγ* methylation in CMV seropositive healthy donors compared to CMV seronegative healthy donors. The effect of chronic CMV infection on DNA methylation is not documented yet, but the change of the composition of the T cell pool with a permanent increase in highly differentiated T cells with a more memory phenotype in CMV seropositive individuals[50] has been demonstrated repeatedly. Therefore, the lower % of *IFNγ* methylation in CMV seropositive individuals might be explained by the fact that memory T cells are less methylated at the *IFNγ* locus (Figure 2 and [12-14]). Nevertheless, also in selected CD8+ memory T cells the methylation of *IFNγ* was significantly lower in the CMV seropositive individuals (Figure 1), indicating that CMV infection not only affects the composition of the T cell compartment but also induces a more aggressive T cell phenotype since demethylation is associated with an increased IFNγ production.

Although we could not identify variations in DNA methylation of either *IFNγ* or *PD1* in CD8+ T cells which could either diagnose or predict allograft rejection after kidney transplantation further research is needed to appreciate the clinical significance of variations in DNA methylation and other epigenetic mechanisms in kidney transplantation. Epigenetic biomarkers, mainly based on variations in DNA methylation, are well established in the diagnosis of cancer and are not only detectable in the affected tissue as well as in the urine or the peripheral blood[51,52]. Currently the application of epigenetic biomarkers is extended to other complex diseases such as autoimmune diseases[30,53,54]. The increasing knowledge on the epigenetic regulation of immune cells will contribute to our understanding of the epigenetic regulation of the complex anti-donor immune response after kidney transplantation. Epigenetic variations precede changes in protein expression and cell function and thereby represent an early indicator of clinical complications. Accordingly, a more comprehensive understanding of the epigenetic regulation of the anti-donor immune response will learn whether variations in DNA methylation can serve as predictive, diagnostic or prognostic markers. Moreover, since DNA methylation is influenced by environmental cues it might serve as a target for therapeutic intervention.

A genome-wide approach instead of selected immunoregulatory genes are a good option for future research. Genome-wide analysis enables the identification of variations in DNA methylation in all promoter regions as well as other gene regions including intragenic and intergenic regions[47,55,56]. Since DNA methylation profiles are cell-type specific[57], selected cell subsets involved in the anti-donor immune response (e.g. CD4+ T-cell subsets, B cells and macrophages), or even better the donor-antigen specific cells, should be analyzed. Another interesting, though technically more challenging option, is to analyze variations in DNA methylation in graft-infiltrating T cells. As variations in DNA methylation occur specifically in donor-antigen specific cells which are more abundantly present in the graft compared to the circulation.

Conclusion

After kidney transplantation the DNA methylation of the promoter of both *IFNγ* and *PD1* increases in the first 3 months and returns to baseline at 1 year after transplantation irrespective of rejection. These variations do not reflect the anti-donor immune response but are more likely the result of the transplantation procedure and the use of immunosuppressive medication.

Methods

Study population

Prior to the selection of kidney transplant recipients, we first determined whether cytomegalovirus (CMV) infection modulates DNA methylation of either *IFNγ* or *PD1*. Peripheral blood mononuclear cells (PBMCs) of 15 CMV seropositive healthy donors (age: 52 years, range 38-71; 5 males and 10 females) and 15 age-matched CMV seronegative healthy donors (age: 52 years, range 44-59; 11 males and 4 females) were studied. Of these 30 healthy donors in total, we selected 5 CMV seropositive and 5 CMV seronegative age-matched individuals to study the methylation status in different CD8+ T-cell subsets. Based on the significant decrease in DNA methylation of *IFNγ* in CMV seropositive healthy donors, we included only CMV seronegative kidney transplant recipients who received their first kidney from a living donor. The DNA methylation of both *IFN*γ and *PD1* was examined in different CD8+ T-cell subsets in 5 recipients who developed a biopsy proven acute cellular rejection within the first 3 months after transplantation (rejectors; Table 1) and 5 age-matched recipients who remained free from rejection the first year after transplantation (non-rejectors) and was compared to 5 age-matched healthy donors (age: 54 years, range 44-59). The different CD8+ T-cell subsets were analyzed at different time points; before transplantation and 3 months and 12 months after transplantation. The selected CMV seronegative recipients all received a kidney from a CMV seronegative donor and received basiliximab as induction therapy. After transplantation, recipients received standard triple maintenance therapy consisting of prednisolone (tapered after 3 months), mycophenalate mofetil (MMF) and tacrolimus. Anti-rejection therapy consisted of methylprednisolone (1 gram per day) on three consecutive days followed in some cases by anti-thymocyte globulin (ATG; n=2) or alemtuzumab (n=1).

Isolation of peripheral blood mononuclear cells and CD8+ T-cell subsets

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by density gradient centrifugation using standard Ficoll-Paque (GE Healthcare, Uppsala, Sweden) procedures. Since DNA methylation profiles are cell type specific[57] we examined naïve (antigen unexperienced; CD27+CD45RA+) CD8+ T cells and memory (antigen experienced) CD8+ T cells separately. The memory CD8+ T cells were subdivided into the differentiated effector memory CD8+ T cells (EMRA: CD27-CD45RA+, with a strong cytolytic activity), CD27+ memory T cells (CD27+CD45RA-; with weak cytolytic potential) and CD27- memory T cells (CD27-CD45RA-; functionally in between CD27+ memory CD8+ T cells and EMRA CD8+ T cells)[44,45]. The different CD8+ T-cell subsets were isolated using cell sorting (BD FACSAria™II SORP, BD Biosciences, San Jose, CA, USA) with a mean purity

Table 1. *Clinical characteristics of kidney transplant recipients*

	Rejectors	Non-rejectors
No of subjects	5	5
Age at transplantation[a] (yr)	47 (43-54)	52 (44-66)
Gender (M/F)	4/1	5/0
Serum creatinin[a,b] (µmol/l)	480 (270-1484)	532 (374-682)
Underlying kidney disease[c]		
HN/PKD/other	3/1/1	0/4/1
Renal replacement therapy[d]		
HD/PD/pre-emptive	1/2/2	1/1/3
Number of HLA-A/B mismatches[e]	2.2±0.4	2.8±0.8
Number of HLA-DR mismatches[e]	2.0±0	1.0±0.7

[a]median with range, [b]before transplantation, [c]HN: hypertensive nephropathy; PKD:polycystic kidney disease, [d]HD: hemodialysis; PD: peritoneal dialysis, [e]mean±SD

of 96%. Total PBMCs were stained with the following monoclonal antibodies: Brilliant Violet 510™ labeled CD3 (Biolegend, San Diego, CA, USA), APC-Cy7 labeled CD8 (BD), PE-Cy7 labeled CD27 (eBioscience, San Diego), APC labeled CD45RA (BD) and 7-amino-actinomycin D (7-AAD, BD) for the exclusion of nonviable cells.

Bisulfite conversion

PBMCs and the FACS-sorted CD8+ T-cell subsets were digested with proteinase K and treated with bisulfite using the EZ DNA Methylation-Direct Kit (Zymo Research from Base Clear Lab products, Leiden, The Netherlands), according to the manufacturer's instructions. During bisulfite treatment unmethylated cytosines were converted into uracil, whereas methylated cytosines remained unchanged.

PCR amplification and pyrosequencing

The DNA methylation of the *IFNγ* promoter was determined at 2 CpGs (CpG-186 and CpG-54) with transcription factor activity[36] and for *PD1* 8 previously described[20] CpG sites ranging between -914 and -738 bp from the start codon were studied (CpG-914, CpG-911, CpG-906, CpG-857, CpG-833, CpG-776, CpG-762, CpG-738). Since the methylation status at adjacent CpGs is correlated[58], the mean % of methylation of either *IFNγ* or *PD1* was calculated. Primers for PCR and pyrosequencing were designed using PyroMark Assay Design 2.0 software (Qiagen, Venlo, The Netherlands; Table 2).

PCR amplifications were performed with the Pyromark PCR Kit from Qiagen with each primer in a concentration of 0.2 µM. The PCR conditions were 15 minutes at 95 °C, 45 cycles

Table 2. *Primers for PCR amplification and pyrosequencing*

Gene	Primers	CpGs
IFNγ	F: 5'-ATGGTATAGGTGGGTATAATGG-3'	
	R: 5'-biotin-CAATATACTACACCTCCTCTAACTAC-3'	
	S: 5'- GGTGGGTATAATGGG-3'	CpG-186
	S: 5'- ATTATTTTATTTTAAAAAATTTGTG-3'	CpG-54
PD1	F: 5'- AGTATAGAATATAAGGAGATAAGTAAGT-3'	
	R: 5'-biotin- CCATAACCACAATTCCAAATCTTT-3'	
	S: 5'-AGAATATAAGGAGATAAGTAAGTT'-3'	CpG-914, CpG-911, CpG-906
	S: 5'-GGATTTTTTGAATTATTTTATTTTG'-3'	CpG-857, CpG-833
	S: 5'- TTAGTTTTATAGTTAGTTTTTG-3'	CpG-776, CpG-762, CpG-738

F: forward primer, R: reverse primer, S: sequencing primer, CpGs: cytosine phosphate guanine sites

of 30 seconds 94 °C, 30 seconds 58 °C for *IFNγ* and 56 °C for *PD1* and 30 seconds 72 °C followed by 10 minutes at 72 °C and on hold at 21 °C. After visualisation of the appropriately sized PCR product on a 1% agarose gel, the PCR product was sequenced using a PyroMark Q24 pyrosequencer (Qiagen) with the following minor revisions to the manufacturer's instructions: to immobilize the PCR product 1µl Streptavidin Sepharose High Performance Beads (GE Healtcare) were used per sequence reaction and annealing of the sequence primers was done for 3 minutes at 80 °C. The bisulfite conversion and the subsequent PCR amplification and pyrosequencing were performed in duplicate. Human low and high methylated DNA from EpigenDx (Hopkinton, MA, USA) were used as controls.

IFNγ and PD1 protein expression

To determine IFNγ and PD1 protein production by the different CD8+ T-cell subsets, total PBMCs were either not stimulated or stimulated in the presence of 1 µg/ml Brefeldin A (GolgiPlug; BD Biosciences) with PMA (50 ng/ml, Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (1 µg/ml, Sigma-Aldrich) for 4 hours at 37 °C in 5 % CO_2. For IFNγ, cells were stained for 30 minutes for the following surface markers: Brilliant Violet 510™ labeled CD3 (Biolegend), APC-Cy7 labeled CD8 (BD Biosciences), PE-Cy7 labeled CD27 (eBioscience), APC labeled CD45RA (BD Biosciences) and 7-amino-actinomycin D (7-AAD, BD Biosciences), fixed, permeabilized and stained with FITC-labeled IFNγ (BD Biosciences) for 30 minutes. Frequencies of IFNγ producing CD8+ T-cell subsets were corrected for background determined with the unstimulated condition. For PD1, cells were stained with the previously described surface markers while PE-labeled PD1 (Biolegend) was added. For PD1 expression a Fluorescence-Minus-One (FMO) was used to correct for background

staining. Samples were measured on the FACSCanto II (BD) and analyzed using FACSDiva software version 6.1.2. (BD).

Statistical analysis

To identify differences between groups the unpaired t-test, Mann-Whitney U test and ANOVA were used as appropriate. To determine differences after kidney transplantation over time between rejectors and non-rejectors we used multilevel analysis with the percentage of DNA methylation as outcome. Predictors were different individuals (rejectors and non-rejectors), time also as categorical predictor (levels 0 (before transplantation), 3 and 12 months after transplantation) and individuals as random intercept. Each model was applied for the 4 different cell types studied; naïve, CD27+ memory, CD27- memory and EMRA CD8+ T-cell subsets. Afterwards we added models with interaction between type of individual and time. The first model describes the same pattern over time for both rejectors and non-rejectors while the second one enables to estimate and test different trends in time for rejectors and non-rejectors. The estimates and standard errors were transformed to CI's and p-values. We used the package R version 3.1.2 and libraries lmer and lmerTest. A p-value of <0.05 was considered statistically significant.

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Chapter 6

Differentially methylated regions in T cells identify kidney transplant patients at risk for de novo skin cancer

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Abstract

Background

Cutaneous squamous cell carcinoma (cSCC) occurs 65-200 times more in immunosuppressed organ transplant patients than in the general population. T cells, which are targeted by the given immunosuppressive drugs, are involved in anti-tumor immune surveillance and are functionally regulated by DNA methylation. Prior to kidney transplantation, we aim to discover differentially methylated regions (DMRs) in T cells involved in de novo post-transplant cSCC development.

Methods

We matched 27 kidney transplant patients with a future de novo cSCC after transplantation to 27 kidney transplant patients without cSCC and studied genome-wide DNA methylation of T cells prior to transplantation. From 11 out of the 27 cSCC patients the DNA methylation of T cells after transplantation was also examined to assess stability of the observed differences in DNA methylation. Raw methylation values obtained with the 450k array were confirmed with pyrosequencing.

Results

We found 16 DMRs between patients with a future cSCC and those who do not develop this complication after transplantation. The majority of the DMRs were located in regulatory genomic regions such as flanking bivalent transcription start sites and bivalent enhancer regions, and most of the DMRs contained CpG islands. Examples of genes annotated to the DMRs are ZNF577, coding for a zinc-finger protein, and FLOT1, coding for a protein involved in T-cell migration. The longitudinal analysis revealed that DNA methylation of 9 DMRs changed significantly after transplantation. DNA methylation of 5 out of 16 DMRs was relatively stable, with a variation in beta-value lower than 0.05 for at least 50% of the CpG sites within that region.

Conclusions

This is the first study demonstrating that DNA methylation of T cells from patients with a future de novo post-transplant cSCC is different from patients without cSCC. These results were obtained before transplantation, a clinically relevant time point for cSCC risk assessment. Several DNA methylation profiles remained relatively stable after transplantation, concluding that these are minimally affected by the transplantation and possibly have a lasting effect on post-transplant cSCC development.

Background

The risk of developing cancer is markedly higher in organ transplant patients than in the general population[1]. The most common cancer in transplant patients is non-melanoma skin cancer whereby cutaneous squamous cell carcinoma (cSCC) occurs most frequently[2], with an increased risk of 65-200 fold[2-4]. Not only the incidence of cSCC increases after organ transplantation, the skin cancer also behaves more aggressively. Transplant patients experience more metastasis and more recurrence of the cSCC: 70% of the patients develop a subsequent skin cancer within 5 years[5,6]. Identification of transplant patients at increased risk for cSCC may allow early intervention and will improve the quality of life for these patients.

Transplant patients are at high risk for cSCC because of their impaired immune system due to lifelong immunosuppressive therapy[7-9]. Immunosuppressive drugs used after organ transplantation suppress T-cell activity[10]. T cells are an important cell type for anti-tumor immune surveillance (CD8+), but can also provide a more immune-tolerant environment for the tumor (regulatory T cells)[11,12]. Carroll et al.[13] showed that high numbers of peripheral regulatory CD_4+$FOXP_3$+ cells predicted the development of a new cSCC in kidney transplant patients who had a previous cSCC. Also the presence of CD8+$CD57^{hi}$ cells, a phenotype associated with T-cell senescence, was shown to predict development of a subsequent cSCC in kidney transplant patients[14]. These studies both predicted recurrence of the cSCC, tools to predict de novo cSCC after transplantation are currently unavailable.

Considering the recurrent nature of cSCC and the increased incidence in immunocompromised transplant patients, we hypothesized that there is a systemic defect in patients who will develop cSCC due to an altered state of T-cell function. Such an altered state of T-cell function is a well-known consequence of loss of kidney function[15]. T-cell function is determined by the chromatin state of its DNA, which is a combination of epigenetic features such as DNA methylation, DNA accessibility, histone modifications and RNA expression[16,17]. DNA methylation is an important epigenetic regulator of cellular function[18,19] and high methylation in the transcription start site (TSS) of a gene is in most cases associated with transcriptional silencing of the corresponding gene[20].

Differential DNA methylation between transplant patients with or without a future post-transplant cSCC might provide insight in the pathogenesis of cSCC. However, DNA methylation is a dynamic feature and significantly influenced by the environment[21]. After kidney transplantation, immunosuppressive therapy is given and the metabolic complications associated with loss of kidney function largely disappear. Therefore, it can be expected that changes in DNA methylation will occur and this may also affect any

DNA methylation profiles identifying patients at risk for de novo post-transplant cSCC. By comparing these DNA methylation profiles before and after transplantation, the extent of their functional effect on post-transplant cSCC development could be assessed.

In this retrospective study, we aimed to identify kidney transplant patients at risk for de novo post-transplant cSCC by studying genome-wide DNA methylation of T cells. We analyzed samples collected before transplantation and compared patients with a future de novo post-transplant cSCC to patients without cSCC. Highly enriched T cell populations were isolated from these patients and genome-wide DNA methylation was measured. We then searched for differentially methylated regions (DMRs) by comparing the future cSCC patients' methylation profiles to the non-cSCC profiles. For a subset of cSCC patients, a post-transplantation sample was available which enabled us to compare DNA methylation before and after transplantation. A technical validation of the raw methylation values on the array was performed with pyrosequencing.

Methods

Patients samples

Anonymized biobank samples were used in this study, this approach had been approved by the local ethical committee (MEC-2015-642). Kidney transplant patients with a future post-transplant cSCC were matched to kidney transplant patients who have not developed an cSCC based on gender, age (±2 years), ethnicity, cytomegalovirus (CMV) status and availability of biobank material. We included patients with at least one cSCC after transplantation and patients with cSCC in situ (Bowen's disease). Patients with a previous kidney transplantation or another donor organ such as liver, heart or lung were excluded, as well as patients with a history of malignancy prior to transplantation. Non-cSCC patients with actinic keratosis, a pre-cancerous lesion, were excluded.

The patient cohort consisted of 27 cSCC patients and 27 non-cSCC patients who had been transplanted between 1997 and 2014. No statistical differences were found between the clinical characteristics of the cSCC and non-cSCC patients, however after cell sorting the composition of CD4+ and CD8+ T cells significantly differed between the cSCC and non-cSCC patients (Table 1). One cSCC patient had received immunosuppressive drugs prior to an ABo-incompatible transplantation.

From 11 cSCC patients, material collected after transplantation was available for a longitudinal analysis, characteristics of this subset of patients are given in Table 2. The post-transplantation samples were collected based on availability of biobank material and are therefore at different time points after transplantation (Table 3). Three of the

Table 1. *Patient characteristics*

	cSCC N = 27	**non-cSCC** N = 27	
Age (years)[a]	61.7 (27-77)	61.3 (27-77)	p=0.802
Gender (male)	19 (70.4%)	19 (70.4%)	p=1
Years between Tx and first cSCC[a]	5.4 (0.9-12.5)	-	-
CMV status			p=0.46
Negative	12 (44.4%)	9 (33.3%)	
Positive	15 (55.6%)	17 (63.0%)	
Unknown	-	1 (3.7%)	
Dialysis pre-transplantation			p=0.783
Yes	16 (59.3%)	15 (55,6%)	
No	11 (40.7%)	12 (44.4%)	
ESRD diagnosis			p=0.058
Polycystic kidney	6 (22.2%)	1 (3.7%)	
Hypertension	6 (22.2%)	3 (11.1%)	
Diabetic nefropathy	1 (3.7%)	6 (22.2%)	
Glomerulonefritis	3 (11.1%)	6 (22.2%)	
Other	11 (40.7%)	11 (40.7%)	
% CD3[a]	97.4 (92.4-99.5)	98.0 (95.1-99.5)	p=0.225
% CD4[a]	73.0 (45.1-91.4)	60.3 (34.8-80.7)	p=0.000
% CD8[a]	20.7 (5.8-46.2)	32.8 (14.8-60.6)	p=0.000

[a]median and range; cSCC: cutaneous squamous cell carcinoma, CMV: cytomegalovirus, ESDR: end stage renal disease

post-transplant samples were taken after diagnosis of the first cSCC. All of these patients received treatment, patient "p1" was treated with a topical chemotherapeutic agent 5-fluorouacil, patient "p2" was treated with photodynamic therapy and surgical excision and patient "p4" was treated with a surgical excision.

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using standard Ficoll-Paque procedures (GE Healthcare, Chicago, IL, US). Isolated PBMCs were stored at -140°C until further use. T cells were isolated from the PBMCs using fluorescence-activated cell sorting (FACS) by the BD FACSAria™ II (BD Biosciences, San Jose, CA, US). PBMCs were stained with anti-CD3 Brilliant Violet 510 (Biolegend, San Diego, CA, US), anti-CD4 Pacific Blue (BD Biosciences), anti-CD8 APC-cy7 (BD Biosciences) and to exclude nonviable cells 7AAD PerCP (BD Biosciences) was used. After cell sorting the purities were >92% for CD3+ cells, samples below 90% were excluded for further analysis.

Table 2. *Patient characteristics longitudinal analysis*

	N = 11
Age at Tx (years)[a]	65.4 (47-75)
Gender (male)	8 (72.7%)
Years between Tx and first cSCC[a]	2.6 (1.1-11.5)
Years between Tx and post-Tx sample[a]	2.1 (0.3-13.0)
CMV acceptor	
Negative	4 (36.4%)
Positive	7 (63.6%)
CMV donor	
Negative	7 (63.6%)
Positive	4 (36.4%)
HLA mismatches[a]	2 (0-6)
Type of immunosuppression directly after transplantation	
Corticosteroids	10 (90.9%)
Tacrolimus	10 (90.9%)
MMF	10 (90.9%)
Cyclosporine	1 (9.1%)
Sirolimus	1 (9.1%)
Basiliximab induction	3 (27.3%)
ATG induction	1 (9.1%)
ESRD diagnosis	
Polycystic kidney	5 (45.5%)
Hypertension	1 (9.1%)
Other	5 (45.5%)
Dialysis pre-transplantation	
Yes	8 (72.7%)
No	3 (27.3%)

[a]median and range; cSCC: cutaneous squamous cell carcinoma, CMV: cytomegalovirus, ESDR: end stage renal disease

Before isolating DNA from the T cells, all patient samples were randomized to minimize batch effects. DNA was isolated using the QIAamp DNA Micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Purity and concentration of the isolated DNA was assessed with the NanoDrop ND-8000 (Isogen Life Science, Utrecht, The Netherlands). DNA degradation was determined by gel electrophoresis, none of the samples showed significant degradation.

Table 3. *Time points longitudinal analysis*

Patient	Time after Tx (y)	Time between Tx and first cSCC (y)	Comment
p1	13	11	Material obtained after diagnosis of first cSCC
p2	7.7	4.1	Material obtained after diagnosis of first cSCC
p3	6.9	7.7	
p4	3.4	2.4	Material obtained after diagnosis of first cSCC
p5	0.9	4.7	
p6	2.1	2.6	
p7	0.3	1.6	
p8	1.1	2	
p9	1.1	1.1	
p10	0.6	2.2	
p11	5	11.5	

Tx: Transplantation, cSCC: cutaneous squamous cell carcinoma, y: years

DNA methylation microarrays

To generate genome-wide DNA methylation data, 500 ng of genomic DNA was treated with sodium-bisulfite to induce methylation-dependent changes in the DNA sequence, using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, US). DNA was then hybridized on Infinium HumanMethylation450 arrays (Illumina, San Diego, CA, US) according to the manufacturer's protocol and IDAT files were generated by the iScan BeadChip scanner (Illumina).

Data quality was examined using the MethylAid R package[22,23]. All samples passed the five quality controls performed using the default MethylAid thresholds. Probes with a detection P value>0.01 were removed from the dataset as well as probes containing single nucleotide polymorphisms. Since our patient population was a mixture of male and female, all probes on the sex chromosomes were also removed. A between-array normalization was applied to the Type I and Type II probes separately using the DASEN method within the wateRmelon Bioconductor R package[23-25]. The methylation level for each cytosine-phosphate-guanine (CpG) site was calculated as the ratio of the methylated probe intensity and the overall intensity. This is presented as a beta-value, a value between 0 (unmethylated) and 1 (fully methylated). After the quality controls and normalization, beta-values of 423,289 CpG sites remained for further analysis. Both the raw and normalized data are available via the NCBI Gene Expression Omnibus (GEO) database with accession number GSE103911.

Data analysis DNA methylation microarrays

To identify DNA methylation differences between the future cSCC and non-cSCC patients, we fitted a linear mixed-effect model using the lme4 R package[26]. The fixed effects included age, percentage CD4, percentage CD8 and CMV status. %CD4 and %CD8 were included in the model because we found that the composition was different between the cSCC and non-cSCC patients after cell-sorting (Table 1). Array IDs were included as a random effect to account for technical variation between the arrays. Single site-specific p-values were obtained and these p-values together with the genomic location of the CpG sites, were used as input into comb-p[27].

Comb-p is a command-line tool based on a python library to spatially correlate p-values[27]. Since DNA methylation at adjacent CpG sites is correlated it strengthens the data to study regions that are differentially methylated instead of single sites[28,29]. Comb-p calculates a weighted correlation between the p-values from the single CpG site-specific analysis and combines adjacent p-values based on this correlation. A sliding window of 500 base pair (bp) was used and the seed was set at $p<0.01$. It then performs a false discovery rate (FDR) adjustment to this new correlation adjusted p-values, finds regions of enrichment at an FDR cut off of 0.05 and assigns significance to those regions. Multiple testing correction in this analysis is done using a Šidák correction (Šidák<0.05)[30]. The resulting DMRs were annotated to ROADMAP reference data of primary CD3+ cells[16] to determine the CpG island content and the chromatin state of the DMRs.

Longitudinal analysis

For 11 cSCC patients (Table 2 and 3), we compared DNA methylation values of the DMRs before and after transplantation. A paired statistical analysis was done per region. To improve clarity, only those CpG sites within a DMR with a Δbeta-value larger than 0.05 (5% methylation) were used for detailed graphical representation and the patients were evenly divided in 4 time segments after transplantation. The CpG sites within a region that increased or decreased less than 0.05 in beta-value per patient were considered stable in time.

Technical validation

Performing methylation arrays for a risk assessment is not easily applicable to clinical practice due to high costs and labor-intensive workflow. Therefore we tested whether we could obtain the same methylation values with bisulfite pyrosequencing, an easy technique to quantitatively measure single-site DNA methylation[31]. CpG sites within the DMRs 2 and 3 were analyzed in the same DNA samples that were used for the array analysis. Of 10

patients, a mixture of cSCC and non-cSCC patients, 200 ng genomic DNA was bisulfite converted using the EZ DNA Methylation-Direct kit (Zymo Research) according to the manufacturer's protocol. The bisulfite treated DNA was then amplified by polymerase chain reaction (PCR) using the Pyromark PCR kit (Qiagen). Primers for PCR and pyrosequencing were designed using PyroMark Assay Design 2.0 software (Qiagen). The PCR primers, melting temperatures and amplicon sizes for the different PCR products can be found in Supplementary Table S1 together with the specific PCR programs for each DMR.

After confirming the amplicon size by gel electrophoresis, the PCR products were sequenced using a PyroMark Q24 pyrosequencer (Qiagen). Minor adjustments were made to the manufacturer's protocol: to immobilize the PCR product 1 µL Streptadivin Sepharose High Performance Beads (GE Healthcare) was used per sequence reaction and annealing of the sequence primers was done for 3 minutes at 80°C. The sequence primers were added at a concentration of 10 µM. Human high and low methylated DNA (EpigenDx, Hopkinton, MA, USA) were used as controls. DNA methylation percentages were calculated by PyroMark Q24 software (Qiagen).

Statistical analysis

Differences in characteristics between the future cSCC and non-cSCC patients were statistically tested using SPSS version 21.0 (IBM Corp., Armonk, NY, US). The Mann-Whitney U test was used for the continuous variables and χ^2 test for the categorical variables. Data processing and statistical analysis of all the microarray data was done in RStudio version 1.0.136 (Rstudio Inc., Boston, MA, US) with R version 3.2.5[24]. Cohen's D was calculated on the residuals of the linear mixed-effect model by the formula $D = (mean_{cSCC} - mean_{non\text{-}cSCC}) / sd_{pooled}$ in R. Analysis of the differences between methylation in pre-transplantation and post-transplantation samples was done using a paired Wilcoxon ranked sum test using R. Correlation between the DNA methylation levels quantified by pyrosequencing and the beta-values of the Illumina 450k arrays was calculated using Spearman's rank correlation coefficient using SPSS. All statistical tests were two-tailed and a $p<0.05$ was considered statistically significant.

Results

Differentially methylated regions

To identify DMRs in T cells between patients who will develop cSCC after kidney transplantation and those without cSSC, we analyzed genome-wide DNA methylation of kidney transplant patients before transplantation. After cell sorting the T cells, we observed a difference in CD4/CD8 composition between the future cSCC and non-cSCC patients' T

cells. The future cSCC patients had a higher percentage of CD4+ cells than the non-cSCC patients ($p<0.001$; Table 1). For this reason we included the percentage CD4+ and CD8+ in the linear mixed model as covariates, thereby avoiding potentially biased results with respect to the differences in DNA methylation. None of the single-site p-values passed the multiple testing correction (Supplementary Figure S1) therefore we continued to DMR analysis.

We found 16 regions significantly differentially methylated between the future cSCC and non-cSCC patients. In Table 4, the genes annotated to the DMRs, the genomic location of the DMRs according to the hg19 genome build (UCSC Genome Browser) and the number of array probes within the regions are presented, and the gene functions are shortly described. Also the Cohen's D is presented per region which is a measure for effect size taking into account the standard deviation in the two groups . Out of the 16 DMRs, 5 were hyper methylated and 11 were hypo methylated in the future cSCC patients.

Table 4. *Resulting differentially methylated regions of the pre-transplantation analysis*

	Genomic location (hg19)	Length DMR	no. of probes	Regional p-value	Cohen's D	DMR state
1	chr19:4531638-4531962	324 bp	4	$3.57 \cdot 10^{-11}$	0.95	Hyper
2	chr5:63461216-63461931	715 bp	10	$5.51 \cdot 10^{-10}$	-0.54	Hypo
3	chr3:44753865-44754399	534 bp	11	$8.18 \cdot 10^{-10}$	-0.6	Hypo
4	chr2:3699195-3699564	369 bp	5	$9.35 \cdot 10^{-10}$	0.81	Hyper
5	chr6:168197177-168197700	523 bp	6	$6.54 \cdot 10^{-9}$	-0.68	Hypo
6	chr4:165898666-165898968	302 bp	8	$1.49 \cdot 10^{-8}$	0.54	Hyper
7	chr5:140305947-140306459	512 bp	10	$2.38 \cdot 10^{-8}$	-0.53	Hypo
8	chr2:177014555-177015126	571 bp	12	$4.35 \cdot 10^{-8}$	0.41	Hyper
9	chr1:185703201-185703689	488 bp	12	$1.89 \cdot 10^{-7}$	-0.42	Hypo
10	chr6:30698584-30698988	404 bp	11	$2.90 \cdot 10^{-7}$	-0.48	Hypo
11	chr19:52391078-52391606	528 bp	12	$6.59 \cdot 10^{-7}$	0.58	Hyper
12	chr8:54164051-54164443	392 bp	8	$1.20 \cdot 10^{-6}$	-0.48	Hypo
13	chr7:51539131-51539584	453 bp	5	$1.61 \cdot 10^{-6}$	-0.64	Hypo
14	chr6:88757302-88757704	402 bp	6	$1.80 \cdot 10^{-6}$	-0.55	Hypo
15	chr2:74875227-74875549	322 bp	8	$1.45 \cdot 10^{-6}$	-0.47	Hypo
16	chr8:96085385-96085690	305 bp	3	$1.22 \cdot 10^{-5}$	-0.74	Hypo

DMR: differentially methylated region, chr: chromosome, bp: base pair

Genomic characteristics of the DMRs

Since CpG islands are often found near transcription start sites (TSS) and are involved in transcription initiation[32], methylation of CpG islands could have a downstream effect on gene activity. Together with the cell-type specific chromatin state of the DNA, this could indicate the biological function of a genomic region. In Figure 1A the CpG island content is depicted for all regions together and the individual DMRs separately, the array content is given as reference. The 16 DMRs are enriched for CpG islands, slightly less CpG sites are within the shores (<2kb flanking CpG islands) and CpG sites within shelves (<2kb flanking shores) are absent in these DMRs. For the chromatin state, we annotated the CpG probes within each DMR to ROADMAP epigenomics reference data of primary T cells using the 15-state model[16] (Figure 1B). Although this might not be an accurate representation of the chromatin state within the T cells we analyzed, it does provide a general perspective on functional and primary T-cell specific characteristics of the genomic region where the DMRs are located. The chromatin states 'flanking bivalent TSS/enh' and 'bivalent enhancer' are enriched in our results, also 7 out of the 16 DMRs are within repressed or weakly repressed polycomb which is a slight enrichment compared to the array content.

DNA methylation of the DMRs after transplantation

To study whether DNA methylation of the 16 DMRs changed after transplantation, we compared beta-values of 11 cSCC patients before and after transplantation. Figure 2A shows the mean difference in beta-value which is an average of all CpG sites per region for all 11 patients together. Overall mean beta-value increased after transplantation. In most regions there were CpG sites that increased and CpG sites that decreased, therefore showing a mean difference close to zero. All differences in beta-value per DMR and per patient can be found in Supplementary Figure S2. A paired Wilcoxon ranked sum test per region resulted in 9 regions that were significantly different after transplantation, after a Bonferroni multiple testing correction (Table 5).

All CpG sites showed variation within all patients, therefore to reduce noise and improve clarity we considered a CpG site that increased or decreased less than 0.05 in beta-value stable. None of the DMRs were 100% stable in time (Figure 2B) however, some regions showed more stability than others. DMRs 1, 5, 9, 14 and 16 showed at least 50% stable CpG sites whereas in DMRs 4, 11 and 13 none of the sites were stable in time. A more detailed graphical representation of the changes in beta-value per region, per patient and in time can be found in Supplementary Figure S3.

We also analyzed the mean methylation differences per patient to examine a possible relationship with time after transplantation and with time to clinical onset of the cSCC

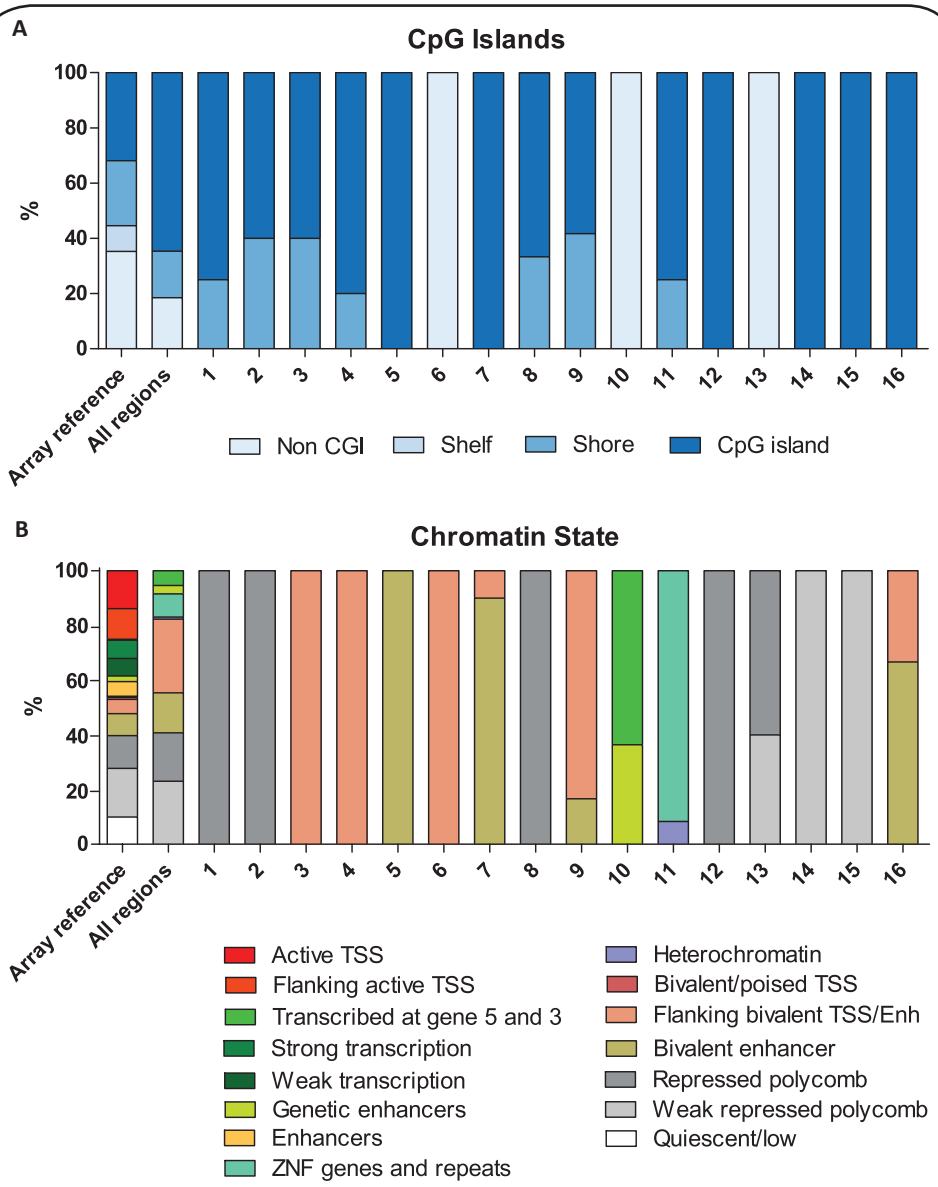

Figure 1. The genomic characteristics of the CpG sites within each DMR. A) *CpG island content for all regions together and the individual DMRs separately, the array content is given as reference. The color represents the CpG island content of each CpG site within that region according to the legend below the graph.* ***B)*** *Primary T-cell specific chromatin state according to the 15-state model of the ROADMAP epigenomics reference data[16] for all regions together and the individual DMRs separately, the array content is given as reference. The color represents the primary T-cell specific chromatin state of the CpG sites within that region according to the legend below the graph.*

Table 5. *Results of statistical tests between pre-transplant and post-transplant beta-values per region*

DMR	P value	Bonferroni correction
1	0.87	
2	$1.83 \cdot 10^{-6}$	$2.92 \cdot 10^{-5}$
3	$2.03 \cdot 10^{-5}$	$3.25 \cdot 10^{-4}$
4	0.002	0.038
5	0.082	
6	0.55	
7	$8.09 \cdot 10^{-8}$	$1.29 \cdot 10^{-6}$
8	0.002	0.033
9	$1.51 \cdot 10^{-5}$	$2.41 \cdot 10^{-4}$
10	$3.71 \cdot 10^{-13}$	$5.93 \cdot 10^{-12}$
11	0.028	
12	$9.42 \cdot 10^{-5}$	0.002
13	0.14	
14	0.32	
15	$5.48 \cdot 10^{-5}$	$8.78 \cdot 10^{-4}$
16	0.33	

(Table 3). These mean differences were relatively small in 5 out of 11 patients (Δbeta-value<0.01) (Figure 3). Mean methylation differences were not significantly correlated to the time between transplantation and clinical onset of cSCC (p=0.46), nor to time after transplantation (p=0.50), nor to time between post-transplant sample and the clinical onset of cSCC (p=0.09).

Technical validation

To confirm the raw beta-values obtained with the 450k array, we performed pyrosequencing analysis of two DMRs (6 CpG sites) on the same DNA samples that were analyzed on the array. The DNA methylation values obtained with pyrosequencing were slightly lower than the beta-values obtained with the arrays, this was a consistent deviation across all samples (Figure 4). There was a strong correlation between the results obtained with the two different techniques; the two sites within DMR 2 had a Spearman correlation coefficient (r) of 0.95 (p<0.0001) and the 4 sites within DMR 3 had an r of 0.88 (p<0.0001).

Discussion

Our results demonstrate that the T cells of patients with a future post-transplant cSCC have different DNA methylation profiles compared to the T cells of kidney transplant patients without cSCC. To our knowledge this is the first study to show DNA methylation differences in peripheral T cells between patients who develop a post-transplant cSCC and those who do not develop cSCC. In addition, we were able to obtain these results at a clinically relevant time point, before transplantation. The retrospective nature of this study allowed us to carefully match the future cSCC patients to non-cSCC patients and examine the DNA methylation within a highly enriched T-cell population.

The observed differences in DNA methylation are predominantly located in CpG islands and bivalent enhancer regions (Figure 1). Since these are both regulatory genomic regions, it is likely that these differences have a downstream effect in T cells and that differential

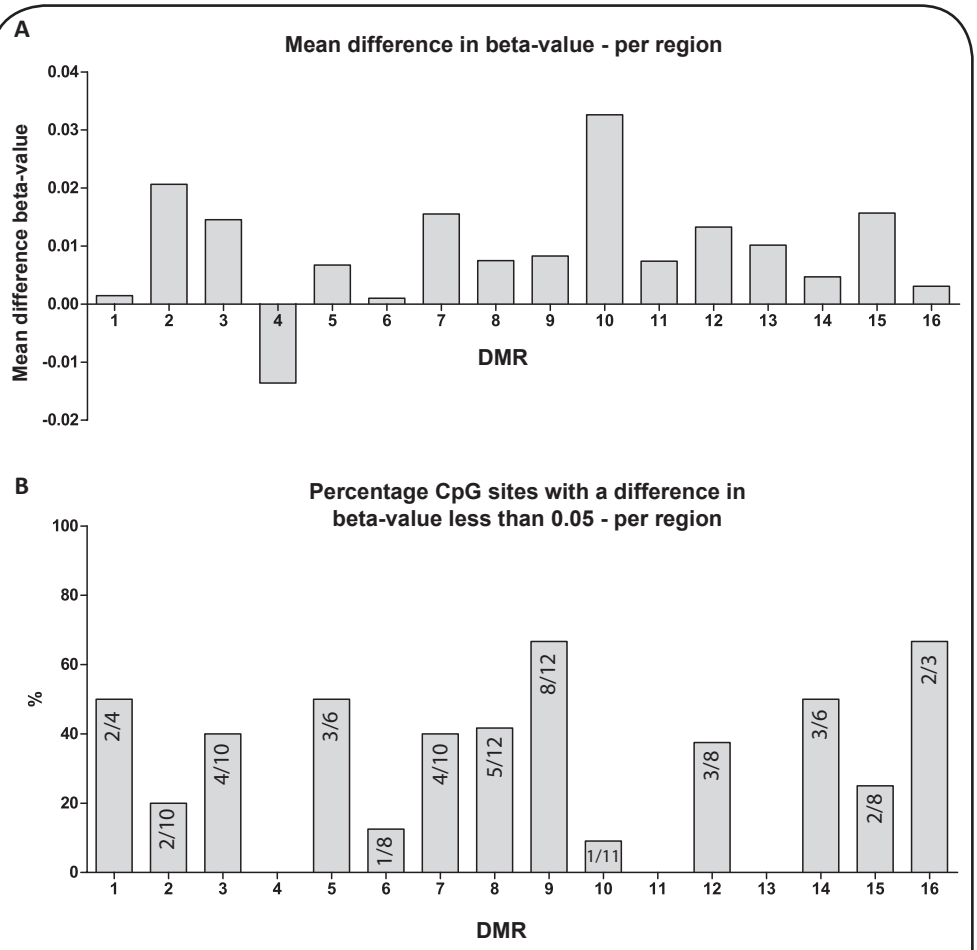

Figure 2. Stability of the 16 DMRs. A) *Mean difference in beta-value per region between pre-transplant and post-transplant samples. The difference is calculated per CpG site for each individual patient and is then averaged over all CpG sites per region for all 11 cSCC patients together.* ***B)*** *Percentage of CpG sites that show a Δbeta-value of less than 0.05 presented per region. The numbers within each bar represent the number of stable CpG sites from the total sites within that region.*

DNA methylation within these regions could affect T-cell function. Though, the effect of differential methylation at enhancer regions is difficult to assess since enhancers can regulate genes at large distances in the genome[33]. RNA sequencing would reveal any distal gene regulation by these enhancers, however that was outside the scope of this study. Here we focus on the genes that were annotated solely on the basis of close proximity to the DMR.

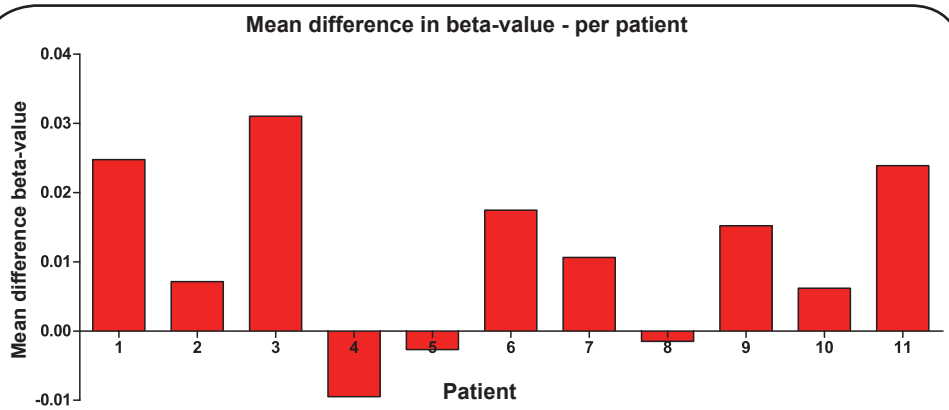

Figure 3. Mean difference in beta-value per patient between pre-transplant and post-transplant sample. *The difference was calculated per CpG site for each individual patient and was then averaged over all CpG sites per patient.*

Out of the 16 DMRs a few could be associated to cancer by studying literature. Even though these studies were not performed in T cells but mostly in the tumor tissue itself, we can speculate on a possible relationship with post-transplant cSCC development. An example is DMR 11 (annotated to ZNF577) which was hypermethylated in our future cSCC patients, showed to be hypermethylated in SCC and adenocarcinoma of the lungs[34]. In addition, an inverse correlation between ZNF577 gene expression and its DNA methylation was found[35]. DMR 10, which was situated within the actively transcribed gene FLOT1, was hypo methylated in our cSCC patients. At first sight an interesting gene due to its involvement in migration of hematopoietic cells[36] and it showed to promote invasion and metastasis of several SCC subtypes when overexpressed[37,38]. However, in the longitudinal analysis this was the most varying region (Table 5) with the majority of CpG sites increasing in DNA methylation after transplantation (Figure S2J). This suggests that this region is greatly influenced by transplantation and it remains unsure how this differential methylation at time of transplantation could affect post-transplant cSCC development.

A kidney transplantation is a procedure with major health effects for an end-stage renal disease (ESRD) patient and these effects influence DNA methylation. Several studies have shown that blood DNA methylation is associated to kidney function[39,40]. In addition to that, we showed in a previous study that DNA methylation of T cells can also be modulated by the immunosuppressive medication that kidney transplant patients receive after transplantation[41]. We therefore expected variation between the pre-transplant and post-transplant DNA methylation values in the longitudinal analysis. Indeed, we see that beta-values were significantly different in 9 of the 16 DMRs (Table 4). More interestingly, all but one region increased in mean DNA methylation after transplantation (Figure 2). This could

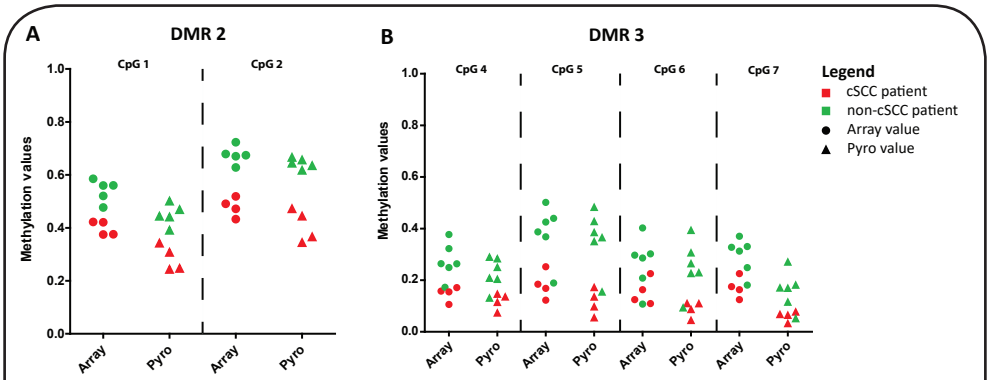

Figure 4. Methylation values on the array and by pyrosequencing of 6 CpG sites within two DMRs. A) *DMR 2 (r=0.95; p<0.0001)* ***B)*** *DMR 3 (r=0.88; p<0.0001). The CpG sites correspond to the CpG sites within the DMRs (Table 4).*

6

be a general effect of the transplantation and is in line with findings by Boer et al.[42] showing increased DNA methylation at the PD1 and IFNγ gene 3 months after transplantation.

To determine which regions could have a lasting effect on post-transplant cSCC development, we examined stability of the 16 DMRs after transplantation and considered the CpG sites that stayed within a Δbeta-value of 0.05 stable. DMRs 1, 5, 14 and 16 have 50% or more stable CpG sites and were also not significantly different in a paired statistical analysis (Table 4), suggesting that these differential methylation profiles might have a prolonged effect after transplantation. Considering the possibility of distal gene regulation by these DMRs, their functional effect could be determined by a genome-wide RNA and protein analysis within these T cells. Additionally, to overcome the variability in sampling time points within this study, a prospective study with sampling at regular intervals after transplantation would further assess stability of these DMRs and their function in post-transplant cSCC development.

The development of post-transplant cSCC is the result of a series of events involving different risk factors[2]. Known examples are age, skin type, gender and possibly immune phenotype[43]. After cell-sorting the T cells, we found significantly higher percentages of CD_4+ T cells and consequently lower percentages of CD8+ T cells in the future cSCC patients (Table 1). This suggests that an altered CD_4/CD8 ratio might be another risk factor for post-transplant cSCC. There is no consensus in literature on the CD_4/CD8 ratio in relation to post-transplant cancer development. In contrast to our findings Thibaudin et al.[44] found, over a 10-year observation period, consistently lower counts of CD_4+ T cells in patients with future post-transplant malignancy. Although this was not evident at time of transplantation but occurred thereafter. Whereas Bottomley et al.[14] found no significant

difference in CD4+ T cell and CD8+ T cell counts or percentages between SCC and non-cSCC kidney transplant patients.

The relative small sample size in this study is a consequence of selective matching and availability of biobank material. This combined with the single-center design of the study leads to cautious interpretation of the findings. Moreover, we acknowledge that patient pairs can never be perfectly matched. Since we are studying T cells and not skin tissue, where the differences between healthy and malignant tissue are much larger, it was expected that the differences would be subtle. Despite these limitations the results of this study are a promising first step towards early risk assessment for post-transplant cSCC. To assess the clinical value of these findings, a validation in a different and larger cohort of transplant patients is necessary in addition to our technical validation[45,46].

Conclusion

The findings presented here demonstrate the potential of studying DNA methylation of the T cells to identify kidney transplant patients at risk for de novo post-transplant cSCC [47]. We showed that there were systemic differences between future cSCC and non-cSCC patients prior to transplantation. A longitudinal analysis showed that several DNA methylation profiles remained relatively stable after transplantation, suggesting a lasting effect on the development of de novo cSCC after transplantation. In the future, identification of patients at increased risk for post-transplant cSCC before transplantation will allow for early clinical interventions such as regular visits to the dermatologist and stricter life-style advise to the patient to minimize additional sun-exposure [48]. Ultimately it may lead to adjustment of the immunosuppressive load but this remains a fine balance between reducing the risk for cancer and causing irreversible damage to the allograft.

Supplementary Tables

Table S1. *PCR primers, sequence primers and PCR programs for technical validation*

DMR	Primers (Forward, Reverse and Sequence)	Amplicon size	CpG sites (Illumina ID)
RNF180	F: 5′-GGTGGAATTTTAGGTATAAGAAGGTAA-3′	229 bp	
	R: 5′-biotin-AAACCACAAAAATTATCCCTATAATCTCC-3′		
	S: 5′-ATTTTAGGTATAAGAAGGTAAG-3′		cg17621438 , cg07850154
	PCR program: 15 min at 95°C, 45 cycles of 30 s 94°C, 30 s 58°C, 30 s 72°C followed by 10 min at 72°C		
ZNF502	F: 5′-TTTAGAGGTGGATTGGGGTTAGGATATTA-3′	159 bp	
	R: 5′-biotin-AAATACCTTCTTCTAAAATCCCATAAAA-3′		
	S: 5′-GGATATTAGTTTTAATTTTTGAAAT-3′		cg21672276, cg10263370, cg11003573, cg15687855
	PCR program: 15 min at 95°C, 45 cycles of 30 s 94°C, 30 s 58°C, 30 s 72°C followed by 10 min at 72°C		

F: Forward primer, R: Reverse primer, S: Sequence primer, bp: basepair, min: minutes, s: seconds

Supplementary Figures

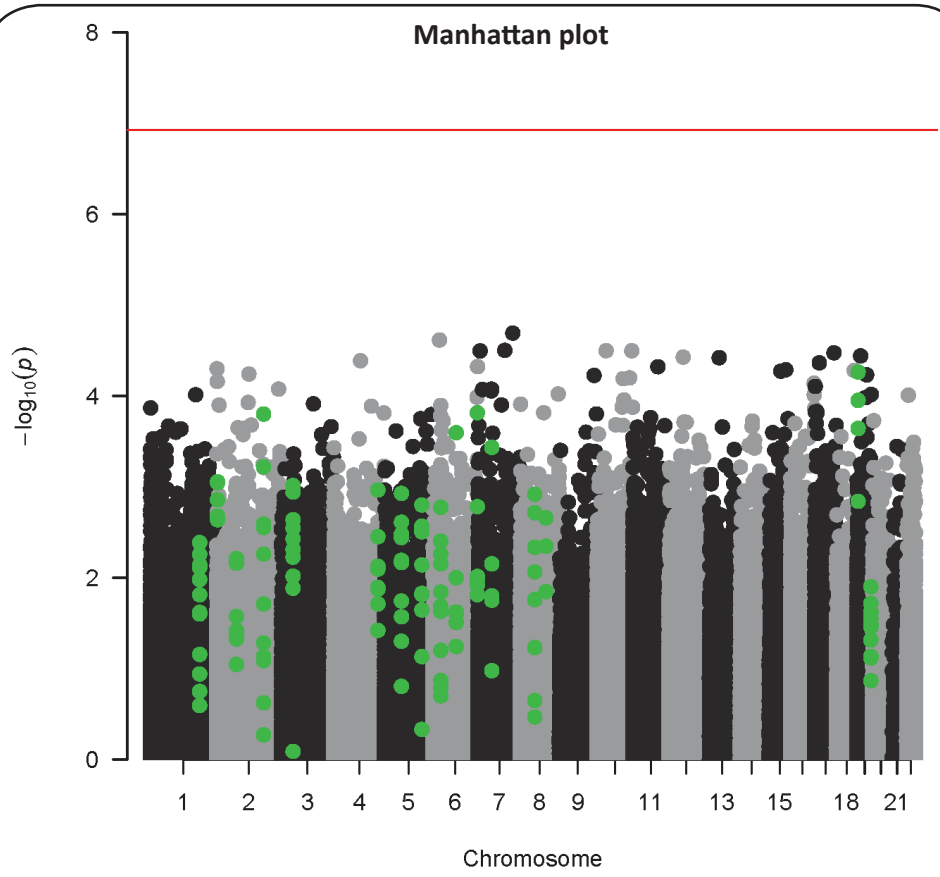

***Figure S1**: **A Manhattan plot** showing all individual CpG sites and their p-values. On the y-axis the $-\log_{10}$ of the p-value is depicted, the genome wide significance line in red is on $-\log_{10}(1.18 \cdot 10^{-7})$, and on the x-axis is the genomic location of all the sites, split up in the different chromosomes. The dots in green represent the CpG sites that are in the significant DMRs.*

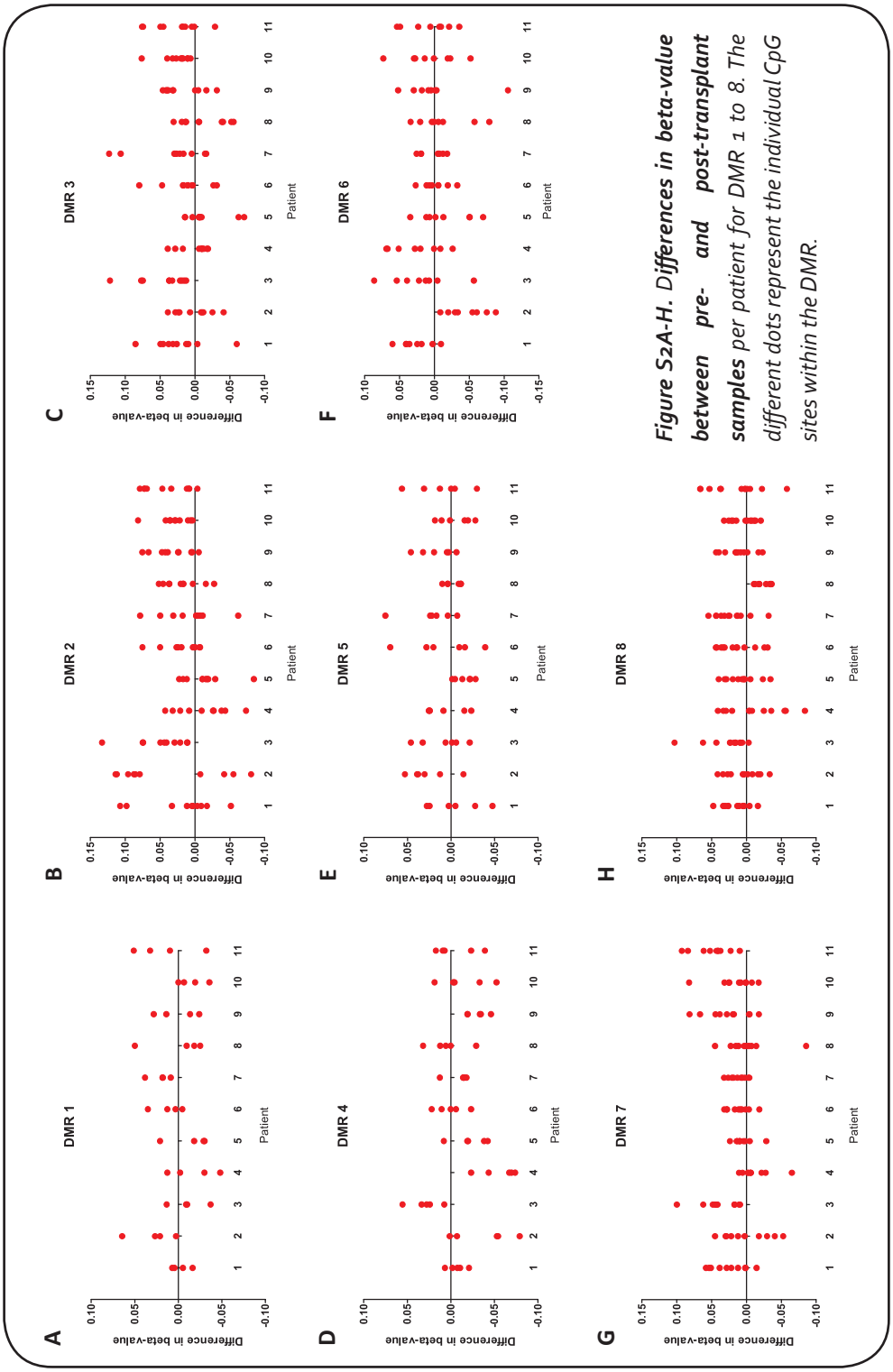

Figure S2A-H. Differences in beta-value between pre- and post-transplant samples *per patient for DMR 1 to 8. The different dots represent the individual CpG sites within the DMR.*

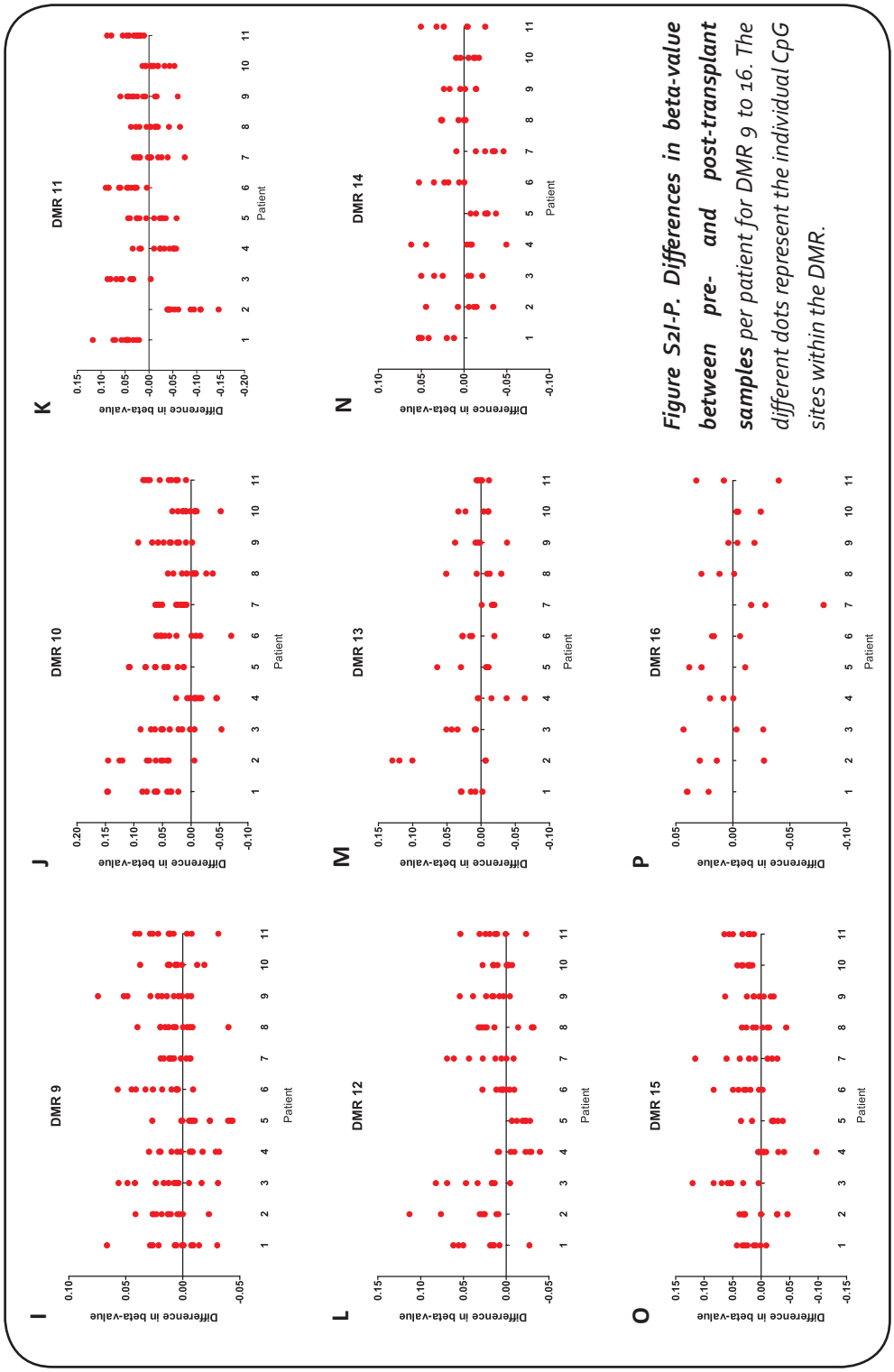

Figure S2I-P. Differences in beta-value between pre- and post-transplant samples *per patient for DMR 9 to 16. The different dots represent the individual CpG sites within the DMR.*

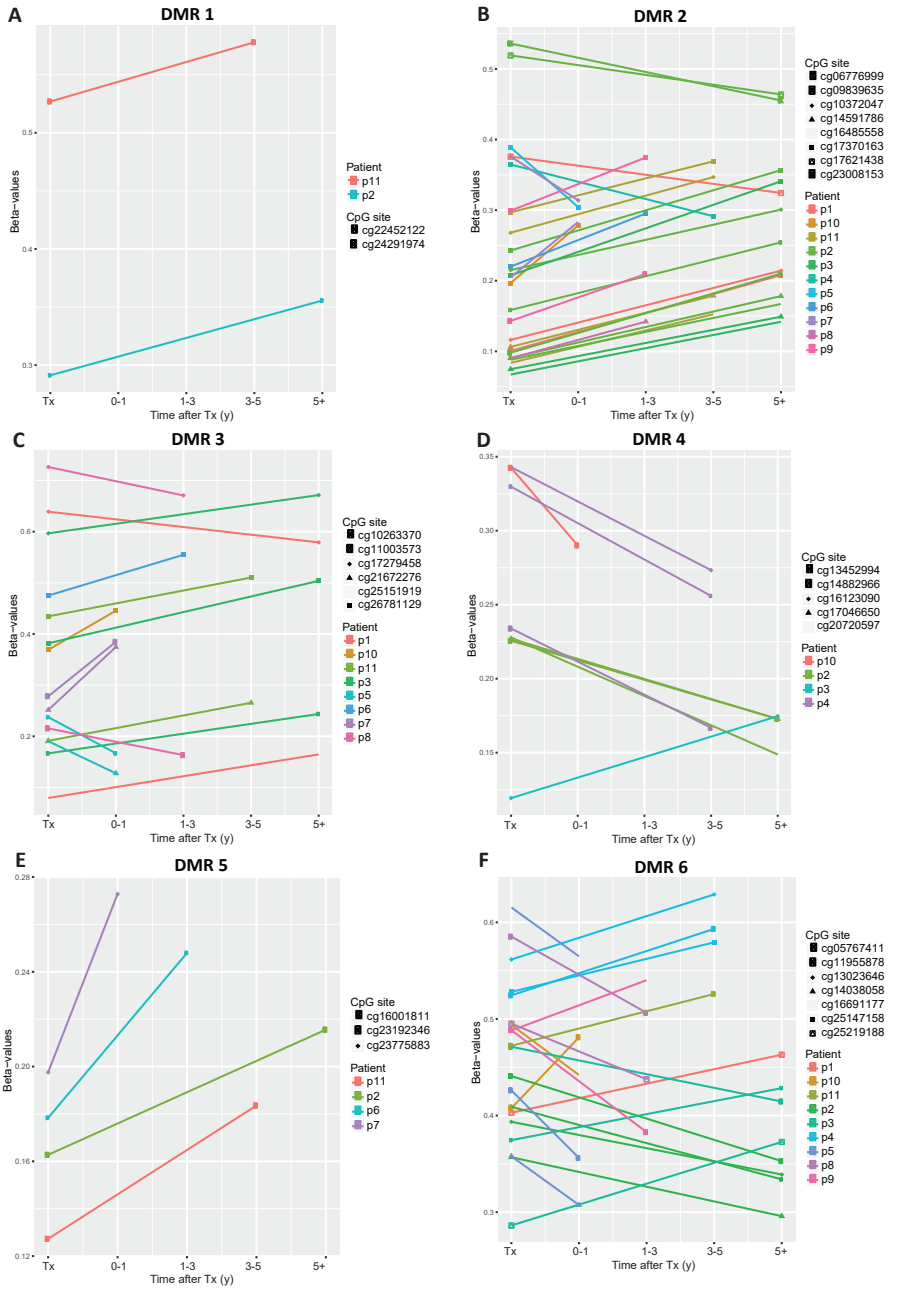

Figure S3A-F. CpG sites within DMR 1-6 that differ more than 0.05 in beta-value, colored per patient. *The y-axis shows beta-value and the x-axis time in years after transplantation. Time points after transplantation are clustered in 0-1 years (N=3), 1-3 years (N=3), 3-5 years (N=2) and 5+ years (N=3).*

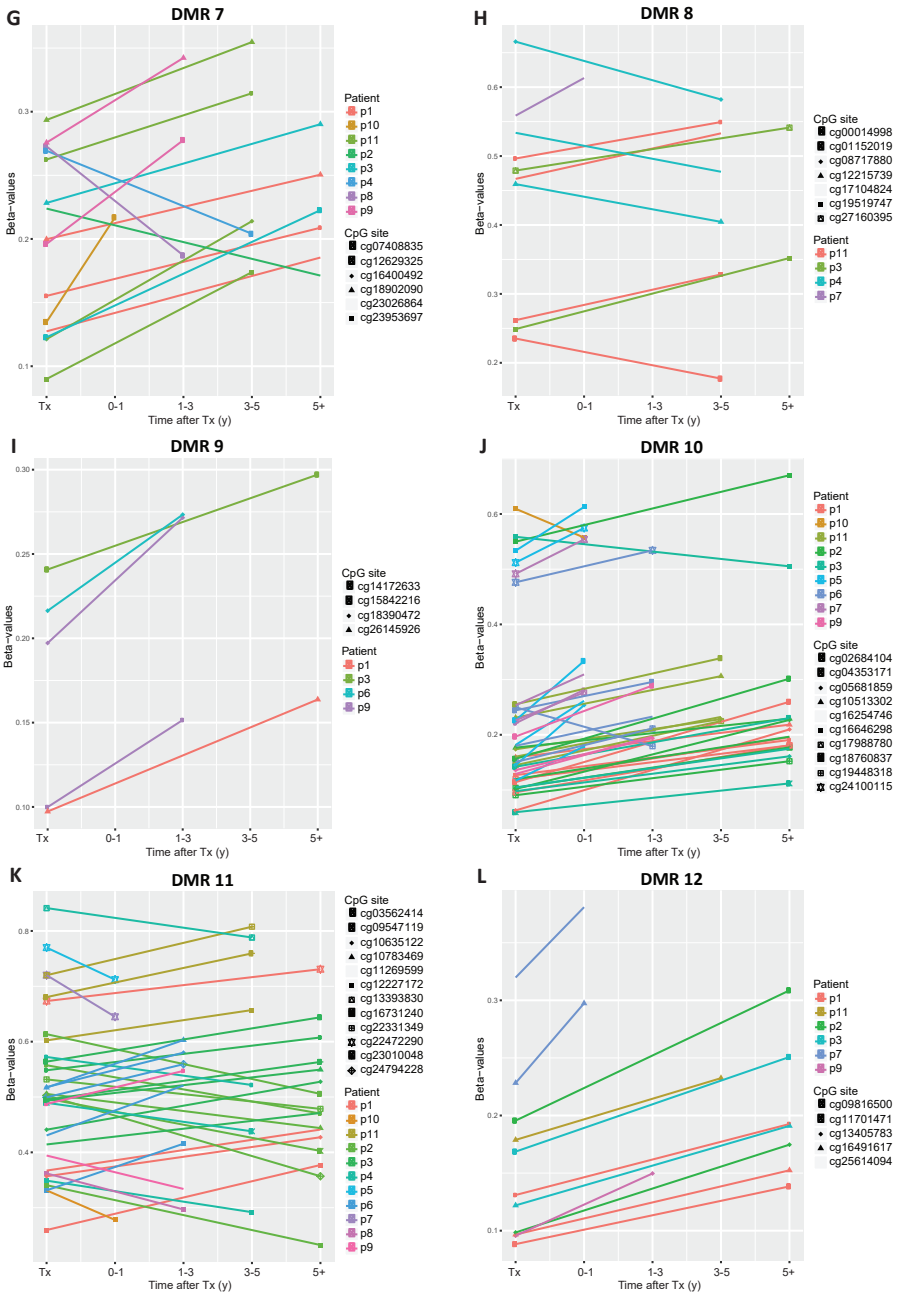

Figure S3G-L. CpG sites within DMR 7-12 that differ more than 0.05 in beta-value, colored per patient. *The y-axis shows beta-value and the x-axis time in years after transplantation. Time points after transplantation are clustered in 0-1 years (N=3), 1-3 years (N=3), 3-5 years (N=2) and 5+ years (N=3).*

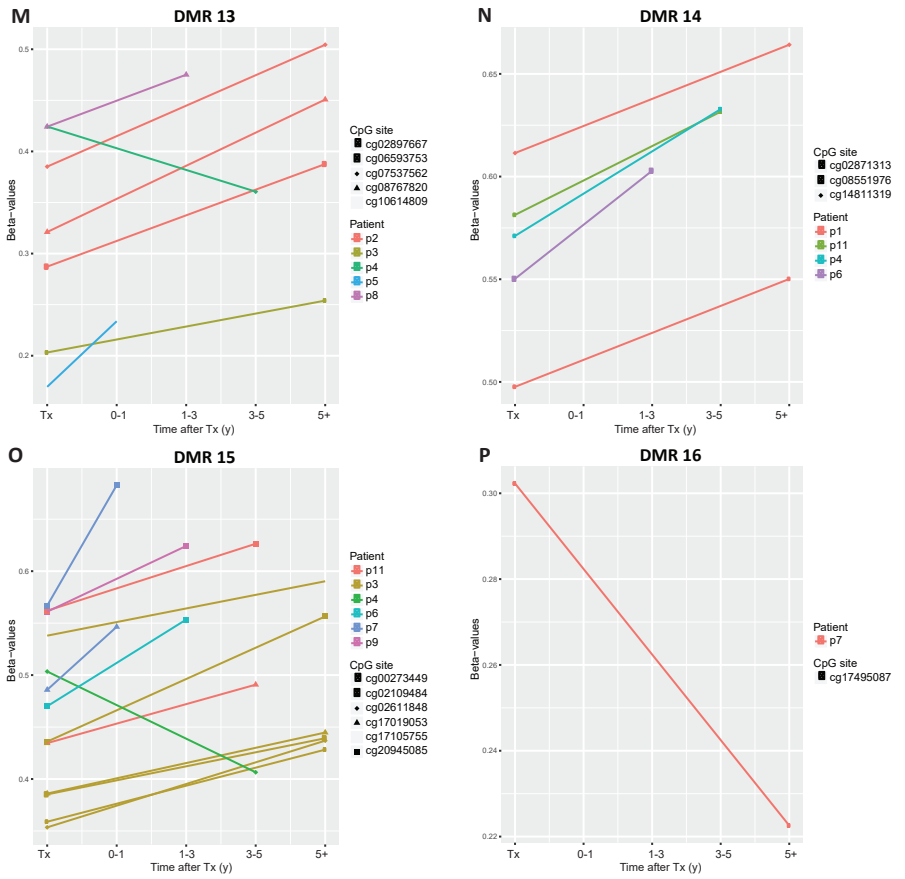

Figure S3M-P. CpG sites within DMR 13-16 that differ more than 0.05 in beta-value, colored per patient. *The y-axis shows beta-value and the x-axis time in years after transplantation. Time points after transplantation are clustered in 0-1 years (N=3), 1-3 years (N=3), 3-5 years (N=2) and 5+ years (N=3).*

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Chapter 7

Disrupted regulation of serpinB9 in circulating T cells is associated with an increased risk for post-transplant skin cancer

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Abstract

Cutaneous squamous cell carcinoma (cSCC) is a serious complication after organ transplantation and patients benefit from an early risk assessment. We hypothesized that functional differences in circulating T cells may represent risk factors for post-transplant cSCC development. Here we analyzed genome-wide DNA methylation of circulating T cells of kidney transplant recipients before the clinical onset of cSCC, to identify differences associated with post-transplant cSCC development. This analysis identified higher DNA methylation of *SERPINB9*, which is an intracellular inhibitor of granzyme B, a protein that induces apoptosis in target cells. High DNA methylation of *SERPINB9* in circulating T cells was confirmed in a second patient cohort, during recurrent cSCC, indicating that high *SERPINB9* methylation represents a persistent risk factor for cSCC development. At the functional level, the inverse correlation between DNA methylation and messenger RNA expression present in non-cSCC patients was absent in the cSCC patients. Also, a significant difference in serpinB9 protein expression between cSCC patients and non-cSCC patients was observed. Concluding that disturbed regulation of serpinB9 in circulating T cells represents a novel risk factor for post-transplant cSCC in kidney transplant recipients.

Introduction

Immunosuppression after organ transplantation is associated with a higher prevalence of cancer[1,2]. Especially non-melanoma skin cancer such as cutaneous squamous cell carcinoma (cSCC) occurs up to 200 times more in the transplanted population than in the general population[3-5]. Transplant recipients also experience more metastasis and over 70% of the patients develop a subsequent cSCC within 5 years[6]. Although immunosuppressive treatment is recognized as an important risk factor for the development of cSCC after solid organ transplantation, not much is known on the immune regulation leading up to formation of cSCC.

Most cSCCs are surrounded by immune cell infiltrates, however, these cells are incapable of mounting an effective immune response directed against the cSCC[7]. The role and phenotype of T cells surrounding an cSCC lesion has been studied extensively[8-10]; high numbers of FOXP3+ regulatory T cells are associated with higher metastasis of cSCCs[11,12] whereas increased activity of effector and cytotoxic T cells often associates with better prognostic outcomes[13,14].

The function of cells, including T cells, is regulated by epigenetic mechanisms such as DNA methylation, which is the addition of a methyl group to a cytosine (C) followed by a guanine (G; CpG dinucleotide) in the DNA. DNA methylation controls gene expression, is a dynamic feature and can be influenced by environmental cues[15]. DNA methylation is also known to be dysregulated in disease such as cancer, however it is often difficult to determine whether it is a driver or a consequence of the disease[16,17].

Here, we hypothesized that functional differences in circulating T cells represent risk factors in the development of a *de novo* post-transplant cSCC. To address this hypothesis, we took an unbiased approach and performed genome-wide DNA methylation analysis of circulating T cells after kidney transplantation but before the clinical onset of cSCC (discovery phase). DNA methylation profiles of kidney transplant recipients with a future cSCC were compared to those of matched kidney transplant recipients without cSCC. The prominent finding of this analysis was higher methylation of a region within *SERPINB9* in cSCC patients. SerpinB9 is an intracellular serine protease inhibitor that inhibits granzyme B[18,19], which is an important protease in the effector function of cytotoxic T cells by inducing apoptosis in target cells[20]. Cytotoxic T cells express serpinB9 to protect themselves against the activity of granzyme B, therefore high expression of serpinB9 in cytotoxic T cells makes them more potent killers[21,22]. Given these data, the finding on *SERPINB9* DNA methylation prompted us to further study *SERPINB9* methylation in a second cohort of kidney transplant recipients with recurrent cSCC, as well as the functional role of *SERPINB9* in cSCC on the level of mRNA and protein expression.

Materials and methods

Study design

Anonymized retrospective biobank samples were used in the discovery phase of the study, this included kidney transplant recipients before the diagnosis of their first post-transplant cSCC. A second cohort of patients was used to confirm findings from the discovery phase and this included kidney transplant recipients during recurrent post-transplant cSCC. The use of biobank material and the inclusion of new patients had been approved by the local medical ethical committee (MEC-2015-642). All kidney transplant recipients with a (future) post-transplant cSCC were matched to kidney transplant recipients who did not develop an cSCC within a similar time period after the first transplant. Matching criteria included gender, age (± 4 years), ethnicity, cytomegalovirus (CMV) status and type of immunosuppressive drugs directly after transplantation. We included patients with at least one cSCC after transplantation and patients with cSCC *in situ* (Bowen's disease). Patients with another donor organ such as liver, heart or lung were excluded, as well as patients with a history of malignancy prior to transplantation. Non-cSCC patients with actinic keratosis, a pre-cancerous lesion, were also excluded.

T cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll-Paque procedures. T cells were isolated from the PBMCs using fluorescence-activated cell sorting (FACS) with the BD FACSAria™ II (BD Biosciences, San Jose, CA, US). Total PBMCs were stained with CD3 Brilliant Violet 510 (Biolegend, San Diego, CA, US), CD4 Pacific Blue (BD Biosciences), CD8 APC-cy7 (BD Biosciences), CD45RO APC (Biolegend), CCR7 PE-cy7 (BD Biosciences), CD25 PE (BD Biosciences), CD127 FITC (eBioscience, Waltham, MA, US) and to exclude nonviable cells Via-Probe 7AAD (BD Biosciences) was used. After cell sorting the purities were >96% for CD3+ cells, samples below 95% were excluded for further analysis.

Genome-wide DNA methylation arrays

Before isolating DNA from the T cells in the discovery cohort, all patient samples were randomized to minimize batch effects. DNA was isolated using the QIAamp DNA Micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Purity and concentration of the isolated DNA was assessed with the NanoDrop ND-8000 (Isogen Life Science, Utrecht, The Netherlands). DNA degradation was determined by gel electrophoresis, none of the samples showed significant degradation.

The Infinium HumanMethylation450 arrays (Illumina, San Diego, CA, US) were performed as described previously[23]. Data quality was examined using the MethylAid R package[24,25]

and all samples passed quality controls using the default MethylAid thresholds. Probes with a detection p-value>0.01, probes containing single nucleotide polymorphisms and probes on the sex chromosome were removed from the dataset. A between-array normalization was applied to the Type I and Type II probes separately using the DASEN method within the wateRmelon Bioconductor R package[25-27]. The methylation level of a CpG site is presented as a beta-value, a value between 0 (unmethylated) and 1 (fully methylated). After the quality controls and normalization, beta-values of 423,289 CpG sites remained for further analysis. Both the raw and normalized data are available via the NCBI Gene Expression Omnibus (GEO) database with accession number GSE117050.

Data analysis DNA methylation arrays

To identify DNA methylation differences between the future cSCC and non-cSCC patients, we performed the data analysis as previously described[23]. First, a linear mixed-effect model was performed using the lme4 R package[28]. The fixed effects included age, percentage CD_4, percentage CD8 and CMV status. Percentage CD_4 and CD8 were included to correct for differences in T-cell composition between individuals and CMV is known to affect DNA methylation at specific genes[29]. Array IDs were included as a random effect to account for technical variation between the arrays. This resulted in single site-specific p-values and these p-values together with their genomic location, were used as input into comb-p[30] to find differentially methylated regions (DMRs). A sliding window of 500 base pair (bp) was used and the seed was set at p<0.01. A stringent multiple testing correction was applied using a Šidák correction (Šidák<0.05)[31].

DNA methylation analysis by pyrosequencing

After the discovery phase we continued measuring DNA methylation of T cells with bisulfite pyrosequencing, an easy technique to quantitatively measure single-site DNA methylation[32], but first we tested whether pyrosequencing resulted in the same methylation values as with the microarrays. Therefore CpG sites within DMR 1 (*SERPINB9*) and DMR 2 (*VTRNA2-1*) were analyzed in the same DNA samples that were used for the array analysis of 10 patients, a mixture of cSCC and non-cSCC patients.

Pyrosequencing was performed as described previously[23,33]. The polymerase chain reaction (PCR) primers, melting temperatures and amplicon sizes for the different PCR products can be found in Supplementary Table S1 together with the specific PCR programs. For *SERPINB9,* 12 CpG sites of which 5 were array probes, were sequenced in two separate reactions and DNA methylation was averaged per sequence reaction (region 1 and region 2). For *VTRNA2-1,* 5 CpG sites of which 3 were array probes, were measured and an average of those 5 sites is presented in the results.

mRNA analysis

Total RNA was isolated from T cells using the High Pure RNA Isolation kit (Roche Applied Science, Pennsburg, Germany) according to the manufacturer's protocol. The quality and purity of the RNA was assessed using the NanoDrop ND-8000 (Isogen Life Science). Samples with a 260/280 ratio below 1.8 and a 260/230 ratio above 1 were excluded for further analysis. Messenger RNA (mRNA) of *SERPINB9* and *GRANZYME B (GZMB)* was quantified by real-time quantitative PCR (qPCR) using a Taqman gene expression assay. Primers used were Hs00394497_m1 (*SERPINB9*; Thermo Fisher Scientific, Waltham, MA, US) and Hs01554355_m1 (*GZMB*; Thermo Fisher Scientific), GAPDH (Hs99999905_m1, Thermo Fisher Scientific) was used as housekeeping gene. qPCR was performed on the StepOnePlus Real-Time PCR system (Applied Biosystems). Gene expression was then calculated by transforming the cycle threshold (Ct) to cDNA copies (2^{40-Ct}). Dividing the number of *SERPINB9* and *GZMB* copies by the number of GAPDH copies resulted in a relative gene expression value.

Protein analysis

Protein levels of serpinB9 and granzyme B within T cells were assessed in cells before and after stimulation for 6 hours at 37°C with or without α-CD3/CD28 coated Dynabeads® (Gibco, Waltham, MA, US). The cells were measured by flow cytometry directly after defrosting the PBMCs. Monensin was added after 1 hour of stimulation, by this newly synthesized granzyme B could be measured. CD107a APC (BD Biosciences) was added to the cell cultures to assess degranulation of the cells. Cells were stained with the following surface antibodies: CD3 Brilliant Violet 510 (Biolegend), CD4 APC-cy7 (Biolegend), CD8 PE (Thermo Fisher Scientific) and Via-Probe 7AAD (BD Biosciences) was used to exclude nonviable cells. After surface staining the cells were fixed, permeabilized and stained for intracellular serpinB9 labelled with Alexa Fluor 488 (Bio-rad, Hercules, CA, US) and granzyme B labelled with Brilliant Violet 421 (BD Biosciences). Isotype controls for AF488 (Bio-rad) and BV421 (BD Biosciences) were used as negative controls for serpinB9 and granzyme B expression. The cells were then analyzed on the FACSCanto II (BD Biosciences) with FACSDiva software. Data was analyzed blind, without knowledge on cSCC status of the samples, using Kaluza software 1.5a (Beckman Coulter, Brea, CA, US).

Statistical analysis

Differences in clinical characteristics, DNA methylation, mRNA and protein expression between the cSCC and non-cSCC patients were statistically tested using SPSS version 21.0 (IBM Corp., Armonk, NY, US). The Mann-Whitney U test was used for the continuous variables and χ^2 test for the categorical variables. Data processing and statistical analysis of

all the microarray data was done in RStudio version 1.0.136 (Rstudio Inc., Boston, MA, US) with R version 3.2.5[26]. Multiple testing correction of the microarray data was done using a Šidák correction (Šidák<0.05)[31]. Correlation between the DNA methylation levels quantified by pyrosequencing and the beta-values of the Illumina 450k arrays was calculated using Spearman's rank correlation coefficient using SPSS as well as correlations between DNA methylation and mRNA expression. All statistical tests were two-tailed and a $p<0.05$ was considered statistically significant.

Results

Patients

The discovery cohort consisted of 19 future cSCC and 19 non-cSCC patients who had been transplanted between 1997 and 2012. All patients in the discovery cohort were Caucasian-European. No statistical differences were found between the patient characteristics of the future cSCC and non-cSCC patients (Table 1). A detailed overview of the time between transplantation, sample and first diagnosis of cSCC can be found in Figure S1A.

The second cohort consisted of 37 non-cSCC and 45 cSCC patients during recurrent cSCC, who had been transplanted between 1976 and 2014. Six cSCC patients and 5 non-cSCC patients received a second kidney transplant. All patients in the second cohort were Caucasian-European. There was a small statistical difference in the end-stage renal disease (ESRD) diagnosis between the cSCC and non-cSCC patients ($p=0.04$; Table 2), no other statistical differences were found. A detailed overview of the time between transplantation, sample and first diagnosis of cSCC can be found in Figure S1B.

Discovery of significant DMRs in circulating T cells before cSCC

To identify differentially methylated regions (DMRs) in circulating T cells associated to future cSCC development, we compared genome-wide DNA methylation of kidney transplant recipients with and without a post-transplant cSCC, before the clinical onset of the cSCC. None of the single-site CpGs were statistically significant after multiple testing correction. However, we found 7 regions significantly differentially methylated. In Table 3 the different DMRs, the genes annotated to these DMRs based on genomic location, the genomic location of the DMRs according to the hg19 genome build (UCSC Genome Browser), the number of probes (CpG sites on the array) within the regions and the effect size is presented. Out of the significant DMRs, 5 were hyper methylated and 2 were hypo methylated in the future cSCC patients.

***Table 1**. Patient characteristics of the discovery cohort before cSCC*

	cSCC N = 19	non-cSCC N = 19	
Age (years)[a]	64.8 (45-77)	63.5 (45-80)	p=0.49
Gender (male)	14 (73.7%)	14 (73.7%)	p=1
Years post Tx[a]	1.5 (0.1-6.9)	1.3 (0.1-6.3)	p=0.93
Years between Tx and first cSCC[a]	5.4 (0.9-12.5)	-	
Biopsy proven rejection	-	3 (15.8%)	p=0.07
Immunosuppressive treatment			
Induction therapy (ATG/ Basiliximab)	7 (36.8%)	5 (26.3%)	
Calcineurin inhibitors (Tacrolimus/ Cyclosporine)	19 (100%)	18 (94.7%)	
Proliferation inhibitors (MMF/ Sirolimus)	18 (94.7%)	19 (100%)	
Antimetabolites (Azathioprine)	1 (5.3%)	-	
Corticosteriods	18 (94.7%)	19 (100%)	
HLA mismatches[a]	3.11 (0-6)	3.11 (0-6)	p=0.94
CMV serostatus acceptor			p=1
Negative	4 (21.1%)	4 (21.1%)	
Positive	15 (78.9%)	15 (78.9%)	
CMV serostatus donor			p=0.11
Negative	12 (63.2%)	7 (36.8%)	
Positive	7 (36.8%)	12 (63.2%)	
ESRD diagnosis			p=0.26
Polycystic kidney	7 (36.8%)	2 (10.5%)	
Hypertension	3 (15.8%)	6 (31.6%)	
Diabetic Nefropathy	1 (5.3%)	1 (5.3%)	
Glomerulonefritis	1 (5.3%)	0 (0%)	
Other	7 (36.8%)	10 (52.6%)	
Dialysis pre-transplantation			p=0.49
Yes (PD/HD)	14 (73.7%)	12 (63.2%)	
No	5 (26.3%)	7 (36.8%)	

[a]median and range; cSCC: cutaneous squamous cell carcinoma, Tx: transplantation, ATG: anti-thymocyte globulin, MMF: mycophenolate mofetil, HLA: human leukocyte antigen, CMV: cytomegalovirus, ESDR: end stage renal disease, PD: peritoneal dialysis, HD: hemodialysis

Table 2. *Patient characteristics of the second cohort during cSCC*

	cSCC N=45	non-cSCC N=37	
Age (years)[a]	66.4 (34-84)	64.0 (28-75)	p=0.20
Gender (male)	30 (66.7%)	25 (67.6%)	p=0.93
Years post Tx[a]	8.5 (0.4-40.5)	9.5 (0.1-35.9)	p=0.89
Years between Tx and first cSCC[a]	4.7 (0-33)	-	
Biopsy proven rejection	12 (26.7%)	13 (35.1%)	p=0.41
Immunosuppressive treatment			
Induction therapy (ATG/ Basiliximab)	1 (2.2%)	6 (16.2%)	
Calcineurin inhibitors (Tacrolimus/ Cyclosporine)	37 (82.2%)	34 (92%)	
Proliferation inhibitors (MMF/ Sirolimus)	27 (60%)	22 (59.5%)	
Antimetabolites (Azathioprine)	9 (20%)	4 (10.8%)	
Corticosteriods	44 (97.8%)	37 (100%)	
HLA mismatches[a]	3.0 (0-6)	3.0 (0-6)	p=0.86
CMV serostatus acceptor			p=0.74
Negative	17 (37.8%)	11 (29.7%)	
Positive	22 (48.9%)	20 (54.1%)	
Unknown	6 (13.3%)	6 (16.2%)	
CMV serostatus donor			p=0.62
Negative	15 (33.3%)	14 (37.8%)	
Positive	18 (40%)	11 (29.7%)	
Unknown	12 (26.7%)	12 (32.4%)	
ESRD diagnosis			p=0.04
Polycystic kidney	11 (24.4%)	5 (13.5%)	
Hypertension	8 (17.8%)	7 (18.9%)	
Diabetic Nefropathy	-	7 (18.9%)	
Glomerulonefritis	7 (15.6%)	4 (10.8%)	
Other	19 (42.2%)	14 (37.8%)	
Dialysis pre-transplantation			p=0.83
Yes (PD/HD)	22 (48.9%)	19 (51.4%)	
No	23 (51.1%)	18 (48.6%)	

[a]median and range; cSCC: cutaneous squamous cell carcinoma, Tx: transplantation, ATG: anti-thymocyte globulin, MMF: mycophenolate mofetil, HLA: human leukocyte antigen, CMV: cytomegalovirus, ESDR: end stage renal disease, PD: peritoneal dialysis, HD: hemodialysis

Table 3. *Resulting differentially methylated regions of the discovery analysis*

	Annotated to	Genomic location (hg19)	Length DMR	no. of probes	Function	Regional p-value	Effect size	DMR state
1	*SERPINB9*	chr6:2891973-2892153	180 bp	5	Granzyme B inhibitor	$1.09 \cdot 10^{-13}$	0.14	Hyper
2	*VTRNA2-1*	chr5:135415948-135416614	666 bp	12	Inhibitor of protein kinase R	$1.40 \cdot 10^{-10}$	0.11	Hyper
3	*VTRNA2-1*	chr5:135414858-135415259	401 bp	4	Inhibitor of protein kinase R	$1.90 \cdot 10^{-8}$	0.07	Hyper
4	*PIF1*	chr15:65116194-65116558	364 bp	3	ATP metabolism	$1.36 \cdot 10^{-7}$	-0.05	Hypo
5	*APC2*	chr19:1465962-1466163	201 bp	2	Signaling pathway regulation	$1.48 \cdot 10^{-7}$	0.08	Hyper
6	*RPH3AL*	chr17:151914-152351	437 bp	6	Tumor suppressor	$3.39 \cdot 10^{-7}$	-0.07	Hypo
7	*AC144450.2*	chr2:1609660-1609833	173 bp	2	LincRNA	$3.15 \cdot 10^{-6}$	0.08	Hyper

DMR: differentially methylated region, chr: chromosome, bp: base pair

Genomic characteristics of DMR 1, 2 and 3

To understand the potential regulatory effect of the DMRs in T cells, the genomic location of the DMR and characteristics of that location are important. In Figure 1 we visualized the top three DMRs with DNA methylation, of both cSCC and non-cSCC patients expressed in beta-value, the genomic location of the DMRs and the primary T-cell specific chromatin state, which is a cell-type specific combination of epigenetic features obtained from the ROADMAP reference data[34]. DMR 1, annotated to *SERPINB9,* is located intragenic and within an actively transcribed region (Figure 1A). DMR 2, annotated to VTRNA2-1, is located in the coding region and a bivalent/poised transcription start site (TSS) of the gene (Figure 1B). DMR 3, also annotated to VTRNA2-1, is located further away from the *VTRNA2-1* gene and partly within a bivalent enhancer region and partly within a repressed area (Figure 1B). All three DMRs overlap with a CpG island and those are often involved in the regulation of gene expression.

Confirmation of microarray methylation values by pyrosequencing

To confirm that the above described findings can also be found with a different technique, DNA methylation of DMRs 1 and 2 was measured by pyrosequencing in the same DNA samples. Correlation between DNA methylation values obtained with the microarray and pyrosequencing was strong in both DMRs (Supplementary Figure S2). Spearman r for *SERPINB9* was 0.86 ($p<0.0001$) and for *VTRNA2-1* r was 0.96 ($p<0.0001$). As a result of this strong correlation, we measured DNA methylation with pyrosequencing throughout the rest of the study.

High intragenic *SERPINB9* methylation during cSCC

To assess the stability of the DNA methylation profiles identified before development of cSCC, we included a second patient cohort during recurrent cSCC (Table 2). *VTRNA2-1* was measured and was not significantly different between cSCC and non-cSCC patients (data not shown). When *SERPINB9* was measured it was significantly different between cSCC and non-cSCC patients. Median DNA methylation of *SERPINB9* was 58.7% (range: 32.5%-81.3%) for region 1 and 54.4% (30.0%-78.5%) for region 2 in the cSCC patients and 50.2% (21.8%-77.5%) for region 1 and 46.4% (22.1%-74.0%) for region 2 in the non-cSCC patients (region 1: $p=0.004$; region 2: $p=0.008$) (Figure 2).

Similar as in our discovery cohort, cSCC patients demonstrated higher *SERPINB9* methylation values than non-cSCC patients. In addition, serpinB9 has a strong relation to T-cell functions, as a regulator of cytotoxicity[21,22]. Together, these findings warranted further investigation into the role of SERPINB9 in controlling the cytotoxic T cells that are

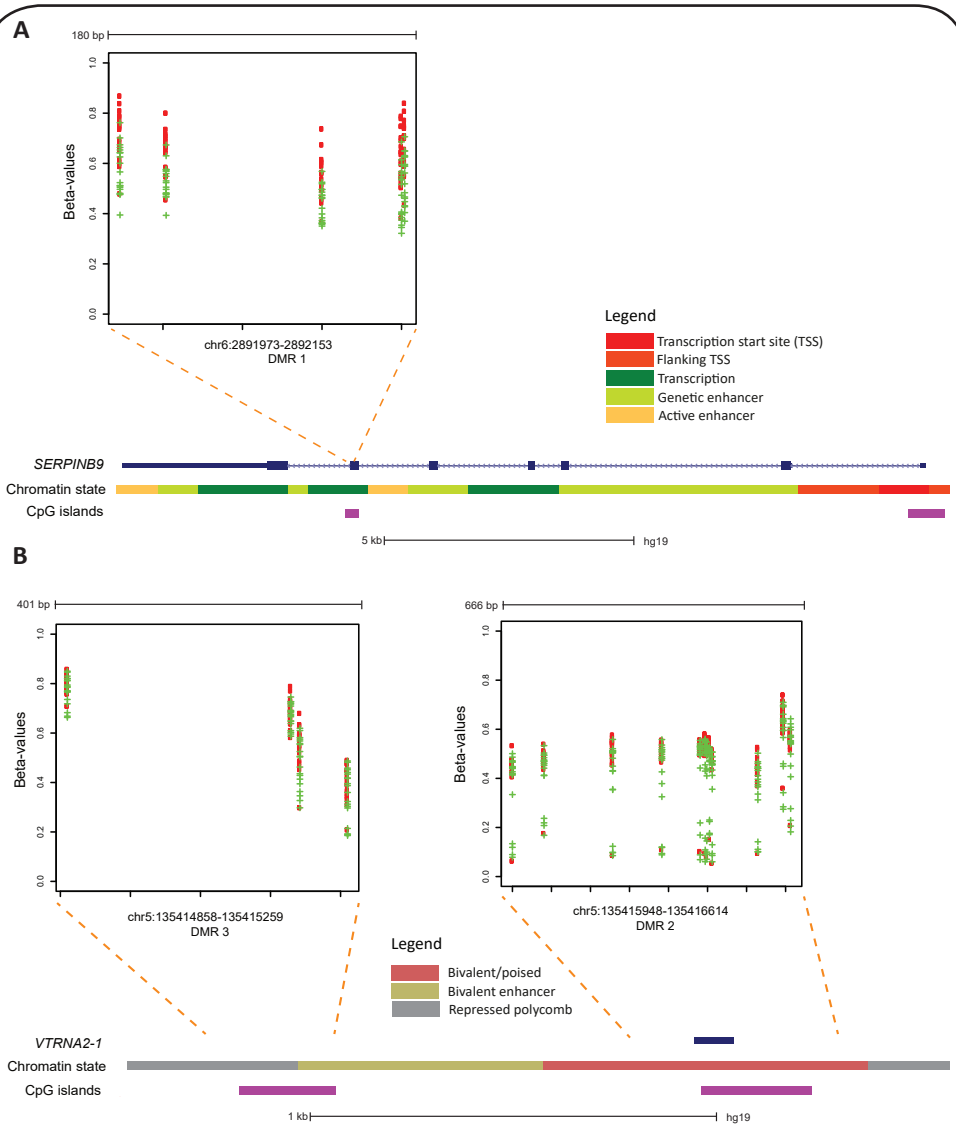

Figure 1. Genomic characteristics of DMR 1 to 3*. The chromatin state specific for primary T cells of SERPINB9* ***A)*** *and VTRNA2-1* ***B)*** *is depicted with the CpG islands below in purple. The location of the DMRs are highlighted by the orange dotted lines. The graphs present the raw beta-values (y-axis) and the genomic location of the single CpGs (x-axis), cSCC patients are depicted in red and the non-cSCC in green. The transcription start site (TSS) is the promoter of a gene, enhancers are locations that bind gene activating or repressing proteins such as transcription factors and repressed polycomb represents inactive DNA.*

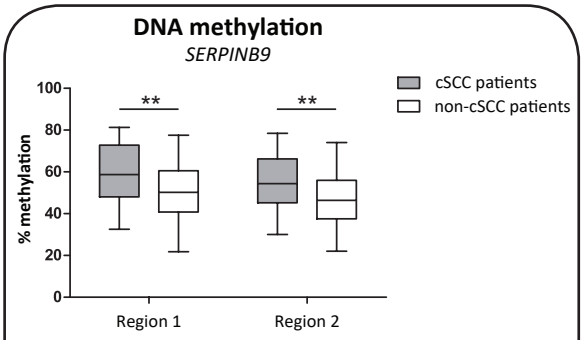

Figure 2. DNA methylation in T cells of cSCC and non-cSCC patients *for region 1 and 2 of SERPINB9 measured by pyrosequencing. **p<0.01*

key for immunosurveillance in post-transplant cSCC.

mRNA expression negatively correlates to DNA methylation only in the non-cSCC patients

To study the translation from DNA to protein of serpinB9, we analyzed mRNA expression of *SERPINB9* in T cells. Relative mRNA expression of *SERPINB9* was not significantly different between cSCC (N=30) and non-cSCC patients (N=27; Figure 3A). When we zoom in and study the correlation between DNA methylation and mRNA expression of *SERPINB9* in the total patient population this was statistically significant (p=0.004,Figure 3B). However, when we stratified the data by cSCC status the correlation remained significant only in the non-cSCC patients (p=0.0003; Figure 3C,D) and not in the cSCC patients, indicating a disrupted transcriptional regulation in the cSCC patients.

Lower serpinB9 expression in circulating T cells of cSCC patients

To investigate the functional impact of differentially methylated *SERPINB9* on cytotoxicity, we analyzed the expression of the following markers: granzyme B (inhibited by serpinB9) and degranulation of T cells by CD107a expression before and after stimulation. Gating strategies for granzyme B and CD107a are presented in Supplementary Figure S3. The percentage of CD3+granzyme B+ cells was not significantly different between the cSCC patients and non-cSCC patients (Supplementary Figure S4A) and neither was degranulation of the T cells as determined by CD107a staining (Supplementary Figure S4B).

SerpinB9 expression was also measured in the T cells. Gating strategy for serpinB9 is presented in Supplementary Figure S5. The percentage of CD3+serpinB9+ cells before stimulation was not significantly different between cSCC and non-cSCC patients (Figure 4A). After stimulation serpinB9 expression in all T cells was upregulated to 98.2% (93.0%-99.0%) for the cSCC patients and 99.1% (97.2%-99.7%) for the non-cSCC patients and this was significantly different even though the differences were small (p=0.006; Figure 4B). When analyzing serpinB9 expression in the CD4+ and CD8+ population separately, we observed that the percentage of CD4+serpinB9+ cells was significantly lower in the cSCC patients than in the non-cSCC patients after stimulation (Figure 4C). In the CD8+

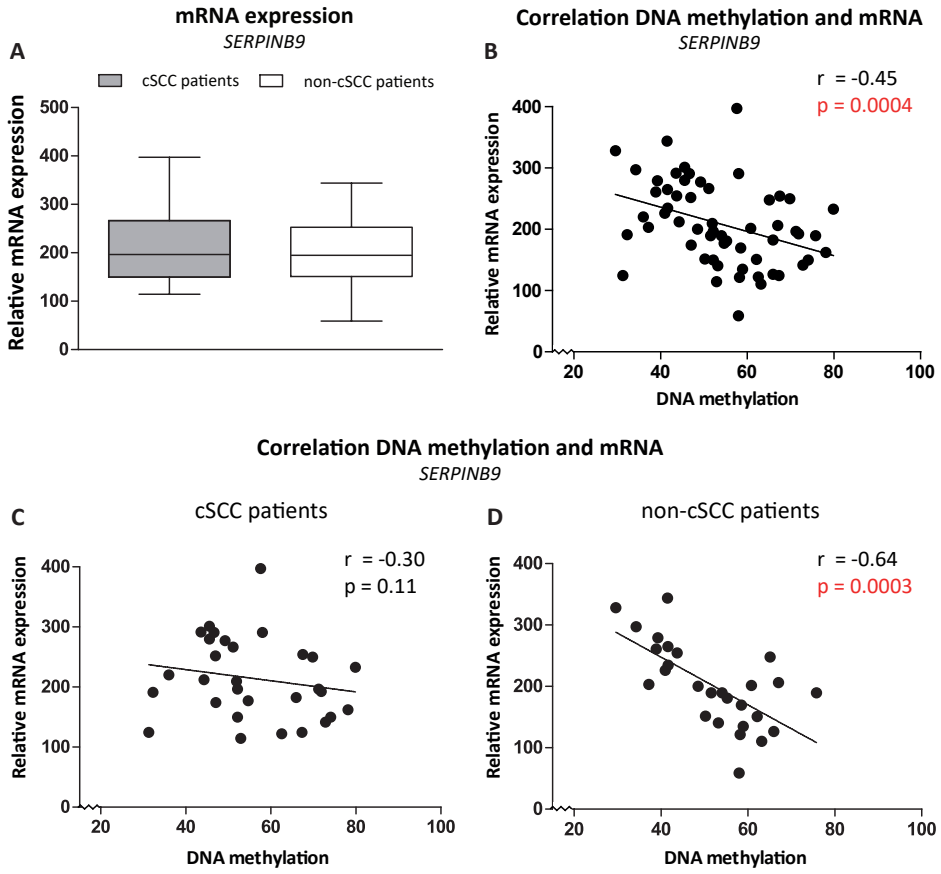

Figure 3. Relative mRNA expression of SERPINB9 A) *in cSCC versus non-cSCC patients,* ***B)*** *as correlated to SERPINB9 DNA methylation (x-axis) within all patients,* ***C)*** *within the cSCC patients and* ***D)*** *within the non-cSCC patients.*

population this difference was not observed (Figure 4D). These results show that the CD4+ population is the main contributor to the difference observed in the total T-cell population.

Discussion

In this study we demonstrate high DNA methylation of *SERPINB9* in circulating T cells before the clinical onset of cSCC in kidney transplant recipients and, in a different patient cohort, during recurrent post-transplant cSCC. These data identify high DNA methylation of *SERPINB9* as a novel risk factor for development of both *de novo* and subsequent post-transplant cSCC. In addition to that, T cells of cSCC patients were unable to fully upregulate serpinB9 expression *in vitro*, which might provide insight in the role of the peripheral

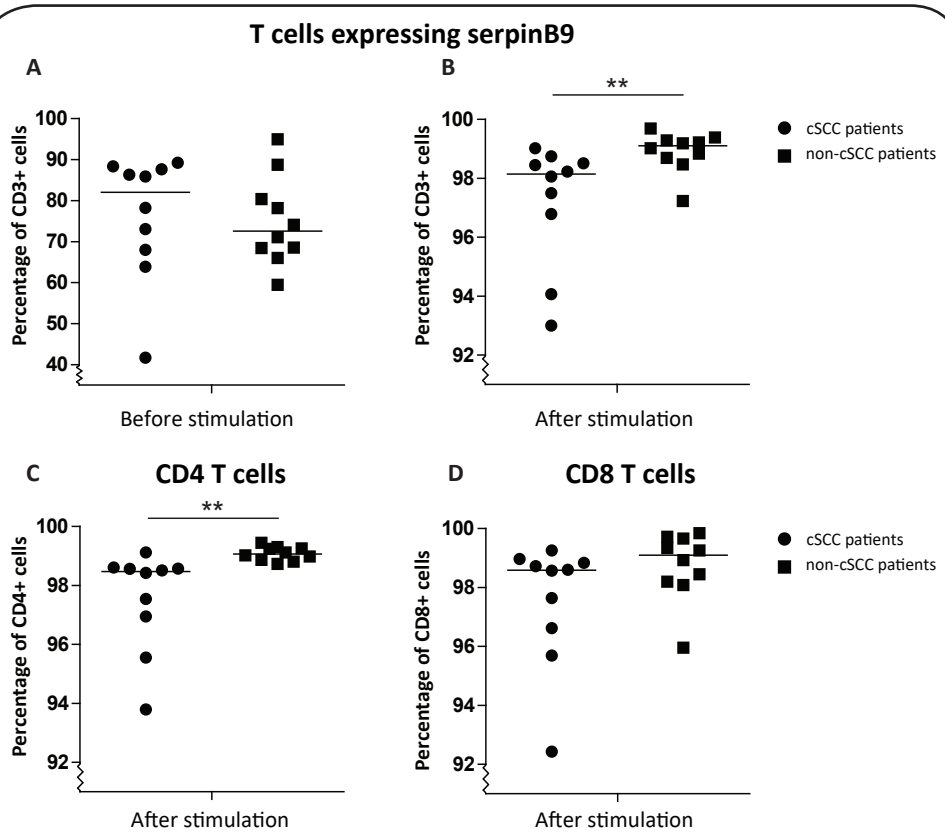

***Figure 4. Quantified flow cytometry data on serpinB9 expression by T cells**. **A)** Percentage of T cells that expressed serpinB9 before stimulation in the cSCC and non-cSCC patients and **B)** after stimulation. C) Percentage of CD4 T cells and D) CD8 T cells that expressed serpinB9 after stimulation in the cSCC and non-cSCC patients. **p<0.01*

immune system in development of an cSCC in kidney transplant recipients.

In a previous study, where we identified DMRs associated with post-transplant cSCC before transplantation[23], *SERPINB9* was not significantly different between T cells of future cSCC patients and non-cSCC patients. Thus, differences in *SERPINB9* DNA methylation that identify patients at risk for cSCC arise after kidney transplantation. Likely kidney transplantation and the use of immunosuppressive therapy affect DNA methylation profiles of the T cells[35]. However, since we demonstrated high *SERPINB9* methylation in patients before and after development of a *de novo* post-transplant cSCC, it seems a persistent risk factor for cSCC after transplantation.

Based on the differential DNA methylation of *SERPINB9*, one could expect differences in

mRNA expression of *SERPINB9*. Nevertheless, when *SERPINB9* mRNA expression was measured in the T cells, this was not significantly different between cSCC patients and non-cSCC patients. This is comparable to findings by Ryer et al.[36], who identified higher methylation of the same region within *SERPINB9* in PBMCs of patients with abdominal aortic aneurysm. Despite the differential DNA methylation, they also did not detect a difference in mRNA expression. This is most likely due to the intragenic location of the DMR, outside of the promoter region of *SERPINB9.* The effect of intragenic DNA methylation on gene expression is still debated[37] though here we demonstrated an inverse correlation between intragenic *SERPINB9* DNA methylation and mRNA expression in the T cells of non-cSCC patients. Surprisingly, this inverse correlation was absent in the T cells of cSCC patients. This illustrates a disturbed transcriptional regulation of *SERPINB9* in cSCC patients and a clear difference between these two patient groups.

Previous studies have shown that higher expression of serpinB9 increased the potency of cytotoxic T cells[21,22]. We observed a slightly lower expression of serpinB9 in the T cells of our cSCC patients, which was mainly due to the CD compartment of the T cells, although it is questionable whether a difference between 98% and 99% serpinB9 positive cells is biologically relevant. In addition, the regulation of serpinB9 seems independent from the regulation of cytotoxic markers of T cells since we did not identify differences in the expression of granzyme B and CD107a between cSCC patients and non-cSCC patients. SerpinB9 is an intracellular protein inactivating granzyme B once it is released into the cytoplasm[38] and therefore serpinB9 exerts its effect on cytotoxicity only after granzyme B is synthesized to its active form. The absence of differences in cytotoxicity, which is in most cases restricted to the CD8+ T cells, and the serpinB9 differences in the CD_4+ T cells may lead to the conclusion that the CD_4+ T cells are the population of interest in post-transplant cSCC.

DNA methylation of *SERPINB9* might represent a future treatment target for cSCC in transplant recipients. It would be interesting to decrease *SERPINB9* DNA methylation in cSCC patients to the level observed in non-cSCC patients and study whether that affects future cSCC development in those patients. DNA methylation can be edited by use of the CRISPR/cas9 system, a technique called epigenetic editing[39]. Although this novel technology is far from a clinical application it is a promising concept for the future. Additionally, this approach will reveal whether *SERPINB9* DNA methylation plays a causal role in cSCC development or whether it is a consequence of another, yet unknown, mechanism leading to post-transplant cSCC development.

We are aware that the single-center design and small sample size may be a limitation of this study. Details such as sun exposure were unknown and dosages of immunosuppression

were often adjusted or immunosuppressive regimens were changed during the course of post-transplant treatment. It was therefore not possible to take these factors into account. Nevertheless, we show a promising proof-of-concept that studying DNA methylation of *SERPINB9* in peripheral T cells can identify kidney transplant recipients at risk for cSCC. The disturbed transcriptional regulation of *SERPINB9* and the lower protein expression of serpinB9 warrant further investigation to fully understand the relation with cSCC development in kidney transplant recipients.

All together these findings demonstrate that DNA methylation, transcriptional regulation and protein expression of serpinB9 differ between cSCC and non-cSCC patients. This identifies a novel risk factor for the development of post-transplant cSCC and may provide mechanistic insight in the role of circulating T cells in cSCC development. Future studies will identify whether serpinB9 plays a causal role in cSCC development and if it is a suitable treatment target to prevent cSCC development after kidney transplantation.

Supplementary Tables

Table S1. *PCR and sequence primers of genes for validation*

Gene	PCR primers	Amplicon size	450k probe names by Illumina
SERPINB9	F: 5′-GGAGGAGTAAAGGTTAGTGTAGA-3′	290 bp	
	R: 5′-biotin-CCCAACRCCAAATACCTACACAAT-3′		
	S1: 5′-GAGTGTTATTTTTATTTTTTATAT-3′		cg20726195, cg10863922, cg01345354
	S2: 5′-AGTTGAGTTTGTTGGT-3′		cg22376758
	S3: 5′-GATGATGTATTAGGAGGT-3′		cg09046168
	PCR program: 15 min at 95°C, 45 cycles of 30 s 94°C, 30 s 57°C, 30 s 72°C followed by 10 min at 72°C		
VTRNA2-1	F: 5′-GGAAGGGGGTAAAATTTATTTATTGG-3′	318 bp	
	R: 5′-biotin-ATACCCTACTAATCACTCATTAATTCATTC-3′		
	S: 5′-GGAGGGGAGGTAGGA-3′		cg08745965, cg16615357, cg18797653
	PCR program: 15 min at 95°C, 45 cycles of 30 s 94°C, 30 s 59°C, 30 s 72°C followed by 10 min at 72°C		

F: forward primer. R: reverse primer, S: sequence primer, bp: basepair, min: minutes, s: seconds

Supplementary Figures

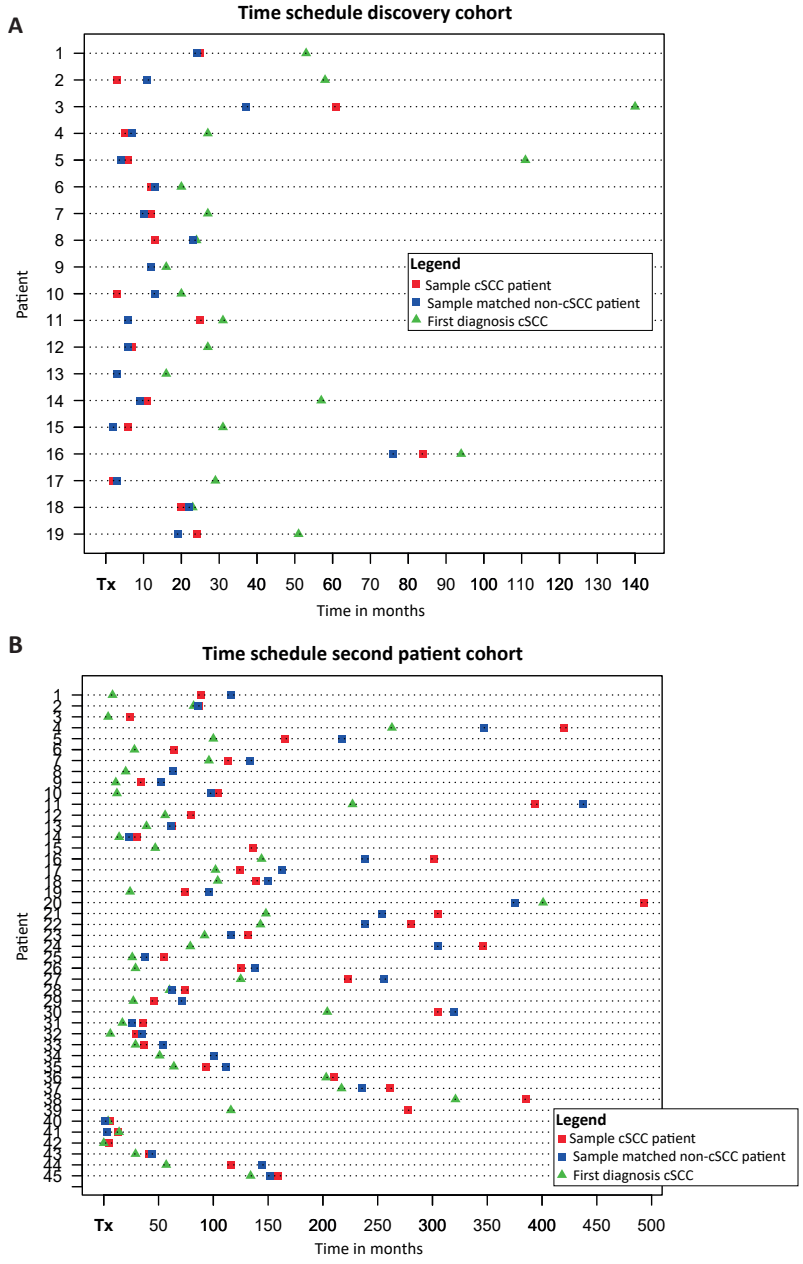

Figure S1. Detailed time schedule *of* ***A)*** *the discovery patient cohort and* ***B)*** *the second patient cohort. On the Y-axis are all the cSCC patients and their matched controls and on the X-axis is the time in months from the transplantation (Tx) onwards.*

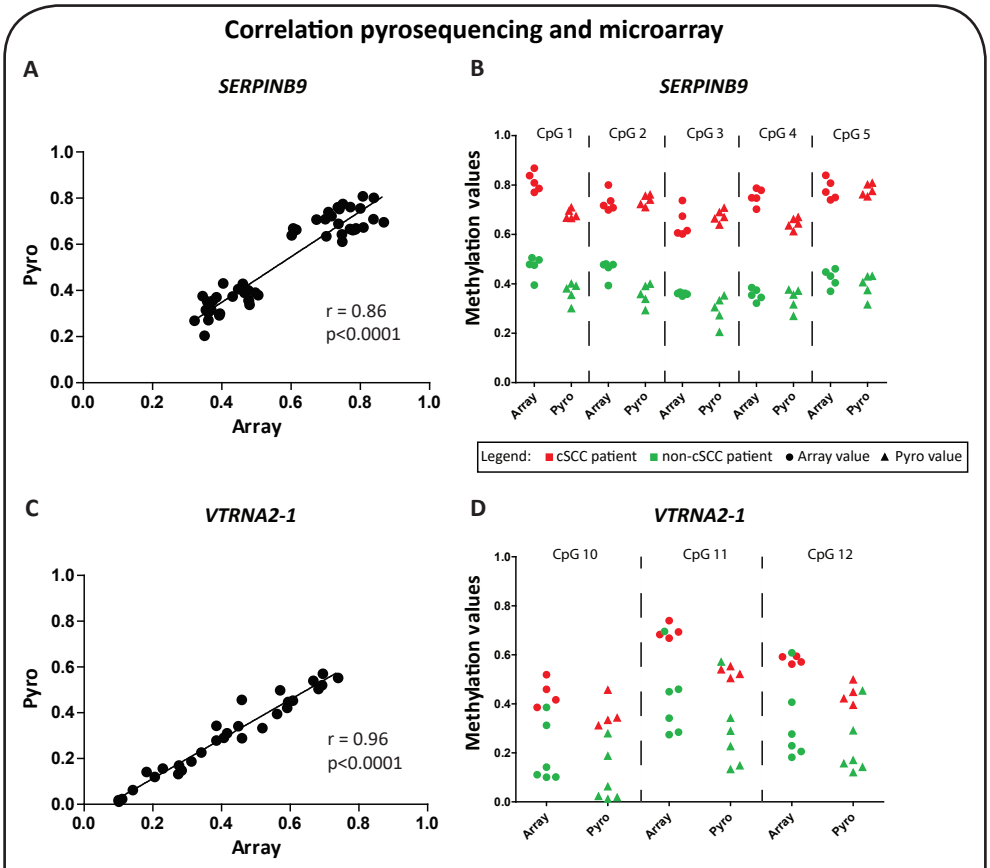

Figure S2. Correlation between methylation values obtained by pyrosequencing and microarray. A) *XY plot of DMR 1 (SERPINB9) with array values on the X-axis and pyro values on the Y-axis.* ***B)*** *Detailed graph for each of the 5 sites within DMR 1 (SERPINB9) with in red the cSCC patients and in green the non-cSCC patients.* ***C)*** *XY plot of DMR 2 (VTRNA2-1) with pyro values on the X-axis and array values on the Y-axis.* ***D)*** *Detailed graph for each of the 3 sites within DMR 2 (VTRNA2-1) with in red the cSCC patients and in green the non-cSCC patients.*

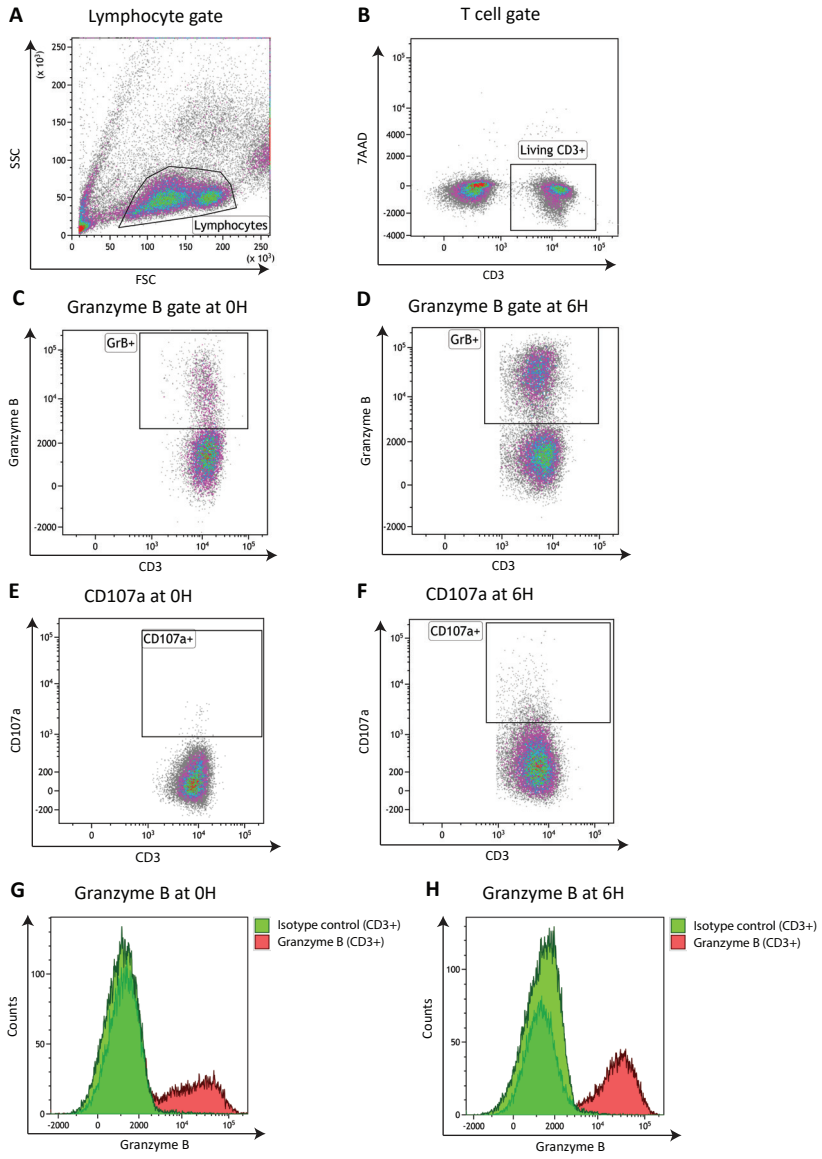

Figure S3. Gating strategy for granzyme B expression and CD107a by T cells. *Representative examples of **A)** lymphocyte gate from forward scatter (FSC) and sideward scatter (SSC), **B)** living T cells gated from 7AAD-CD3 staining, **C)** granzyme B+ cells gated within the living T cells at 0 hours, **D)** granzyme B+ cells gated within the living T cells at 6 hours, **E)** CD107a+ cells gated within the living T cells at 0 hours , **F)** CD107a+ cells gated within the living T cells at 6 hours, **G)** isotype control in green and granzyme B stained sample in red at 0 hours and **H)** isotype control in green and granzyme B stained sample in red at 6 hours.*

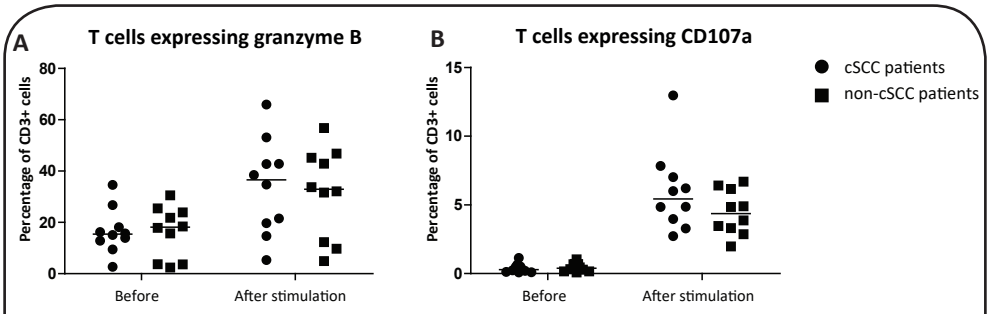

Figure S4. Quantified flow cytometry data on granzyme B and CD107a expression by T cells. A) *Percentage of T cells that expressed granzyme B before and after stimulation in the cSCC and non-cSCC patients.* ***B)*** *Percentage of T cells that expressed CD107a before and after stimulation in the cSCC and non-cSCC patients.*

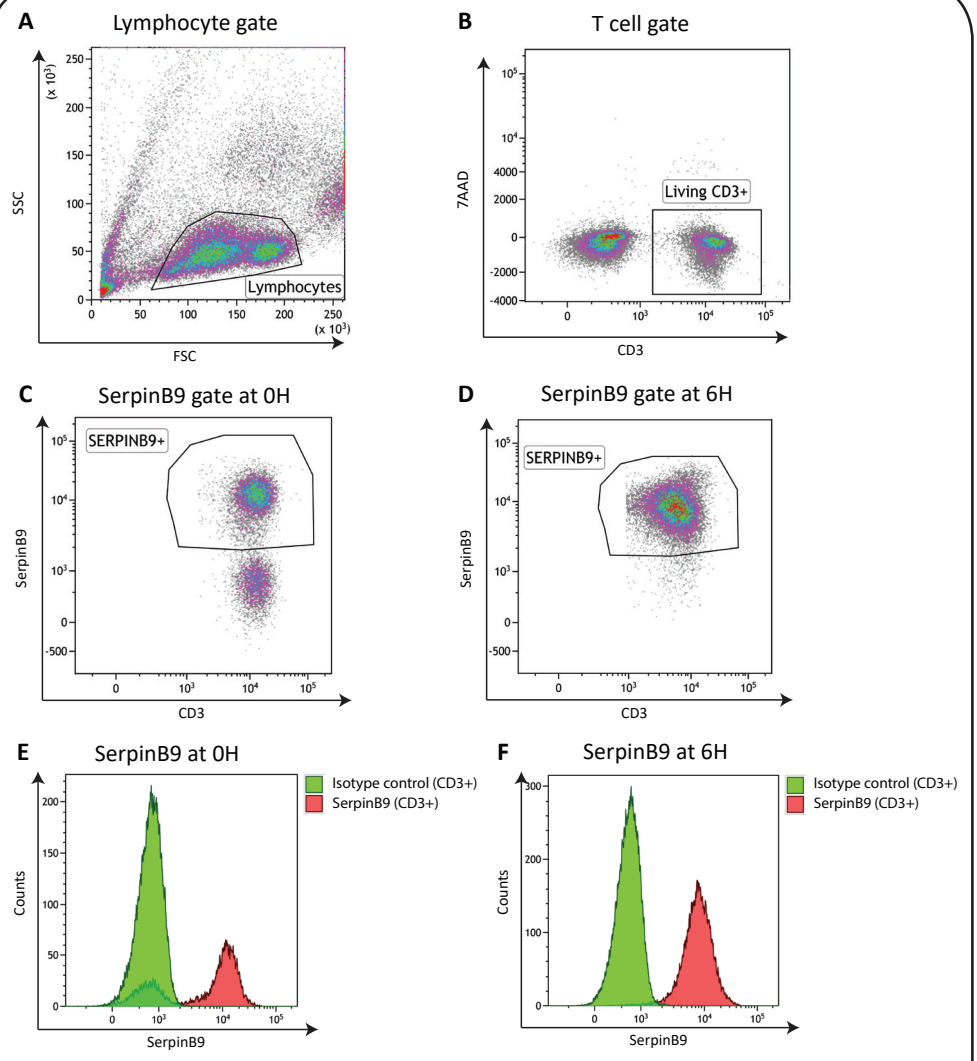

Figure S5. Gating strategy for serpinB9 expression by T cells. *Representative examples of* ***A)*** *lymphocyte gate from forward scatter (FSC) and sideward scatter (SSC),* ***B)*** *living T cells gated from 7AAD-CD3 staining,* ***C)*** *serpinB9+ cells gated within the living T cells at 0 hours,* ***D)*** *serpinB9+ cells gated within the living T cells at 6 hours,* ***E)*** *isotype control in green and serpinB9 stained sample in red at 0 hours and* ***F)*** *isotype control in green and serpinB9 stained sample in red at 6 hours.*

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Part IV

Summary and discussion

Summary

Epigenetic mechanisms determine the gene expression levels within the cell without changing the underlying DNA sequence. DNA methylation is currently the best understood epigenetic mechanism because it is biochemically stable and an easily measured epigenetic mark. DNA methylation profiles are crucial in determining the gene expression profiles of different cell types and are extensively studied in cell differentiation. This includes T cell differentiation, where antigen-naive T cells are characterized by high methylation of effector genes, which are demethylated upon antigen recognition and subsequent differentiation into effector T cells. DNA methylation profiles are dynamic and represent an interface between genomic information and the environment. It is known that DNA methylation is altered in disease and DNA methylation can therefore function as a biomarker for disease diagnosis, disease prognosis or risk assessment. A well-known and clinically applied example is methylation of the *MGMT* gene promoter which predicts whether patients with glioblastoma, an aggressive brain tumor, respond to a specific chemotherapy.

Organ transplantation is the preferred treatment option for patients with end-stage organ failure. Despite immunosuppressive treatment, approximately 20% of kidney transplant recipients experience a rejection episode. Rejection is a complex interplay of both innate and adaptive immune cells where T cells play an important role. Since rejection may cause irreversible damage to the transplanted organ one of the current challenges is to find a biomarker that precedes tissue damage and identifies recipients at increased risk for rejection. Besides rejection, transplant recipients may experience other complications after transplantation and these often relate to the general suppression of the immune system. Common complications are infections and the development of malignancies. The most common post-transplant malignancy is skin cancer, specifically cutaneous squamous cell carcinoma (cSCC), which is associated with high morbidity and increased mortality in transplant recipients. The large incidence of cSCC in immune suppressed individuals indicates the critical role of the immune system in the development of cSCC.

In general, there is a demand for innovative methods to identify patients at increased risk for developing complications after transplantation such as rejection or cSCC. If patients at an increased risk of complications are closely monitored, early clinical intervention may prevent negative consequences of the complication and thereby benefit the patients well-being. One such clinical intervention might be adjustment of the immunosuppressive load, however, there is a fine line between reducing the risk on cSCC and increasing the risk on rejection and vice versa.

In this thesis we aim to identify patients at increased risk for rejection or skin cancer by

studying DNA methylation profiles of the circulating T cells. To substantiate the validity of the DNA methylation profiles, we also investigated the stability of DNA methylation in experimental systems, evaluating the effect of immunosuppressive drugs and cytokines on DNA methylation profiles.

Tacrolimus and mycophenolate mofetil (MMF; active ingredient MPA) are most often prescribed as maintenance immunosuppressive therapy in our clinic. These compounds are designed to suppress T-cell activity within the recipient. However, it is not known whether they initiate changes on the epigenetic level to perform their function. To investigate this, we cultured total T cells, naive T cells and memory T cells in the presence of tacrolimus or MPA and measured *interferon gamma* (*IFNγ)* DNA methylation, T cell phenotype and IFNγ protein expression. In chapter 2 we describe that MPA affected *IFNγ* DNA methylation of the naive T cells but not that of memory T cells after *in vitro* stimulation. Tacrolimus showed no effect on *IFNγ* DNA methylation of the T cells after stimulation.

To further investigate environmental effects on DNA methylation, mesenchymal stromal cells (MSCs) were cultured in the presence of cytokines, IFNγ, transforming growth factor β (TGFβ) and a combination of factors, that are known to induce phenotypic and functional changes in MSCs. Also methylation profiles before and after 14 days of culture were studied to infer the effect of culture expansion. Chapter 3 describes that the changes in genome-wide DNA methylation induced by the cytokines IFNγ, TGFβ or a multi-factor combination (MC; IFNγ, TGFβ and retinoic acid) were minor. The stimulation with IFNγ and MC resulted in decreased methylation of a single CpG site. Interestingly, culture expansion led to differential methylation of >4,000 CpG sites. These sites were located within or near genes associated to membrane composition, cell adhesion and transmembrane signaling.

In the second section of this thesis the possible clinical applications of DNA methylation in organ transplantation are described. Chapter 4 reviews the current literature on DNA methylation and describes how it could be applied as an early biomarker for complications such as rejection in a non-invasive and quantitative manner. We speculated that DNA methylation testing in a clinical setting will improve future treatment of transplant recipients.

The study into the relation between DNA methylation profiles and rejection in kidney transplantation is described in chapter 5. Here we measured *IFNγ* and *programmed death 1* (*PD1)* DNA methylation in $CD8^+$ T cells before, at 3 months and 12 months after transplantation in rejecting and non-rejecting kidney transplant recipients. We observed an increase in DNA methylation for both genes within the EMRA $CD8^+$ T cell subset and for *PD1* also in the $CD27^-$ and $CD27^+$ memory subsets. In addition, the increase in *PD1* DNA methylation in the $CD8^+CD27^-$ memory population was more prominent in the rejecting

patients than in the non-rejecting patients. There was no difference between rejecting and non-rejecting patients before transplantation, thus predicting rejection was not possible with these data.

Due to the suppressed immune system, kidney transplant recipients are more prone to develop cancer, especially cSCC is a common complication after transplantation. We hypothesized that there is a systemic defect in the circulating T cells in patients that develop cSCC after transplantation. Chapter 6 demonstrates that genome-wide T-cell DNA methylation was different between kidney transplant recipients with a future post-transplant cSCC and those without cSCC. Sixteen differentially methylated regions (DMRs) were found prior to transplantation, which is a clinically relevant time point for risk assessment. For a subset of cSCC patients a post-transplant sample was available which allowed us to identify several DMRs that were stable after transplantation. These stable DMRs might have a lasting effect on the development of cSCC after kidney transplantation.

In chapter 7 we continued to study differential methylation associated to cSCC after kidney transplantation. Here we identified, after transplantation, a DMR within *SERPINB9*, a protein-coding gene that acts as an intracellular inhibitor of granzyme B. This genomic region was higher methylated in T cells of patients before they developed a first cSCC as well as in T cells of patients that already developed cSCC. At the functional level, we observed a disturbed transcriptional regulation of *SERPINB9* and a lower protein expression of serpinB9 in the patients with cSCC.

Discussion

This thesis describes differences in DNA methylation associated to complications after kidney transplantation and explores whether DNA methylation profiles can be used as a tool for risk assessment. Before DNA methylation analysis can be clinically utilized it is important to know to which extent DNA methylation is influenced by environmental factors that are relevant in organ transplantation. Therefore, we first assessed the changes in DNA methylation induced by immunosuppressive drugs, cytokines and culture expansion in activated cells.

For several medical drugs it is known that these agents alter DNA methylation. Examples are the antihypertensive drug hydralazine[1,2] and valproate which is used in treatment of epilepsy[3]. When we studied the effect of the two commonly prescribed immunosuppressive drugs tacrolimus and MPA on *IFNγ* promoter DNA methylation in T cell cultures, only the lymphocyte proliferation inhibitor MPA had an effect (**chapter 2**). Also, the suppression of IFNγ protein production by tacrolimus was not mediated by DNA methylation changes. Since this was a targeted analysis focusing on a single gene promoter, there is a chance

that other genomic regions are affected more prominently by these compounds. Nevertheless, when umbilical-cord derived MSCs (ucMSCs) were primed with IFNγ and in combination with soluble factors (IFNγ, TGFβ and retinoic acid) that are known to affect phenotype and function of MSCs[4,5], the genome-wide changes in DNA methylation were minor (**chapter 3**). Similar findings have been described for vitamin D. *In vitro* exposure of vitamin D on immune cells altered gene expression of known vitamin D responsive genes without substantial genome-wide DNA methylation changes and without DNA methylation changes in the vitamin D responsive genes[6]. It is likely that in the absence of DNA methylation changes, other epigenetic mechanisms, such as histone modifications[7], may play a leading role in changing gene expression patterns. This would also explain why we observed a discrepancy between T-cell phenotype and *IFNγ* DNA methylation after stimulation (**chapter 2**), and functional and phenotypical changes upon priming of MSCs without major DNA methylation changes (**chapter 3**).

MSCs have great therapeutic potential due to their regenerative capacities, immunosuppressive effect and low immunogenicity as demonstrated *in vitro*[8]. The number of MSCs that can be isolated from human tissue is low, therefore *in vitro* expansion is necessary to generate sufficient cell numbers for therapeutic purposes. Surprisingly, our results show that culture expansion leads to widespread changes in genome-wide DNA methylation (**chapter 3**). In literature DNA methylation changes during culture expansion are often attributed to cellular senescence[9,10] and aging of the MSCs[11,12] and those epigenetic changes associated with a declining function of the MSCs. However, in our study, surface marker expression and immunosuppressive capacities of the MSCs were similar before and after 14 days of culture expansion. Indicating that culture-induced epigenetic changes do not necessarily affect the intended function of the cells. It is therefore important to know the effect of epigenetic changes on cellular function before a cellular product can be clinically utilized. Stability and standardization of the cellular product are crucial and DNA methylation analysis may serve as an additional quality control for the cellular end-product. An example of this can be found in another form of cellular therapy: regulatory T (Treg) cell therapy[13]. Tregs have immunosuppressive capacities and are therefore proposed as a cellular immunotherapy in transplantation. An important characteristic of stable Tregs is a demethylated region within the transcription factor FOXP3[14], but often only surface marker expression is assessed after culture expansion of Tregs[15]. Concluding that analyzing DNA methylation changes during culture expansion of cells in parallel with cell function, could improve standardization of the cellular product.

In the second section of this thesis we explored the value of DNA methylation for kidney transplantation. There are several examples available where DNA methylation profiling is successfully applied in a clinical framework, most of these are in the field of oncology.

Examples are the methylation of the *MGMT* promoter in glioma[16], which is a crucial factor in clinical decision-making[17], and methylation of *SHOX2* which is used as a biomarker for lung cancer[18] and is explored for other tumor types as well[19]. Recently, it was described that ischemia during kidney transplantation induced genome-wide hyper methylation measured in kidney biopsies[20]. It is known that procedures during kidney transplantation such as cold ischemia time and ischemia-reperfusion-injury (IRI) negatively affect the outcome of the transplantation[21]. In the study by Heylen et al.[20], the time of cold ischemia directly correlated with the degree of hyper methylation. The degree of hyper methylation also predicted reduced allograft function 1 year after transplantation, thereby outperforming established clinical variables. This is strong evidence that DNA methylation is one of the molecular mechanisms underlying functional behavior of the cells and shows that DNA methylation could be a tool to predict risk for post-transplant complications. Unfortunately, this study was performed on kidney biopsies and therefore issues with cellular heterogeneity and sampling error still remain. Ideally, risk on post-transplant complications can be assessed non-invasively in blood or urine (**chapter 4**). In our studies we focused on DNA methylation profiles of peripheral T cells associated with acute rejection and skin cancer.

A pilot study on DNA methylation of the *IFNγ* and *PD1* promoters in peripheral CD8+ T cells before, at 3 months and 12 months after kidney transplantation identified only a minor difference between rejecting patients and non-rejecting patients (**chapter 5**). Since T cells play a crucial role in the rejection process, a difference in epigenetic regulation of T cell function between rejecting patients and non-rejecting patients may be expected. However, our results demonstrate that the promoter regions of these two well-known genes are not differentially methylated at the time of sampling. Possibly, at the exact time of rejection, DNA methylation changes take place at the promoter regions of *IFNγ* and *PD1*, since both molecules play a role in the rejection process[22]. DNA methylation changes preceding a rejection may be more subtle and probably occur at different genomic regions. This pleads for moving from a targeted approach to an unbiased genome-wide approach to find the regions of interest, thereby including DNA methylation outside promoter regions. The functional effect of DNA methylation outside promoters is not fully elucidated[23] but studies show that inter-individual variation in DNA methylation is much higher in gene bodies than in gene promoters[24,25]. It may be those variable regions[26] where we could find the subtle differences in DNA methylation that identify kidney transplant recipients at increased risk for rejection.

Besides rejection, a common complication after transplantation is cSCC, affecting up to 30% of the transplant population[27-29]. Biomarkers for post-transplant cSCC described in literature are most often related to T-cell phenotypes. T regulatory (Treg) cells, identified as $CD3^{+}CD4^{+}FOXP3^{+}CD25^{hi}CD127^{lo}$, were associated to higher cSCC risk[30] as well as Tregs

identified by demethylation of the Treg specific demethylated region (TSDR)[31]. Also the presence of senescent T cells defined as $CD8^+CD57^+$ was described as a strong predictor for recurrence of cSCC[32]. In these studies high risk patients were identified as those with a previous cSCC and T-cell phenotypes of these high risk patients were associated to a recurrent cSCC. In contrast, our study was designed in a retrospective manner which allowed us to analyze T cells before development of a first cSCC (**chapter 7**) and even before transplantation (**chapter 6**), a novel approach in the field of post-transplant cSCC. In addition, the genome-wide approach identified DNA methylation differences of the T cells in an unbiased manner. Of the 16 identified DMRs before transplantation, several regions remained relatively stable after transplantation and these present interesting targets to study in relation to cSCC development (**chapter 6**).

When comparing DNA methylation of the pre- and post-transplant samples within the same patients, we observed an overall increase in DNA methylation after transplantation (**chapter 6**). This is similar to what we observed in the gene promoters of *IFNγ* and *PD1*, where DNA methylation also increased in patients after kidney transplantation (**chapter 5**). In addition, even though measured in a very different compartment, kidney biopsies also showed increased methylation induced by ischemia injury during the transplantation procedure[20]. Apart from the ischemia induced hyper methylation, attributing this increase in DNA methylation of T cells to a specific component of the transplantation is difficult since these patients experience many changes, ranging from improved kidney function, to the surgical procedure and the immunosuppressive therapy they receive after transplantation. Though these changes could explain why we did not identify the same cSCC-associated DMRs before and after transplantation (**chapter 6 and 7**).

None of the genes annotated to the pre-transplant DMRs showed a clear link to T cell function. Also all DMRs were outside promoter regions which makes it difficult to predict their function solely based on the DNA methylation results[23]. This was also evident when we identified differential methylation in an intragenic region of *SERPINB9* after transplantation but before the clinical onset of the cSCC (**chapter 7**). Despite the difference in DNA methylation, there was no significant difference in mRNA expression of *SERPINB9* between the cSCC and non-cSCC patients. However, upon closer examination of the data we observed an inverse correlation between DNA methylation and mRNA expression of *SERPINB9* in the non-cSCC patients, but not in the cSCC patients. An inverse correlation between DNA methylation and mRNA expression is normally observed in the context of promoter methylation but these data indicate that intragenic DNA methylation can also work as a repressor for gene expression. Since this inverse correlation was not observed in the cSCC patients, we speculate that other epigenetic mechanisms may overrule this effect. To reliably assess the effect of genome-wide differential methylation, RNA

sequencing would be a useful addition to DNA methylation analysis. Combining these two technologies will shed light on possible distal gene regulation and makes it more straightforward to interpret DNA methylation findings on a functional level.

Our findings on the disturbed regulation of serpinB9 in cSCC patients are a first step towards unraveling the pathogenesis of post-transplant cSCC (**chapter 7**). SerpinB9 has not previously been described in relation to cSCC but many reports are available on its function both in T cells as well as in tumor cells. Bladergroen et al.[33] demonstrated the expression of serpinB9 in several types of lymphoma and proposed this as a novel protective mechanism for tumor cells to escape cytotoxic elimination via granzyme B-induced apoptosis. SerpinB9 expression also showed an association with unfavorable outcome in metastatic melanoma[34], demonstrating its prognostic value. On the other hand, serpinB9 is an essential protein in cytotoxic T cells to perform its function. Endogenous serpinB9 protects the cells from self-inflicted damage by misdirected granzyme B. Transgenic upregulation of serpinB9 in T cells significantly improved their cytotoxic potency[35], in theory increasing their ability to eliminate tumor cells[36]. This dual role makes serpinB9 an interesting target to study further. Our study demonstrated that transcriptional regulation was significantly different between cSCC and non-cSCC patients (**chapter 7**). Additional experiments will unravel which epigenetic mechanism is leading in regulating expression of *SERPINB9* and what this means for the development of post-transplant cSCC. Also the relation between peripheral T cells and T cells surrounding the cSCC lesion is unclear. The histological analysis of an cSCC showed hardly any serpinB9 positive T cells surrounding the cSCC lesion, suggesting that the tumor-specific T cells that migrate to the tumor are serpinB9 negative and cannot perform any cytotoxic activity. Whilst the peripheral T cells, a pool of all T cells, expressed serpinB9 at levels between 40-90% (**chapter 7**). If serpinB9 can be induced in tumor-specific T cells, it would be interesting to see if, as a result, the cytotoxic activity of these T cells increases.

Although there are many reviews published on the potential of epigenetics in transplantation[37-41], the actual research papers are scarce. With this thesis we hope to have demonstrated the potential that DNA methylation analysis holds for improving transplantation research and patient care. Hopefully this work leads to increased recognition for the wide range of possibilities of DNA methylation research in the field of transplantation.

Future directions

The results discussed in this thesis are representing a novel tool in transplantation research. We believe that DNA methylation analysis in the field of transplantation will improve the research and patient care throughout the coming years. Due to the explorative nature

of the research, the sample sizes were small and the studies were performed in a single-center study design. To build upon these promising results, we recommend to validate the findings in a larger cohort and preferably in a multi-center setting. In addition, our studies have been focused on peripheral T cells but the frequency of antigen-specific T cells (e.g. to the allograft or to the cSCC) is low in the total T-cell population. Analyzing the parameters studied in this thesis in antigen-specific T cells, will further unravel the role of DNA methylation in the process of rejection and development of post-transplant cSCC since these are the actual cells that will target either the allograft or the cSCC.

We have shown that analyzing DNA methylation in a targeted manner, by studying DNA methylation of genes known to play a role in the process of interest, may not identify the differences we are looking for. This could be due to timing, differences may occur during an event but not ahead of the event, making an early risk assessment difficult. Also, by studying DNA methylation of targeted genes, important variations in genomic regions of yet unknown genes or outside promoters could be missed. For these reasons, genome-wide DNA methylation analysis should be the method of choice until it is well-established which genomic regions are of interest to study in relation to post-transplant complications. This genome-wide discovery process will reveal potential biomarkers that, after rigorous validation, could be implemented in the transplantation clinic. These DNA methylation biomarkers could be combined with well-established clinical risk factors in a computational model to generate personalized risk profiles for each transplant recipient.

If we can reliably asses the risk on cSCC before or shortly after transplantation, patients could receive personalized life-style advice or a therapeutic intervention. Several studies demonstrated a beneficial effect of switching from a calcineurin inhibitor to sirolimus on the recurrence of the skin cancer[42,43]. It could also be considered to lower the dosage of immunosuppression but only if a patient has a low risk profile for rejection simultaneously. Future studies will reveal whether these interventions also have an effect on DNA methylation profiles, which due to their dynamic nature, could be a potential monitoring tool for treatment responses.

Besides the potential to serve as a biomarker, analyzing DNA methylation profiles will help understand the mechanisms that lead to complications after transplantation. In our search for DNA methylation differences associated to post-transplant cSCC, we identified *SERPINB9* as a genomic region of interest. Due to the important role of serpinB9 in cytotoxic T cell function, we propose two additional studies to further unravel the role of serpinB9 in cSCC development.

First, the correlation between *SERPINB9* DNA methylation, mRNA expression and protein expression of the peripheral T cells and the T cells present around the cSCC lesions is

unknown. It could be that the serpinB9 negative cells are the tumor specific T cells that are recruited to the cSCC lesion, or that the T cells lose their serpinB9 expression upon migration to the cSCC. Identifying the tumor specific T cells in the pool of peripheral T cells and comparing the functional and molecular characteristics with those of the T cells surrounding the cSCC will reveal the similarities or dissimilarities between the two T-cell compartments.

Second, we observed higher *SERPINB9* DNA methylation in two different patients cohorts, one cohort after transplantation and before development of the first cSCC, and one cohort after development of a first cSCC. However, no such differences in *SERPINB9* DNA methylation were found in a pre-transplant cohort, suggesting that these differences arise after transplantation. Measuring *SERPINB9* DNA methylation in a prospective cohort before and at regular intervals after transplantation, will unravel the dynamics of DNA methylation in this specific genomic region. This prospective study would also address whether *SERPINB9* DNA methylation could serve as a tool for early risk assessment for post-transplant cSCC.

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Samenvatting

In iedere cel van het menselijk lichaam zit DNA. Dit DNA bevat alle informatie die nodig is voor het opbouwen, in stand houden en functioneren van een organisme, dus ook voor de mens. Het DNA is opgebouwd uit vier bouwstenen die we aanduiden met de letters A (adenine), T (thymine), C (cytosine) en G (guanine). In het DNA bevinden zich regio's die de code bevatten voor het maken van eiwitten, moleculen die belangrijk zijn voor de functie van een cel. Deze regio's noemen we genen. De volgorde van de vier letters in een gen bepaalt bijvoorbeeld of je blauwe of bruine ogen hebt. Het is belangrijk om het aflezen van deze genen, de zogenoemde genexpressie, goed te reguleren, want dit zorgt ervoor dat elke cel in het lichaam de juiste functie uitvoert.

Een belangrijke manier om genexpressie te reguleren is het koppelen van moleculen aan het DNA, zoals bijvoorbeeld een methyl-groep. Deze vorm van regulatie wordt epigenetica genoemd. Epigenetische mechanismen reguleren genexpressie door te bepalen of deze regio's in het DNA beschikbaar zijn om afgelezen te worden. Van alle epigenetische mechanismen wordt DNA-methylatie het meest bestudeerd. Voornamelijk omdat dit een biochemisch stabiel kenmerk is en relatief makkelijk te meten is. DNA-methylatie is de toevoeging van een molecuul, een methyl-groep, op het DNA. Deze methyl-groep zit altijd op een C die gevolgd wordt door een G in het DNA, dit noemen we een CpG-site.

DNA-methylatie profielen reguleren dus genexpressie in verschillende cel types en zijn belangrijk in de differentiatie van cellen. Een voorbeeld hiervan is de T-cel rijping waarbij naïeve T-cellen, welke nog geen lichaamsvreemde stoffen (antigenen) zijn tegen gekomen, gekarakteriseerd worden door hoge methylatie van genen die coderen voor signaalstoffen, ook wel cytokines genoemd. Deze hoge DNA-methylering zorgt ervoor dat naïeve T cellen deze cytokines niet produceren. Zodra naïeve T-cellen een antigeen herkennen, zullen de T-cellen veranderen naar T-cellen die actief cytokines gaan produceren. Dit proces gaat gepaard met een vermindering van de methylatie op de genen die coderen voor de cytokines.

DNA-methylatie profielen zijn beïnvloedbaar door factoren van buitenaf zoals voeding, medicijnen en chemische stoffen en door factoren van binnenuit zoals hormonen en cytokines. Om deze reden geeft DNA-methylatie een raakvlak weer tussen de genetische informatie van een individu en de omgeving waar een individu, en dus ook de cellen van het individu, zich in bevinden. Ook weten we dat er vaak veranderingen plaatsvinden in DNA-methylatie voorafgaand aan of ten tijde van een ziekte. Hierdoor kan DNA-methylatie toegepast worden als meetbare biologische indicator (biomarker) voor de diagnose, prognose of risicobepaling voor verschillende ziektes. Een bekend voorbeeld van een

klinische toepassing van DNA-methylatie onderzoek is de analyse van methylatie van het *MGMT*-gen. Methylatie van dit gen bepaalt welke behandeling het beste werkt bij een agressieve vorm van hersenkanker.

Niertransplantatie is momenteel de beste behandeloptie voor mensen met eindstadium-nierfalen. Ondanks dat transplantatiepatiënten medicijnen krijgen die het afweersysteem onderdrukken, ontwikkelt ongeveer 20% van de patiënten een afstotingsreactie tegen de nier. Een afstoting is een complex samenspel van verschillende cellen van het afweersysteem, waarin de T-cellen een belangrijke rol spelen. De T-cellen herkennen het lichaamsvreemde weefsel en starten een immuunreactie. Hierbij gaan de T-cellen cytokines produceren, vermeerderen ze in aantal en zullen ze differentiëren van de naïeve T-cel naar actieve en geheugen T-cellen. Een afstotingsreactie na transplantatie kan leiden tot onomkeerbare schade aan het getransplanteerde orgaan en daarom is het belangrijk om een biomarker te vinden die een afstoting in een vroeg stadium kan voorspellen.

Naast het ontwikkelen van een afstotingsreactie, zijn er meerdere complicaties die transplantatiepatiënten kunnen ontwikkelen. Vaak zijn deze gerelateerd aan de afweeronderdrukkende medicijnen die deze patiënten moeten slikken om het getransplanteerde orgaan te behouden. Hierdoor hebben patiënten vaker infecties en zijn ze gevoeliger voor het ontwikkelen van kanker. Het meest voorkomende type kanker na transplantatie is huidkanker, specifiek het plaveiselcelcarcinoom (PCC). Dit type kanker komt 65 tot 200 keer vaker voor bij transplantatiepatiënten dan bij andere mensen. PCC zorgt voor groot ongemak bij patiënten en verlaagt de kwaliteit van leven. Deze sterk verhoogde kans op PCC na een transplantatie geeft aan dat het onderdrukte afweersysteem een essentiële rol speelt in de ontwikkeling van een PCC.

Er is veel vraag naar nieuwe methodes die patiënten identificeren met een verhoogd risico op het ontwikkelen van complicaties na een orgaantransplantatie, bijvoorbeeld een afstotingsreactie of PCC. Als patiënten met een verhoogd risico op complicaties nauwlettend gevolgd worden door de arts, kan er in een vroeg stadium worden ingegrepen door bijvoorbeeld de dosis afweeronderdrukkende medicijnen aan te passen. Alhoewel voorzichtigheid hierbij geboden is want er is een dunne lijn tussen het verlagen van risico op een PCC en het verhogen van het risico op afstoting en andersom.

In dit proefschrift zijn DNA-methylatie profielen van T-cellen in het bloed bestudeerd in de hoop hiermee patiënten te kunnen identificeren met een verhoogd risico op afstoting of PCC na niertransplantatie. Voordat dit toegepast zou kunnen worden in de kliniek is het belangrijk om de stabiliteit van DNA-methylatie profielen te weten. Daarom hebben we eerst DNA-methylatie gemeten in experimentele systemen en bepaald wat de invloed is van afweeronderdrukkende medicijnen en cytokines op DNA-methylatie profielen.

Tacrolimus en mycofenolate mofetil (MMF; actieve ingrediënt MPA) zijn de meest voorgeschreven afweeronderdrukkende medicijnen in ons centrum. Deze medicijnen worden voorgeschreven om de activiteit van T-cellen te onderdrukken, maar het is onbekend of ze ook een effect hebben op DNA-methylatie. Om dit te bestuderen hebben we T-cellen gekweekt, zowel totale T-cellen als geïsoleerde naïeve en geheugen T-cellen, in de aanwezigheid van tacrolimus of MPA. Op verschillende tijdspunten hebben we DNA-methylatie van *interferon-gamma* (*IFN*γ) gemeten. IFNγ is een ontstekingsbevorderende (pro-inflammatoir) cytokine dat een belangrijke rol speelt in de functie van T-cellen. Ook hebben we verschillende oppervlaktekenmerken van de T-cellen gemeten en de productie van het IFNγ-eiwit door de T-cellen. In hoofdstuk 2 beschrijven we dat enkel MPA de *IFN*γ DNA-methylatie van de naïeve T-cellen beïnvloedde na het stimuleren van de cellen. De DNA-methylatie in de geheugen T cellen veranderde niet door het toevoegen van MPA. Tacrolimus had geen effect op *IFN*γ DNA-methylatie van de T-cellen.

Vervolgens zijn we het effect van omgevingsfactoren op DNA-methylatie verder gaan bestuderen. Hiervoor hebben we mesenchymale stam cellen (MSC) gekweekt samen met cytokines waarvan we weten dat ze de functie van MSC beïnvloeden: IFNγ, transformerende-groeifactor β (TGFβ) en de combinatie IFNγ, TGFβ met retinol. Daarnaast hebben we ook de veranderingen in DNA-methylatie gemeten voor en na een periode van 14 dagen kweken, om het effect van vermenigvuldiging van de cellen op methylatie te bepalen. DNA methylatie werd op 850.000 CpG-sites gemeten, verdeeld over het gehele DNA (genoom-breed). Hoofdstuk 3 beschrijft dat er minimale veranderingen plaatsvonden in genoom-brede DNA methylatie onder invloed van de cytokines IFNγ, TGF-β of een combinatie van factoren (IFNγ, TGF-β en retinol). De toevoeging van IFNγ en de combinatie van factoren aan de MSC leidde tot een verlaging van de methylatie op een enkele CpG-site, terwijl het bekend is dat deze factoren de functie van MSC kunnen beïnvloeden. Een opvallende bevinding was dat de vermenigvuldiging van de cellen voor een periode van 14 dagen leidde tot een verschil in methylatie op meer dan 4.000 CpG-sites. Deze plekken in het DNA reguleren waarschijnlijk genen die te maken hebben met samenstelling van het celmembraan, het vermogen van de cel zich te hechten en signalen van buiten de cel naar binnen door te geven.

In het tweede deel van dit proefschrift gaan we in op de mogelijke klinische toepassingen van DNA-methylatie in relatie tot orgaantransplantatie. Hoofdstuk 4 bevat een overzicht van de huidige literatuur over DNA-methylatie. We beschrijven hoe dit kan worden toegepast als biomarker voor complicaties zoals afstoting op een manier waarbij de patiënt weinig last ondervindt van het onderzoek, zoals bijvoorbeeld enkel de afname van een buisje bloed. We speculeren dat DNA-methylatie onderzoek in een klinische context de toekomstige behandeling van transplantatiepatiënten zal verbeteren.

Het onderzoek naar de relatie tussen DNA-methylatie profielen en afstoting na niertransplantatie wordt beschreven in hoofdstuk 5. In deze studie hebben we DNA-methylatie gemeten van *IFN*γ en *programmed-death 1* (*PD1*). PD1 is een eiwit wat zich op de oppervlakte van T-cellen bevindt en een immuunreactie kan reguleren. *IFN*γ en *PD1* DNA methylatie werd gemeten in $CD8^+$ T-cellen vóór de transplantatie, en drie en vervolgens 12 maanden ná de transplantatie. Dit hebben we zowel bij patiënten die een afstotingsreactie ondergaan, als bij patiënten die niet afstoten, gemeten. We vonden een verhoging van DNA-methylatie op beide genen in de EMRA $CD8^+$ T-cel populatie, dit zijn ver doorgedifferentieerde geheugen T-cellen. Bij het gen *PD1* was er ook een verhoging van DNA-methylatie in de $CD27^-$ en $CD27^+$ T-cel populaties, twee type geheugen T-cellen. Vóór transplantatie was er geen verschil tussen patiënten die later een afstotingsreactie ontwikkelden en patiënten zonder afstoting, het voorspellen van een afstotingsreactie was met deze gegevens dus niet mogelijk.

PCC komt erg veel voor na een niertransplantatie. Wij denken dat er een defect is in de circulerende T-cellen in het bloed van patiënten die PCC ontwikkelen na transplantatie. Hoofdstuk 6 laat zien dat de genoom-brede DNA methylatie verschillend was tussen niertransplantatiepatiënten met een toekomstige PCC en niertransplantatiepatiënten die geen PCC ontwikkelden na transplantatie. Zestien gebieden in het DNA vertoonden verschillen in DNA-methylatie. Deze verschillen werden gemeten vóór de transplantatie, wat een klinisch relevant tijdspunt is voor een risicobepaling bij transplantatiepatiënten. Van een deel van de PCC-patiënten was ook materiaal aanwezig van ná de transplantatie. Hierdoor konden we DNA-methylatie in de tijd volgen. Van een aantal van de 16 gebieden bleef DNA-methylatie stabiel na transplantatie. Dit zou kunnen betekenen dat deze gebieden een rol spelen in de ontwikkeling van PCC na een transplantatie.

In hoofdstuk 7 gaan we verder met het bestuderen van DNA-methylatie profielen die geassocieerd zijn met PCC na een niertransplantatie. Hier vonden we, ná de transplantatie maar vóór het ontwikkelen van de PCC, een verschillend gemethyleerde gebied in het gen *SERPINB9* in T-cellen. *SERPINB9* codeert voor een eiwit dat de werking van granzyme B remt en granzyme B, wat voornamelijk geproduceerd wordt door $CD8^+$ T-cellen, kan celdood veroorzaken in cellen die herkend worden door het immuunsysteem. Het geïdentificeerde gebied in het *SERPINB9* gen had een hogere methylering in patiënten die een PCC ontwikkelden, zowel voor als na het ontwikkelen van de PCC. Verder onderzoek toonde aan dat de relatie tussen DNA-methylatie en genexpressie verstoord was en dat er een lagere eiwit-expressie van serpinB9 was in de patiënten met PCC.

In het eerste deel van dit proefschrift wordt beschreven dat afweeronderdrukkende medicijnen en cytokines een minimale invloed hebben op DNA-methylatie, terwijl deze

factoren de celfunctie wel kunnen beïnvloeden. De verandering in celfunctie kan misschien verklaard worden doordat, in sommige gevallen, andere epigenetische mechanismes een belangrijkere rol spelen dan DNA methylatie. Daarnaast leidde het kweken van MSC tot grote veranderingen in DNA methylatie. Deze bevinding kan belangrijk zijn als MSC worden toegepast als celtherapie. Een goede kwaliteitscontrole is belangrijk voordat een celtherapie aan de patiënt gegeven wordt en DNA methylatie kan hier in de toekomst wellicht een rol in spelen.

In het tweede deel van dit proefschrift beschrijven we verschillen in DNA-methylatie tussen patiënten die wel of geen PCC ontwikkelden na niertransplantatie. Deze resultaten zijn nieuwe bevindingen binnen het transplantatieveld en zullen hopelijk het onderzoek en patiëntenzorg in de komende jaren verbeteren. De verschillen in DNA-methylatie vonden we door een genoom-brede analyse van DNA-methylatie uit te voeren. In de studie waarbij we op een toegespitste manier naar DNA-methylatie keken konden we de complicatie afstoting niet voorspellen. Om die reden moet er in de toekomst meer onderzoek gedaan worden naar genoom-brede veranderingen van DNA-methylatie die associëren met complicaties na transplantatie, in plaats van te focussen op specifieke genen. Dit kan leiden tot ontdekking van nieuwe gebieden in het genoom die, na uitgebreide validatie, kunnen functioneren als biomarker voor complicaties na transplantatie.

Naast de potentie die DNA-methylatie heeft om te functioneren als biomarker, zal het onderzoeken van DNA-methylatie ook helpen de mechanismes te begrijpen die voorafgaan aan complicaties na transplantatie. De studie naar verschillen in DNA-methylatie geassocieerd met PCC, leidde tot de bevinding dat *SERPINB9*, een molecuul dat een belangrijke functie heeft in T-cellen, anders gemethyleerd was in patiënten met PCC. SerpinB9 inactiveert granzyme B, een molecuul dat celdood kan veroorzaken in cellen die herkend worden door het immuunsysteem en dus belangrijk is in de afweer tegen kankercellen. Vervolgstudies waarbij de rol van serpinB9 in de huid wordt bestudeert en studies naar de dynamiek van *SERPINB9* DNA-methylatie na transplantatie zullen leiden tot meer kennis over welke rol dit molecuul speelt in de ontwikkeling van PCC na transplantatie.

Part V

Appendices

List of abbreviations

ACR	acute cellular rejection
APC	antigen presenting cell
ATG	anti-thymocyte globulin
bp	base pairs
BPAR	biopsy proven acute rejection
CAV	cardiac allograft vasculopathy
cDNA	complementary DNA
cfdDNA	cell free donor-derived DNA
CKD	chronic kidney disease
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CpG	cytosine-phosphate-guanine
cSCC	cutaneous squamous cell carcinoma
DMR	differentially methylated region
DMS	differentially methylated site
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DSA	donor specific antibody
EMB	endomyocardial biopsy
ESRD	end-stage renal disease
EWAS	epigenome-wide association study
FACS	fluorescence-activated cell sorting
IFNγ	interferon gamma
IL	interleukin
IMPDH	inosine monophosphate dehydrogenase
IRI	ischemia-reperfusion injury
HD	hemodialysis
HLA	human leukocyte antigen
HPV	human papilloma virus
MFI	median fluorescence intensity
MMF	mycophenolate mofetil
MPA	mycophenolate acid
mRNA	messenger RNA
MSC	mesenchymal stromal cell
NFAT	nuclear factor of activated T cells
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells

Abbreviations

PCR	polymerase chain reaction
PD	peritoneal dialysis
PD1	programmed death 1
RNA	ribonucleic acid
TCR	T cell receptor
TET	ten-eleven translocating
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor α
Treg	regulatory T cell
TSS	transcription start site
TSDR	Treg-specific demethylated region
Tx	transplantation
ucMSC	umbilical cord-derived MSC
UV	ultraviolet

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Portfolio

PhD portfolio

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General courses

Basic Course on R (MolMed)	2015
Biostatistical Methods I: Basic Principles AB (Nihes)	2015
Advanced Immunology (MolMed)	2016
Workshop Photoshop and Illustrator CS6 (MolMed)	2016
Biomedical English Writing and Communication	2016 – 2017
Research Integrity	2017
Workshop InDesign CS6 (MolMed)	2018

National and international conferences

FederaDag, *Rotterdam, The Netherlands*	2014	
Science Days, Dept. of Internal Medicine, *Antwerp, Belgium*	2015	
Bootcongres, Joint Dutch and British Transplant Society, *Bournemouth, UK*	2015	
Epigenetics Discovery Congress, *London, UK*	2015	
Science Days, Dept. of Internal Medicine, *Antwerp, Belgium*	2016	Poster
20th Molecular Medicine day, *Rotterdam, The Netherlands*	2016	Poster
Bootcongres, Dutch Transplant Society, *Groningen, The Netherlands*	2016	Poster
Epigenetics Discovery Congress, *London, UK*	2016	Poster

Egenomics of Common Disease, *Cambridge, UK*	2016	Poster
Science Days, Dept. of Internal Medicine, *Antwerp, Belgium*	2017	Poster
Bootcongres, Dutch Transplant Society, *Zeist, The Netherlands*	2017	Presentation (2x)
21st Molecular Medicine day, *Rotterdam, The Netherlands*	2017	Presentation
PLAN dag, *Rotterdam, The Netherlands*	2017	Presentation
The Transplantation Society (TTS) Basic Science meeting, *Victoria, Canada*	2017	Poster (2x)
European Society for Organ transplantation (ESOT) meeting, *Barcelona, Spain*	2017	Presentation (2x)
Science Days, Dept. of Internal Medicine, *Antwerp, Belgium*	2018	Poster
Bootcongres, Dutch Transplant Society, *Rotterdam, The Netherlands*	2018	Poster
TTS meeting, *Madrid, Spain*	2018	Poster & Presentation

Awards and travel grants

Travel grant Erasmus Trustfonds	2017
Mentor-Mentee Award by the NTV/TTS	2017
NTV Scholingsbeurs	2017

Memberships

Nederlandse Transplantatie Vereniging (NTV)	2014 – present
European Society for Organ Transplantation (ESOT)	2017 – present
The Transplantation Society (TTS)	2017 – present

Additional activities

Supervising a biomedical sciences student for his bachelors thesis	2016
Board member Young Professionals NTV	2017 – 2018

Curriculum Vitae auctoris

Fleur Susanne Peters was born on February 19th 1990 in Amsterdam, the Netherlands. She attended the VWO from 2002 to 2008 at Keizer Karel College in Amstelveen. In September 2008 she started studying at the University of Amsterdam. She followed the BSc Bio-Exact, an inter-disciplinary program focusing on cellular and systems biology. During her bachelor studies she went on an Erasmus Exchange program to Portsmouth, UK where she followed courses on Forensic Biology. After finishing the BSc in 2011, she enjoyed a gap year and travelled through Southeast Asia and Australia. In September 2012, she continued her studies at the University of Amsterdam attending the MSc program Forensic Science, specializing in molecular biology, from which she graduated in August 2014. She started her PhD project in October 2014 at the Transplantation laboratory within the department of Internal Medicine, section Nephrology and Transplantation at Erasmus MC, under supervision of prof. Carla Baan, dr. Karin Boer and dr. Michiel Betjes. In this project she studied DNA methylation in relation to complications after kidney transplantation. This thesis is a representation of this research. She will continue her career as a postdoctoral researcher at the Amsterdam UMC (location AMC).

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