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Epigenetic profiles of monocytes, monocyte-derived macrophages and dendritic cells

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Molecular Pathology, Institute of Biomedicine and Translational Medicine, University of Tartu.

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Supervisors: Pärt Peterson, PhD, Professor of Molecular Immunology, Molecular Pathology, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Kai Kisand, MD, PhD, Senior Research Fellow of Immunology, Molecular Pathology, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Ana Rebane, PhD, Senior Research Fellow, RNA Biology Research Group, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Reviewers: Maris Laan, PhD, Professor of Human Molecular Genetics, Chair of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Sirje Rüütel Boudinot, PhD, Associate Professor, Molecular Immunology, Department of Gene Technology, Tallinn University of Technology, Estonia

Opponent: Ola Winqvist, MD, PhD, Professor, Translational Immunology Unit, Karolinska Institutet, Sweden

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Contribution of Liina Tserel to original publications:

Study I: Performed the experiments and participated in the data analysis, figure preparation and paper writing.

Study II: Performed the experiments and participated in the data analysis and figure preparation, with the exception of the cloning, as well as Luciferase assays.

Study III: Participated in the study design, material collection, experimental procedures, data analysis and paper writing.

ABBREVIATIONS

Acetyl-CoA	acetyl coenzyme A
ADP	adenosine diphosphate
BSA	bovine serum albumine
caC	carboxylcytosine
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
cDC	classical dendritic cell
cDNA	complementary deoxyribonucleic acid
CDP	common dendritic cell progenitor
ChIP	chromatin immunoprecipitation
CpG	cytosine guanine dinucleotide
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EGTA	ethylene glycol tetraacetic acid
FACS	fluorescent activated cell sorting
fC	formylcytosine
FCS	foetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HLA	human leukocyte antigen
hmC	hydroxymethylcytosine
HMT	histone methyltransferase
HSC	hematopoietic stem cell
IFN	interferon
IL	interleukin
iNOS/NOS	inducible nitric oxide synthase/nitric oxide synthase
kb	kilobase
LC	Langerhans cell
LNA	locked nucleic acid
LP	lymphoid progenitor
LPS	lipopolysaccharides
LUC	luciferase
MACS	magnetic activated cell sorting
mC	methylcytosine
MDP	macrophage and dendritic cell progenitor
MF	macrophage
MHC	major histocompatibility complex

MO	monocyte
moDC	monocyte-derived dendritic cell
MP	myeloid progenitor
MPS	mononuclear phagocyte system
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
NRP1	neuropilin 1
NRXN2	neurexin 2
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PCR	polymerase chain reaction
pre-cDC	preclassical dendritic cell
pre-miRNA	precursor micro ribonucleic acid
pri-miRNA	primary micro ribonucleic acid
PRMT	protein arginine methyltransferase
RISC	ribonucleic acid induced silencing complex
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	real time polymerase chain reaction
SAM	S-adenosyl-L-methionine
siRNA	small interfering ribonucleic acid
TET	Ten-11 translocation family protein
Th cell	T helper cell
Tip-DC	TNF/iNOS producing dendritic cell
TLR	Toll-like receptor
TNF	tumour necrosis factor
TSS	transcription start site
TTP	tristetraprolin
UTR	untranslated region

1. INTRODUCTION

The immune system is divided into the innate immune system, which provides the first non-specific response, and the adaptive immune system, which elicits a highly specific response that involves immunological memory. Monocytes, macrophages and dendritic cells (DCs) are essential components of the innate immune system that recognise and mediate the clearance of harmful agents and have crucial roles in maintaining immune homeostasis in organisms. DCs are the main antigen-presenting cells and provide the link between the immune responses.

In normal conditions, DCs are generated from common DC precursors (CDPs), but in the case of infection, DCs are rapidly generated in large numbers during the monocyte stage. Monocyte-derived DCs are under going extensive study and are currently the most promising cell type to be used in immunotherapy for cancer and various autoimmune diseases. However, many challenges remain in the introduction of these therapies to the clinic (Lesterhuis *et al.*, 2008; Eubel and Enk, 2009; Van Brussel *et al.*, 2013).

As DCs are rare in human blood, *in vitro* generated monocyte-derived dendritic cells are used as substitutes for their study as they are easy to generate in large numbers. In our model, we used monocyte-derived macrophages and DCs generated in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) alone or in combination with interleukin 4 (IL-4).

Recent evidence shows the importance of epigenetics in the regulation of gene expression, affecting processes such as cell cycle regulation, development, differentiation, ageing, X chromosome inactivation and the response to environmental and biological modifications. Epigenetic mechanisms include: histone modifications, which mark active or inactive genes; microRNAs, which mainly negatively regulate gene expression by inhibiting translation or degrading of mRNA; and DNA methylation, a cytosine methylation that occurs in context of CpG dinucleotides and represents gene silencing (Tammen *et al.*, 2012). Epigenetic patterns change gradually in response to cellular and environmental stimuli. Aberrant epigenetic patterns are described in several different biological processes, including cancers, various diseases and ageing (Portela and Esteller, 2010).

This thesis focuses on the study of different epigenetic aspects affecting monocytes, monocyte-derived macrophages and DCs, including correlations of gene expression with histone modifications in promoters and the expression of microRNAs in these cells. In addition, the DNA methylation patterns of monocytes during ageing were studied.

2. REVIEW OF LITERATURE

2.1. The mononuclear phagocyte system

The immune system encompasses several different cells and molecules that protect the host organism by activating cascades of defence reactions that ensure the recognition and elimination of potentially harmful agents. The latter include microbe components, macromolecules (proteins and polysaccharides) and small chemicals that are recognised as foreign, regardless of their physiological or pathological origin. The immune response consists of innate and adaptive immunity that together provide defence against pathogens. Innate immune cells (monocytes, macrophages, dendritic cells, natural killer lymphocytes) provide a rapid but less specific response to pathogens when compared to adaptive immune cells (B and T lymphocytes). Adaptive immunity provides a highly antigen-specific response and also immunological memory (Abbas and Janeway, 2000).

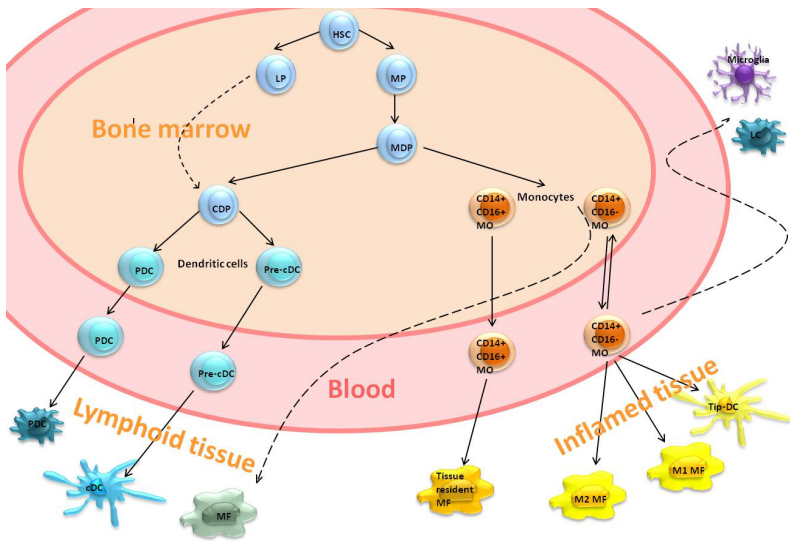

Figure 1. Monocyte, macrophage and dendritic cell (DC) differentiation lineages. Hematopoietic stem cells (HSC) produce myeloid (MP) and lymphoid progenitors (LP). MPs differentiate into monocyte, macrophage and DC progenitors (MDP), which further differentiate into monocytes or move into the population of macrophage and common DC progenitors (CDP). Monocytes leave the bone marrow and under normal conditions they differentiate into tissue-resident macrophages. During inflammation, monocytes differentiate into monocytes-derived DCs, for example tumour necrosis factor and inducible nitric oxide synthase DCs (TipDCs), and to classically (M1 MF) and alternatively activated macrophages (M2 MF). In some conditions, they differentiate into the microglia and Langerhans cells (LC). In the other branch of differentiation, CDPs differentiate into pre-classical DCs (pre-cDCs) and plasmacytoid dendritic cells (pDC) that enter lymphoid tissues through the blood, where pre-cDCs differentiate into classical DCs (cDC). Modified from (Geissmann *et al.*, 2010; Lawrence and Natoli, 2011).

DCs act as connective bridges between innate and adaptive immune systems. They recognise pathogens by pattern-recognition receptors and mediate clearance through activating T cells and potentiating antibody secretion (Steinman, 2011). DCs as well as monocytes and macrophages have a crucial role in maintaining homeostasis in organisms. DCs, monocytes and macrophages originate from the same type of hematopoietic stem cell (HSC) in bone marrow and are differentiated in the mononuclear phagocyte system (MPS) (Figure 1). Commitment to a mononuclear phagocyte lineage is determined at the macrophage and DC progenitor (MDP) stage. By this stage, erythroid, megakaryocyte, lymphoid and granulocyte fates have been excluded. MDPs differentiate into monocytes and CDPs (Fogg *et al.*, 2006; Auffray *et al.*, 2009). CDPs are proliferative cells that differentiate into plasmacytoid dendritic cells (pDC) and pre-classical dendritic cells (pre-cDC), but they have lost the ability to differentiate into monocytes (Naik *et al.*, 2007; Onai *et al.*, 2007; Liu *et al.*, 2009). Pre-cDCs migrate via the blood into lymphoid organs and peripheral tissues, where they acquire a cDC phenotype. In contrast, pDCs develop fully in the bone marrow before they enter the bloodstream and migrate into multiple tissues (Shortman and Naik, 2007). MDPs also have the potential to differentiate into a monocyte lineage that can further differentiate into macrophages or DCs.

2.1.1. Monocytes

Monocytes are circulating white blood cells that originate from myeloid progenitors in bone marrow (Fogg *et al.*, 2006) and can further differentiate into tissue macrophages and DCs (Auffray *et al.*, 2009) (Figure 1). They have several roles, including phagocytosis, cytokine production, "patrolling" the vascular endothelium, antigen processing and antigen presentation. Monocytes also differentiate into monocyte-derived DCs and macrophages, which are rapidly recruited to sites of infection, where they can phagocytose pathogens and initiate tissue repair (Gordon and Taylor, 2005; Auffray *et al.*, 2009).

Monocytes in peripheral blood are a heterogeneous population that can be divided into three subpopulations by their CD14 and CD16 surface molecules: classical $CD14^{++}CD16^{-}$ monocytes that express high levels of CD14, but not CD16; intermediate $CD14^{++}CD16^{+}$ monocytes that express high levels of CD14 and a lower level of CD16; and non-classical $CD14^{+}CD16^{++}$ monocytes that express very high levels of CD16 and a lower level of CD14 on their surface (Ziegler-Heitbrock *et al.*, 2010). All monocyte cell types are developmentally linked and differentiate from classical monocytes through intermediates to non-classical subpopulations. Classical monocytes either leave the circulation and differentiate into DCs and macrophages or further differentiate into intermediate monocytes that can also either leave the circulation or become non-classical monocytes. During an infection, there is an increase of classical, then intermediate and finally non-classical monocytes (Weiner *et al.*, 1994).

Classical $CD14^{++}CD16^{-}$ monocytes are the most prevalent subset of monocytes in human blood (approximately 90%). They express CCR2 (Geissmann *et*

al., 2003) and are rapidly recruited to sites of infection and inflammation (Serbina *et al.*, 2008). Chemokine receptor CCR2 has a crucial role in the trafficking of monocytes to the site of inflammation; monocyte trafficking is clearly reduced when CCR2 is deficient (Kurihara *et al.*, 1997; Kuziel *et al.*, 1997). Classical monocytes have high phagocytic activity but low cytokine producing properties (Ziegler-Heitbrock, 2000). The $CD16^{+}$ monocyte population, consisting of $CD14^{++}CD16^{+}$ intermediate and $CD14^{+}CD16^{++}$ non-classical monocytes (Ziegler-Heitbrock *et al.*, 2010), represent approximately 10% of the total monocyte population. $CD14^{+}CD16^{++}$ non-classical monocytes adhere to and migrate along the endothelial cell surface of blood vessels in a "patrolling" process and produce proinflammatory cytokines, such as tumour necrosis factor α (TNF-α), interleukin-1β (IL-1β) and CCL3, in response to viruses and nucleic acids (Cros *et al.*, 2010). The third subpopulation, $CD14^{++}CD16^{+}$ intermediate monocytes, are found at low frequency and have distinct features. The number of these cells increases after cytokine treatment and during inflammation (Skrzeczynska-Moncznik *et al.*, 2008; Moniuszko *et al.*, 2009). This subset of monocytes also express pro-angiogenic markers and have pro-angiogenic properties (Zawada *et al.*, 2011).

2.1.2. Macrophages

Tissue macrophages have an important role in regulation of tissue homeostasis through the elimination of senescent cells and the remodelling and repair of tissues after inflammation. In normal conditions, macrophages have anti-inflammatory properties, as they maintain tissue homeostasis by removing toxic molecules and dead or dying cells (Mosser and Edwards, 2008; Biswas and Mantovani, 2010; Rivollier *et al.*, 2012). Macrophages express pattern-recognition receptors that identify pathogen- or damage-associated patterns expressed by pathogens or by host cells during cellular senescence (Gordon and Taylor, 2005). Under inflammatory conditions, macrophages become activated and produce several mediators that recruit neutrophils and promote the inflammation process (Denning *et al.*, 2007; Mosser and Edwards, 2008; Rivollier *et al.*, 2012). There are several subpopulations of macrophages that have been described based on their location and function: osteoclasts in bone, alveolar macrophages in lungs, histiocytes in interstitial connective tissue, Kupffer cells in liver, secondary lymphoid organ macrophages in lymph nodes and spleen and multiple types of macrophages in the gut and central nervous system (Gordon and Taylor, 2005). Although the names and the phenotypes vary, they all have macrophage properties and have similar functional capabilities when activated.

Macrophages are also divided into functional subpopulations based on their specific activities (Biswas and Mantovani, 2010) (Figure 1). Classically activated macrophages (M1 macrophages) are activated by Toll-like receptor ligands and interferon-γ and mediate defence against bacteria, protozoa and viruses and antitumour immunity. M1 macrophages are pro-inflammatory and

antimicrobial (Sindrilaru et al., 2011). They produce large amount of TNF, IL-12 and IL-23 and are important drivers for T helper 1 (Th1) and Th17 cell responses (Krausgruber *et al.*, 2011). Classically activated macrophages play an important role in chronic inflammation and autoimmune diseases, including rheumatoid arthritis, atherosclerosis, pulmonary fibrosis and Crohn's disease (Smith *et al.*, 2009; Wilson *et al.*, 2010; Woollard and Geissmann, 2010).

Alternatively activated macrophages (M2 macrophages) are activated by signals from the Th2 type cytokines IL-4 and IL-13 (Gordon, 2003). M2 macrophages promote the encapsulation of parasites (Noel *et al.*, 2004), tissue repair and remodelling (Wynn, 2004). In wound healing, M2 macrophages are rapidly recruited to the site of tissue injury, where they secrete a wide variety of cytokines, chemokines, matrix metalloproteinases and their inhibitors, which in turn regulate the deposition of extracellular matrix components (Wynn, 2004). M2 macrophages antagonise the functions of M1 macrophages, thereby starting wound healing and restoring the tissue homeostasis process (Sindrilaru *et al.*, 2011). In addition to M1 and M2 macrophages, regulatory macrophages, tumour-associated macrophages and the monocytic subset of myeloid-derived suppressor cells have been described (Mosser and Edwards, 2008).

2.1.3. Dendritic cells

DCs are a migratory group of leukocytes that are specialised for the uptake, transport, processing and presentation of antigens to B and T lymphocytes (Steinman, 1991; Matzinger, 1994; Hart, 1997). In normal situations, DCs are at an immature stage of development. An encounter with a microbe or with tissue damage initiates migration of the DC to the lymph nodes (Randolph *et al.*, 2005). Encountered antigenic samples are processed and presented on the cell surface by the major histocompatibility complex (MHC) II molecules of mature and activated DCs (Guermonprez *et al.*, 2002; Trombetta and Mellman, 2005). DCs can be activated by a series of microbial products, such as bacterial DNA, viral RNA, lipopolysaccharides (LPS) and products from dying cells (Hartmann *et al.*, 1999; Verdijk *et al.*, 1999; Singh-Jasuja *et al.*, 2000). Antigen-presenting DCs can efficiently trigger an immune response by activating T lymphocytes carrying the specific receptor (Cyster, 1999). The main function of DCs is to initiate antigen-specific adaptive immune responses to foreign antigens (Steinman and Banchereau, 2007) and to maintain tolerance to self antigens (Steinman *et al.*, 2003).

DCs are categorised into two main cell populations consisting of classical dendritic cells (cDC) and plasmacytoid dendritic cells (pDC) (Heath and Carbone, 2009). cDCs, which mainly populate lymphoid and non-lymphoid organs, are specialised to process and present antigens (Villadangos and Schnorrer, 2007; Segura and Villadangos, 2009; Joffre *et al.*, 2012). cDCs are mobile cells that can move from tissues to the secondary lymphoid organs, which contain B and T cells, and initiate the adaptive immune response (Banchereau

and Steinman, 1998; Randolph *et al.*, 2005). In an immature state, cDCs have high phagocytic activity but low expression of MHC-I and -II proteins. After activation, cDCs transport MHC complexes to the cell surface and acquire co-stimulatory molecules and the capacity to produce cytokines (Banchereau and Steinman, 1998; Mellman and Steinman, 2001).

pDCs are rare (0.3–0.5% of human peripheral blood) cells that also originate from bone marrow progenitors (Naik *et al.*, 2007) and reside in steady state in the lymphoid organs. They develop fully in bone marrow and enter lymph nodes by circulation in the blood (Randolph *et al.*, 2008; Sozzani *et al.*, 2010). In steady state, they have an impaired capacity to populate non-lymphoid organs and their phagocytosis, processing and presentation of extracellular antigens is limited (Villadangos and Young, 2008). Upon activation, they produce abundant quantities of type I interferons (Liu, 2005) and gain antigen presentation properties (Colonna *et al.*, 2004). Together, these signals lead to the recruitment and activation of almost all immune cell types (Jego *et al.*, 2003; Krug *et al.*, 2004).

In the case of infection, numerous monocytes are recruited to the site of infection or inflammation (Shi *et al.*, 2011) and generate a large population of monocyte-derived or inflammatory DCs that can outnumber other tissue-resident DCs (Iijima *et al.*, 2011). Autoimmune diseases are usually accompanied by large numbers of inflammatory DCs (Rescigno and Di Sabatino, 2009; Zaba *et al.*, 2009) that can modify the local immune environment by secreting proinflammatory cytokines and chemokines (Connolly *et al.*, 2009; Weber *et al.*, 2011), which cause direct tissue damage due to production of TNFα and inducible nitric oxide synthases (iNOS/NOS). These cells are designated as TNF/iNOS producing dendritic cells (Tip-DCs) (Serbina *et al.*, 2003). This term has been extended to all inflammatory monocyte-derived DCs.

Another interesting subtype of DCs are the Langerhans cells (LC), which populate the epidermal layer of the skin and can migrate to lymph nodes to present antigens (Schuler and Steinman, 1985; Wilson and Villadangos, 2004). However, unlike conventional DCs, they are generated from embryonic precursor cells that are recruited to the skin before birth and are maintained throughout life (Chang-Rodriguez *et al.*, 2005; Chorro *et al.*, 2009). LCs mediate contact-hypersensitivity responses (Kaplan *et al.*, 2005), skin antimicrobial immunity and play a role in the induction of tolerance to peripheral antigens (Merad *et al.*, 2008).

DCs can be easily generated from monocytes *in vitro* in the presence of GM-CSF and IL-4. In the presence of GM-CSF, only macrophages are generated (Sallusto and Lanzavecchia, 1994). These *in vitro* generated cells are called monocyte-derived DCs and monocyte-derived macrophages. Monocyte-derived DCs are at immature state and can be matured by stimulation with pro-inflammatory cytokines or microbial products. *In vitro* generated monocyte-derived DCs are widely used as a substitute for *in vivo* DCs as they are easy to generate in large numbers compared with separating infrequent DCs from blood. Although *in vitro* systems are very widely used, the *in vivo* equivalent

has been difficult to identify, as GM-CSF driven cells do not exactly resemble the DCs identified *in vivo*. It has been suggested that culturing monocytes with GM-CSF and IL-4 generates dermal $CD1a^+$ cDCs (Sallusto and Lanzavecchia, 1994; Grassi *et al.*, 1998; Palucka *et al.*, 1998; Merad *et al.*, 2013).

2.2. Epigenetics

Epigenetics is the study of changes that regulate gene expression without affecting the DNA sequence. Epigenetics aims to clarify how a limited number of genes (approximately 21000) (Pennisi, 2012) can define differentiation into specific cell types. The category of epigenetics consists of several sub-categories, including histone modifications, DNA methylation and microRNAs (miRNA) (Table 1). As miRNA expression profiles are not inherited to the daughter cell and are controlled through similar mechanisms to mRNA expression, their inclusion in the epigenetics field is still being questioned. Epigenetic control is important in a wide variety of processes, including the cell cycle, development, X chromosome inactivation, imprinting and response to environmental and biological modifications and ageing (Brooks *et al.*, 2010).

Table 1. Epigenetic modifications and the correspondent enzymes

Epigenetic modification	Best known types	Location of action	Enzymes
DNA methylation	5-methylcytosine	CpG	DNMT
	5- hydroxymethylcytosine	dinucleotide	TET
Histone	Acetylation of histone tails	N-terminal tails	HAT
modifications	Methylation of histone tails	of histones	HMT
Non-coding RNAs	miRNAs	Complementary target mRNA	Pol II, Drosha, Dicer, Ago

2.3.1. Histone posttranslational modifications

In the nucleus, genomic DNA is complexed with several classes of chromosomal proteins and packed into chromatin. Basic chromosomal proteins, histones, form a nucleosome that consists of two copies of each core histone, H2A, H2B, H3 and H4 (Figure 2). Nucleosomes consisting of 146 bp of DNA wrapped around a histone octamer are the fundamental structural units of chromatin (Patel and Wang, 2013). The fifth histone, H1, binds to DNA at the intranucleosomal spacer region and thereby further packs nucleosomes into a high-order structure (Woodcock *et al.*, 2006). Histones are proteins that have protruding N-terminal tails, which can influence interactions with other nucleosomes and other non-histone regulatory factors (Patel and Wang, 2013). Several amino acid residues within histone tails and the histone can be modified by different post-translational modifications, including methylation, acetylation,

phosphorylation, ubiquitination, sumoylation, citrullination, ribosylation and proline isomerisation (Figure 2) (Lee *et al.*, 2013). Histone modifications are dynamic and influence several cellular processes, including transcription, replication, DNA repair and cell cycle progression (Ehrenhofer-Murray, 2004; Cosgrove and Wolberger, 2005; Shilatifard, 2006; Groth *et al.*, 2007; Kouzarides, 2007; Li *et al.*, 2007).

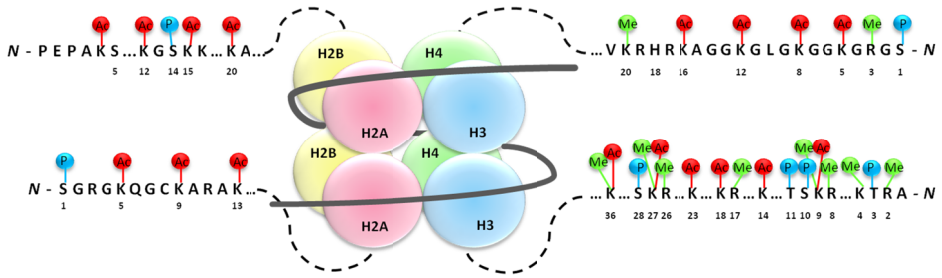

Figure 2. Post-translational modifications on histone tails. N-terminal tails of histones (H2A, H2B, H3, H4) are modified. Ac: acetyl group, Me: methyl group, P: phosphate group. Modified from (Lee *et al.*, 2013).

Two of the best-characterised histone modifications are histone methylation and histone acetylation. Histone acetylation is almost always associated with active transcription. It is catalysed by histone acetyltransferases (HATs) and is reversed by histone deacetylases (HDACs). Thus, the cellular level of histone acetylation is determined by counteraction between HATs and HDACs. In the acetylation reaction, the acetyl group is carried over from acetyl-CoA to a lysine residue by HAT, which leads to chromatin decondensation and transcription activation (Marmorstein and Roth, 2001). On the contrary, HDACs remove acetyl group from histones, which leads to formation of heterochromatin and transcription repression.

Histones can be methylated at lysine or arginine residues by histone methyltransferases (HMTs) (Shilatifard, 2006; Kouzarides, 2007) or protein arginine methyltrasferases (PRMTs) (Wolf, 2009), respectively. Lysines are methylated by the SET-domain containing proteins (Rea *et al.*, 2000) and DOT1-like proteins (Feng *et al.*, 2002). Histone-related lysine demethylation is carried out by lysine-specific demethylase 1 (LSD1) and members of the Jumonji C (JmjC) protein family (Rice *et al.*, 2003). LSD1 carries out only mono- and dimethylated substrate demethylation, whereas the JmjC family members can also demethylate trimethylated lysines (Klose and Zhang, 2007; Shi, 2007).

Epigenetic regulation of chromatin structure is a complex process as it is regulated by different grades of modifications at lysine and arginine residues. Methylation of histone H3 at lysine 4 (H3K4), lysine 36 (H3K36) and lysine 79 (H3K79) results in transcriptional activation, whereas methylation of histone H3 at lysine 9 (H3K9), lysine 27 (H3K27) and histone H4 at lysine 20 (H4K20)

is associated with transcriptional repression (Wu *et al.*, 2007; Hublitz *et al.*, 2009). Complexity is added because methylation groups can be inserted in mono-, di- or trimethyl forms for lysines and mono- or dimethyl forms for arginines. The potential to activate or repress transcription is also determined by the genomic context. For example, di- and tri-methylation at H3K9 has an activation potential when at the coding regions and a negative potential when at the promoter regions (Vakoc *et al.*, 2005).

2.2.2. DNA methylation

DNA methylation is the best-characterised chemical modification of chromatin. DNA methylation is a covalent addition of a methyl group to the fifth carbon of the cytosine ring to form 5-methylcytosine (5-mC)(Klose and Bird, 2006). DNA methylation mainly occurs in the context of CpG dinucleotides, although there is also evidence that methylation can be found outside of CpG context in embryonic stem cells (Lister and Ecker, 2009; Yan *et al.*, 2011). There are approximately 30 million CpG dinucleotides in the human genome that can be in methylated or unmethylated state (Cocozza *et al.*, 2011). Up to 80% of the total number of CpG sites in the genome are methylated (Lister *et al.*, 2009). CpG dinucleotides are not distributed uniformly along mammalian genomes; CpG sites are found in higher densities in gene-rich regions compared with gene-poor regions (Weber *et al.*, 2005).

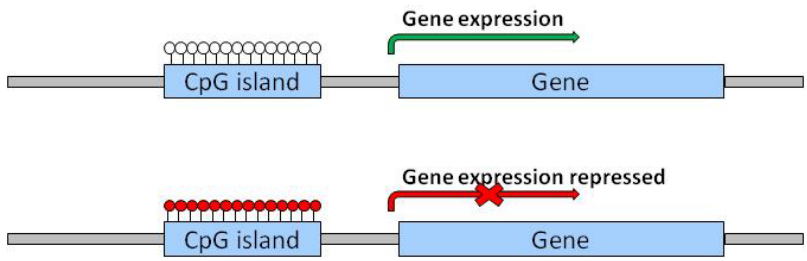

Figure 3. DNA methylation. DNA methylation occurs in the context of CpG dinucleotides. Many genes have upstream CG-rich regions called CpG islands. DNA methylation on CpG islands (marked with red dots) represses gene expression.

There are dense CpG rich areas at the promoters of more than a half of all genes. These areas, called CpG islands, are usually unmethylated (Figure 3) (Suzuki and Bird, 2008; Cedar and Bergman, 2009). Approximately 60% of gene promoters have CpG islands, including most housekeeping genes and half of the tissue-specific genes (Cheong *et al.*, 2006; Deaton and Bird, 2011). CpG islands are approximately 0.5–2 kb long DNA sequences, with a CG content greater than 55%. Methylation of promoter CpG islands leads to gene inactivation by allowing the binding of repressors and alteration of the DNA confor-

mation and local histone structures (Meehan *et al.*, 1992; Ng and Bird, 1999; Valinluck *et al.*, 2004; Cedar and Bergman, 2009). Although it is known that methylation in promoters suppresses gene expression, the role of methylation in gene bodies is less clear and requires further investigation (Lorincz *et al.*, 2004; Flanagan and Wild, 2007; Cokus *et al.*, 2008; Ball *et al.*, 2009; Rauch *et al.*, 2009). DNA methylation plays a vital role in several normal cellular functions and in a wide variety of pathological functions, including cancer (Jones and Baylin, 2007), imprinting disorders (Feinberg, 2007; Jones and Baylin, 2007) and even in the development of neuropsychiatric phenotypes (Mill *et al.*, 2008).

DNA methylation is carried out by the DNA methyltransferase (DNMT) family members, DNMT1, DNMT3A, DNMT3B and DNMT3L (Table 2) (Denis *et al.*, 2011; Arand *et al.*, 2012). These enzymes function by transferring a methyl group from S-adenosyl-L-methionine (SAM) to deoxycytosine. SAM is a modified amino acid that is produced in the carbon metabolism pathway. The process is reversed by DNA demethylases (Auclair and Weber, 2012). The transmission of DNA methylation patterns to daughter cells during cellular replication is executed by DNMT1, which methylates the nascent hemi-methylated strand after DNA synthesis and prevents passive demethylation during cell division (Denis *et al.*, 2011). DNMT1 deficiency leads to chromosomal instability and DNA repair defects (Kim *et al.*, 2004). It also has a key function maintaining stem cells; DNMT1 disruption leads to instability of the stem cell pool (Trowbridge *et al.*, 2009). Enzymes DNMT3A and DNMT3B are responsible for *de novo* methylation; they are capable of methylating both unmodified cytosines and hemimethylated cytosines, producing new methylation patterns (Gowher and Jeltsch, 2001). The last member of the DNMT family is catalytically inactive DNMT3L, which is important in *de novo* methylation and imprinting. DNMT3L reorganises DNMT3A and DNMT3B complexes into defined sub-complexes that have a higher affinity to SAM, increased catalytic properties and higher processivity (Holz-Schietinger and Reich, 2010; Denis *et al.*, 2011).

Table 2. DNA methyltransferases and their role in DNA methylation

DNA methyltransferase	Role in DNA methylation	Preference
DNMT1	Maintenance	Hemimethylated DNA
DNMT3A	*De novo* methylation	Un- and hemimethylated DNA
DNMT3B	*De novo* methylation	Un-and hemimethylated DNA
DNMT3L	Imprinting, enhances methylation on DNMT3s	Non-catalytic

While 5-mC is the predominant epigenetic marker in higher eukaryots, it can be further oxidised to 5-hydroxymethylcytosine (5-hmC) by the Ten-11 translocation family proteins (TET1–3) (Laird *et al.*, 2013). It is considered an inter-

mediate in DNA demethylation pathway (Wu and Zhang, 2011), but whether its role is passive or active still needs to be confirmed. The demethylation pathway can also further oxidise 5-hmC to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). Many of the commonly used assays to measure DNA methylation level cannot distinguish between 5-mC and 5-hmC, although the level of 5-hmC in genome is approximately 10% of measured 5-mC and approximately 0.4% of all cytosines (Branco *et al.*, 2011).

Methylation has been extensively studied (Fernandez *et al.*, 2012), and it has been shown that differentiated cell types have specifically methylated CpG sites (Ji *et al.*, 2010; Bocker *et al.*, 2011; Calvanese *et al.*, 2011). There is also growing evidence that DNA methylation plays a pivotal role in several pathological processes, including cancer (Kriaucionis and Bird, 2003; Feinberg, 2007; Jones and Baylin, 2007; Richardson, 2007; Urdinguio *et al.*, 2009; Rakyan *et al.*, 2011). In addition, multiple age-related methylation changes have been reported in multiple tissues and organisms (De Haan and Gerrits, 2007; Gronniger *et al.*, 2010; Rakyan *et al.*, 2010; Teschendorff *et al.*, 2010; Bocker *et al.*, 2011; Bocklandt *et al.*, 2011).

Many different processes in mononuclear phagocytes are regulated or tuned through epigenetic mechanisms. For example, in monocytes the production of pro-inflammatory cytokine IL-1β is regulated through DNA methylation (Wessels *et al.*, 2010). In macrophages, histone modifications are involved in the production of pro-inflammatory cytokines and polarisation towards M1 or M2 subtypes (Medzhitov and Horng, 2009; Takeuch and Akira, 2011). In monocyte differentiation to DCs, an increase in DC-SIGN (CD209) expression is correlated with the loss of DNA methylation and the acquisition of active histone modifications instead of repressive histone modifications. In another example, the reduction of CD14 expression in monocyte-derived dendritic cells is correlated with the loss of active histone modifications (Bullwinkel *et al.*, 2011).

2.2.3. MicroRNAs

miRNAs are small (~22 nucleotides) non-coding RNAs that regulate target gene expression by fine-tuning rather than completely shutting down mRNA translation in cells (Guo *et al.*, 2010). They can act by directly binding to their target sequence in the 3'UTR, forming degradable double-stranded mRNA, or through the formation of a RNA-induced silencing complex (RISC) (Bartel, 2004). miRNAs play a crucial role in several biological processes, including cell proliferation, differentiation, development, apoptosis and metabolism (Bushati and Cohen, 2007). To date, more than 2000 mature miRNAs have been described in humans (miRNA registry in http://www.mirbase.org/). miRNAs have numerous roles in eukaryotes, including the regulation of immune responses. In addition to their intracellular location, miRNAs have been found in various body fluids, including serum (Chen *et al.*, 2008; Gilad *et al.*, 2008; Lawrie *et al.*, 2008), plasma (Mitchell *et al.*, 2008), saliva (Park *et al.*, 2009), urine (Hanke *et al.*, 2010) and milk (Chen *et al.*, 2010; Kosaka *et al.*,

2010). miRNA expression patterns are significantly altered in pathological conditions such as cancer, diabetes and tissue injury (Zen and Zhang, 2010).

miRNA biogenesis is a complex process involving several enzymes. miRNA genes can be located in the introns of protein-coding genes, but many are also derived from independent transcriptional units (Rodriguez *et al.*, 2004). miRNAs are transcribed as long primary transcripts (pri-miRNAs) containing one or several miRNA-encoding hairpin structures (Figure 4) (Lee *et al.*, 2004). Pri-miRNA is processed to precursor miRNA (pre-miRNA), which is then exported to the cytosol by Exportin-5. In the cytosol, pre-miRNA is further processed by Dicer to a double-stranded RNA duplex containing mature miRNA and its antisense strand (Kim *et al.*, 2009). In the Dicer cleavage process, miRNA is coupled with RISC, which directs RISC to its target mRNA through partial sequence complementarity (Thieme *et al.*, 2012). The RISC complex mediates the inhibition of translation and/or deadenylation and degradation of mRNA. miRNAs target the 3'UTR of mRNA, as actively translating ribosomes abolish the binding of the RISC complex to the coding region (Bartel, 2004; Kim, 2005; Chi *et al.*, 2009). Each miRNA is predicted to target many mRNAs, and several miRNAs can control one mRNA at the same time. miRNAs can also target transcription factors, which adds complexity to their regulatory functions (Xiao and Rajewsky, 2009). Under certain conditions there is evidence that miRNAs can upregulate the expression of target genes (Vasudevan *et al.*, 2007) and directly interfere with gene transcription (Kim *et al.*, 2008); they can also be imported from the cytoplasm to the nucleus to regulate their own expression (Chen *et al.*, 2012). miRNAs are secreted into the extracellular space, where they function as signalling molecules and mediate communication between cells (Chen *et al.*, 2012). For instance, it has been demonstrated that miRNAs can serve as ligands to Toll-like receptors (Fabbri *et al.*, 2012; Lehmann *et al.*, 2012), which normally mediate the host immune response to invading pathogens.

Several miRNAs regulate the differentiation and function of MPS cells (Xiao *et al.*, 2007; Lodish *et al.*, 2008; O'Connell *et al.*, 2010). Two well-studied miRNAs that have roles in the immune system are miR-155 and miR-146a; both have several functions in macrophages and DCs. miR-155 acts mainly as an activator and miR-146 as a suppressor of immune responses. miR-155 controls T cell differentiation and germinal centre reactions (Thai *et al.*, 2007). In macrophages, miR-155 is involved in macrophage polarisation and apoptosis (O'Connell *et al.*, 2007; Cai *et al.*, 2012; Ghorpade *et al.*, 2012; Koch *et al.*, 2012). In pDCs, it targets transforming growth factor beta (TGF-β) activated kinase 1-binding protein 2, regulating IFN-α/β expression and DCs TLR/IL-1 inflammatory pathways related to monocyte-derived DCs (Ceppi *et al.*, 2009; Zawada *et al.*, 2011). In peripheral blood monocytes, miR-146 controls the response to inflammatory challenge by targeting RelB, a member of the NF-κB/Rel family (Etzrodt *et al.*, 2012).

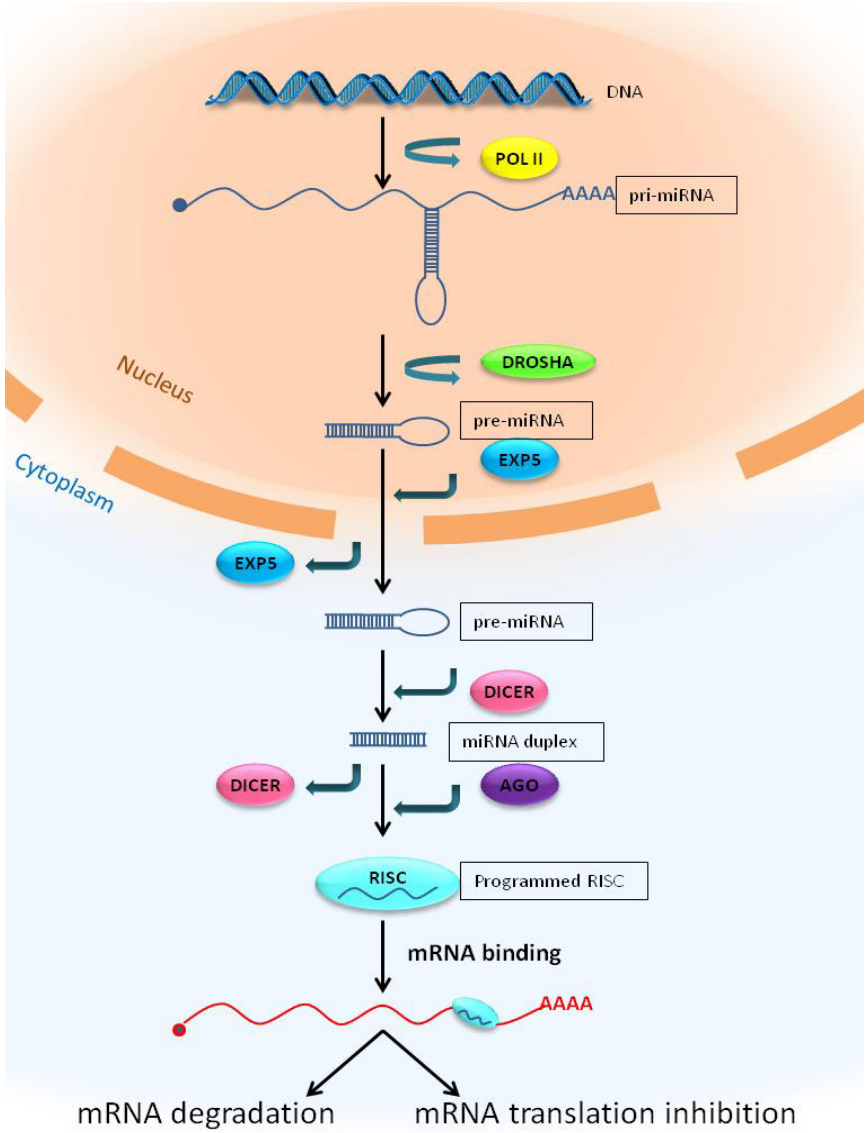

Figure 4. The miRNA processing pathway. The primary miRNA transcript (pri-miRNA) is transcribed by RNA polymerase II and then cleaved to the pre-miRNA by the microprocessor protein complex Drosha in the nucleus. Pre-miRNA is exported from the nucleus by Exportin-5–Ran-GTP (EXP5). In the cytoplasm, the functional strand of the mature miRNA, together with Argonaute (AGO2) proteins, is loaded into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs. Modified from (Cullen, 2006; Winter *et al.*, 2009).

2.3. Ageing in the immune system

Ageing is a complex physiological process that is contributed to by the accumulation and progression of several degenerative processes, including cellular senescence, DNA repair and oxidative stress (Vijg and Campisi, 2008). Ageing is associated with gradual declines in mechanisms that maintain tissue structure and function. It is a process of physical, psychological and social changes that are associated with characteristic epigenetic changes, including DNA methylation and histone post-translational modifications (Krishnamoorthy *et al.*, 2006; Fraga and Esteller, 2007; Oberdoerffer and Sinclair, 2007).

Many clinical disorders associated with ageing can be ascribed to the immune system; ageing is described as overall deterioration of immune system (DeVeale *et al.*, 2004). Both adaptive and innate immune systems are affected in the ageing process. In the adaptive immune system, ageing is associated with a reduced proportion of naïve T cells compared to their memory counterparts (Chakravarti and Abraham, 1999). This is mainly the result of thymic involution, which leads to a reduction of naïve T cells, and lifelong accumulating exposure to foreign antigens, which leads to an increase of activated memory T cells (Jamieson *et al.*, 1999; Koch *et al.*, 2008). In addition to changes in numbers and composition, immune cells also acquire diminished proliferative responses, altered cytokine production and responsiveness, aberrant phenotypes, decreased antigen recognition and aberrations in signal transduction (Chakravarti and Abraham, 1999; Miller, 2000; Weksler, 2000; Szakal *et al.*, 2002; McGlauchlen and Vogel, 2003; Gupta *et al.*, 2004; Shilatifard, 2006; Koch *et al.*, 2008). In antibody-mediated immune responses, the quantity and production of serum antibodies declines with age (Weksler, 2000). The antibody repertoire of elderly individuals is composed of different isotypes and has reduced affinity compared to that of young individuals (Weksler, 2000; McGlauchlen and Vogel, 2003).

Unlike the adaptive immune system, which exhibits reduced functions with age, the impairment of the innate immune system results in elevated level of basal inflammation. This is also referred as inflamm-ageing and is characterised as having elevated levels of circulating pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα, IL-15) and reduced levels of anti-inflammatory cytokines (IL-10) (Franceschi *et al.*, 2007). Whereby, centenarians have been shown to maintain the cytokine profile of younger adults (Di Bona *et al.*, 2009). Ageing exhibits harmful effects on the cells of the innate immune system; the phagocytic capacity of neutrophils, reactive oxygen intermediate synthesis and intracellular killing efficiency are impaired (Wenisch *et al.*, 2000; Fulop *et al.*, 2004), although the number of neutrophils does not change in the elderly (Gomez *et al.*, 2008). NK cell cytotoxicity and cytokines and chemokine production are also dysfunctional (Mariani *et al.*, 2002; Mocchegiani *et al.*, 2003). The absolute numbers of monocytes are not changed during ageing, but the subpopulation proportions are changed; $CD16^{+}$ monocyte numbers are increased and $CD16^{-}$ monocyte numbers are decreased (Nyugen *et al.*, 2010).

Nevertheless, monocytes display age-related alterations in their function. The ability of monocytes to activate the TLR pathway or to express cytokines is often impaired; the expression of TLR1 and TLR2 increases with age, whereas the functions of TLR4 and TLR8 decrease (van Duin *et al.*, 2007; Agarwal and Busse, 2010). Although the number of macrophage precursors in bone marrow is lower, the macrophage number, size, DNA content and expression of cell-surface markers are unaltered (Sebastian *et al.*, 2005). The ability of macrophages to migrate towards the site of infection is declined, which may contribute to a delayed immune response (Fietta *et al.*, 1993). In addition, intracellular killing of pathogens is decreased due to a lower production rate of reactive oxygen intermediates (Ding *et al.*, 1994). The number of DCs do not change during ageing (Agrawal *et al.*, 2007), but in elderly people, their ability to activate naïve T cells is decreased; this has been observed in both monocyte-derived DC and pDCs (Agrawal *et al.*, 2008; Agrawal *et al.*, 2012). Endocytosis, chemotaxis and the production of IL-12 are also impaired (Agrawal *et al.*, 2012). DCs also contribute to the constant inflammatory status of elderly by increasing the production of IL-6 and TNF-α without specific stimulation signals (Agrawal and Gupta, 2011).

During the lifetime epigenome can be influenced by genetic, stochastic (random mutation) and systematic (response to environmental changes) factors. It is well established that some genes that are in early life epigenetically down-regulated become activated in ageing (Salpea *et al.*, 2012). Genetically identical twins have indistinguishable epigenome early in life, whereas older individuals exhibit significant differences in their epigenetic patterns (Fraga *et al.*, 2005). A gradual age-associated DNA hypomethylation has been described in several genomic copartments, including promoters, exons, introns and intergenic regions (Heyn *et al.*, 2012). In addition to hypomethylation, also hypermethylation of specific promoters across lifespan has been described, including also estrogen receptor, genes involved in DNA binding and regulation of transcription (Post *et al.*, 1999; Oakes *et al.*, 2003). Ageing is characterised by changes in type and combinations of histone modifications and gradual reduction of histone level that drastically affects chromatin structure (D'Aquila *et al.*, 2012). In addition, the activity of several enzymes responsible for the establishment and removal of epigenetic marks is changed in ageing. For example, the activities of DNMT1 and DNMT3A (Casillas *et al.*, 2003) and deacetylase SIRT1 (Marton *et al.*, 2010) are reduced and the activities of demethylases Jmjd3 and Jarid1b are increased (Jung *et al.*, 2010; Nijwening *et al.*, 2011). All this contributes to changed epigenetic patterns and thereby changed gene expression facilitating in ageing phenotype.

In sum, ageing-associated changes in the immune system encompass both the number of immune cells and their functionality. These alterations lead to cumulative effects in the immune response, resulting in chronic inflammation and contributing to age-related diseases, including infections, cardiovascular diseases and cancers.

3. AIMS OF THE STUDY

The aims of this study were:

1. To compare the histone modification and gene expression profiles of human monocytes, monocyte-derived DCs and macrophages.
2. To identify microRNA expression profiles of human monocytes, monocyte-derived DCs and macrophages, to find DC-specific microRNAs and study their functional role.
3. To study changes in DNA methylation, which occur in human monocytes during ageing.

4. MATERIALS AND METHODS

4.1. Monocyte extraction and cell culture

The whole blood and buffy coats were obtained from donors at the Estonian Genome Center and Tartu University Hospital's Blood Center. All participants were older than 18 years and signed a written informed consent form. Studies were approved by the Ethics Review Committee on Human Research of the University of Tartu, Estonia (protocol numbers 170/T-7; 182/M-1 and 206/T-4).

PBMCs were prepared by density gradient centrifugation on Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB). Monocytes were enriched using magnetically activated cell sorting (MACS; Miltenyi Biotec) with anti-CD14-conjugated magnetic microbeads and either a manual or automatic separation system (Miltenyi Biotech). Monocytes were differentiated into MFs using 50 ng/ml GM-CSF and into DCs using 50 ng/ml GM-CSF and 25 ng/ml IL-4 (both from PeproTech); differentiation was performed for 6 days at 1 million cells/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml PENICILLIN, 100 μg/ml streptomycin and 10% FCS (all from PAA). The expression of surface proteins on monocytes, macrophages and DCs was analysed using fluorescence-conjugated antibodies to CD14, DC-SIGN, CD80 and CD83 (Miltenyi) and FACSCalibur (BD Biosciences) was used to confirm the characteristic phenotypes.

4.2. Quantitative real-time PCR

For quantitative mRNA RT-PCR, cDNA was synthesised using oligo-dT and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative PCR was carried out using Maxima SYBR green/Rox Master Mix (Fermentas). miRNA expression was analysed using Taqman MicroRNA Assays, a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and 5X HOT FIREPol Probe RT-PCR Mix Plus (Solis Biodyne). All primers used in the study are listed in Table 3. All RT-PCRs were carried out using ABI Prism 7900; the relative gene expression levels were calculated using the comparative C_t ($\Delta\Delta C_t$) method (Applied Biosystems).

4.3. Gene expression arrays

RNA was extracted using a miRNeasy Mini Kit (Qiagen) or the Trizol reagent (Invitrogen) combined with a RNeasy Mini Kit (Qiagen). The concentration of RNA was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and the quality was assessed with an Agilent RNA 6000 Nano Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was amplified and labelled using an Illumina TotalPrep RNA Amplification Kit (Ambion Inc.) or a TargetAmp-Nano Labeling Kit (Epicentre). mRNA and miRNA profiling

was carried out in the Core Facility at the Department of Biotechnology, University of Tartu using Illumina Human-6 v2 BeadChips and Illumina miRNA Universal-16 BeadChips (miRBase version 12.0) (both from Illumina) respectively. The data were analysed with BeadStudio Gene Expression Module v3.3.7 (Illumina) using Illumina's custom rank invariant method or average normalisation. Microarray data are available in the ArrayExpress databank with accession numbers E-TABM-976 and E-TABM-968.

4.4. Transfection of anti-miR inhibitors and siRNAs

For miRNA inhibition experiments, pre-designed anti-miRNA inhibitors, locked nucleic acid (LNA)-based miR-511 inhibitor and unlabelled control A (Exiqon) were used. For DICER inhibition, Silencer Select Validated DICER1 siRNAs (s23754, s23756) and respective negative controls (Applied Biosystems) were used. All transfections were carried out at the concentration of 120 nM siRNA or miRNA inhibitors using 3 μl of siPORT *NeoFX* Transfection agent for 10^6 cells/1 ml medium (Applied Biosystems). After the transfection procedure, monocytes were differentiated as usual (Paragraph 0). Transfection efficiency was controlled by fluorescence microscopy of separate transfections using Cy3-labelled negative control miRNA inhibitors or negative control siRNAs; efficiency was estimated to be between 90–100%.

4.5. Chromatin immunoprecipitation (ChIP) and ChIP-on-ChIP assay

ChIP with some modifications was performed according to the Upstate Chromatin Immunoprecipitation Assay protocol (Upstate). Briefly, formaldehyde crosslinking was carried out in PBS containing 1% formaldehyde and 0.5 mM EGTA with the density of $7x10^7$ cells in 70 ml. To process all samples in parallel, monocyte samples were crosslinked, lysed and subjected to sonication on day one and the lysates were kept at 4ºC until macrophage and DC samples were collected. Approximately of $1x10^7$ cells, 30 μl of packed protein G sepharose beads (GE Healthcare; pre-absorbed with 100 μg/ml BSA and 500 μg/ml of sheared salmon sperm DNA) and 4 μg of H3 (Abcam, ab1791), H3K4me3 (Abcam, ab8580), H3K27me3 (Upstate, 07–449) or AcH3 (Upstate, 06–599) antibodies were used in each immunoprecipitation.

DNA probes from ChIP were amplified using the GenomePlex Complete Whole Genome Amplification (WGA) Kit and reamplified using the GenomePlex WGA Reamplification Kit (both from Sigma-Aldrich). Sample labelling, hybridisation and data extraction was performed by NimbleGen Systems using 385K RefSeq Promoters array set. The NimbleGen RefSeq Promoter array data are available at ArrayExpress databank with accession number E-TABM-979.

4.6. DNA isolation and DNA methylation determination

Genomic DNA was isolated from cell pellets using a QIAmp DNA Micro Kit (Qiagen) according to the manufacturer's protocol. DNA concentration was measured with NanoDrop ND-1000 spectrophotometry. Approximately 500 ng of genomic DNA was bisulfite-converted with an EZ-96 DNA Methylation Kit (Zymo Research Corporation), according to the manufacturer's protocol. DNA methylation analysis was performed using Infinium Human Methylation 450K bead chip technology (Illumina).

Quantitative analyse of DNA methylation was determined using Sequenom's EpiTYPER T Complete Reagent Set (Sequenom, Inc.). DNA was amplified using bisulfite-converted DNA, Hot Start DNA Polymerase (Solis BioDyne) and primers, according to the EpiTYPER protocol. Primers were designed with Sequenom's EpiDesigner program and are listed in Table 3.

4.7. Cloning and Luciferase assay

For the Luciferase (LUC) assay, the following 3'UTR fragments of TLR4 (NM_138554.3) were used: TLR4-I (3096–3736), TLR4-II (4588–5000) and TLR4-III (5028–5384). The fragments were PCR amplified, digested with FseI (New England Biolabs) and XbaI (Fermentas) and inserted into the same restriction sites of pGL3-Control (Promega) downstream the LU coding region. The cloned plasmids were verified by sequencing. The cloning primers are listed in Table 3.

For Luciferase assay 8×10^4 HEK293 cells were plated into 24-well plates and transfected after 24 h with 20 ng of renilla encoding pRL-TK (Promega), 100 ng of the pGL3-3'UTR reporters and with either 50 nM of pre-miR-511 precursor or the FAM labelled pre-miR-control (Applied Biosystems); the siPORT NeoFX Transfection Agent (Applied Biosystems) was used in these experiments. Cells were harvested after 48 h or 96 h in contact inhibition conditions (Vasudevan *et al.*, 2007) and analysed using the Promega dual luciferase assay. Luminescence was counted using a Wallac 1420 (Perkin Elmer).

4.8. Protein expression

For western blot analyses, rabbit polyclonal anti-human TLR4 (sc-10741, Santa Cruz Biotec) in 2% milk, mouse monoclonal anti-human GAPDH (ab8245, Abcam) in 5% milk and DICER1 (ab14601 Abcam) in 5% milk were used. Signals were detected with an ECL Advance Western Blotting Detection Kit (GE Healthcare) and captured and quantified using an ImageQuant™-RT ECL image analysis system. For FACS, fluorescence-conjugated antibodies to CD83, DC-SIGN, CD86 (BD Biosciences), CD14 and HLA-DR (Miltenyi Biotec) were used with the FACSCalibur system (BD Biosciences). Data were analysed and visualised with FlowJo v. 7.6.

4.9. Primer sequences

The sequences of primers (TAG Copenhagen, Copenhagen, Denmark) used in the study are listed in Table 3.

Table 3. Primers used in the study together with the corresponding sequences, genes and applications used

Oligo name	Sequence	Gene	Application
DICER F	GGCTGTAAAGTACGACTACC	Dicer	qPCR
DICER R	GATCTCCTAAGCTCAGAATCC	Dicer	qPCR
TLR4-1 F	ATCCCCTGAGGCATTTAGGC	TLR4	qPCR
TLR4-1 R	TCAATTGTCTGGATTTCACACCTG	TLR4	qPCR
TLR4-2 F	TCCCTCCCCTGTACCCTTCT	TLR4	qPCR
TLR4-2 R	AGCATTGCCCAACAGGAAAC	TLR4	qPCR
TLR4-3 F	ATCCCTGGGTGTGTTTCCAT	TLR4	qPCR
TLR4-3 R	TGCGGACACACACACTTTCA	TLR4	qPCR
HPRT F	GACTTTGCTTTCCTTGGTCAGG	TLR4	qPCR
HPRT R	AGTCTGGCTTATATCCAACACTTCG	TLR4	qPCR
TLR4-I F	ATATCTAGAAAAGACAGAGAAAA CAGAAAGAGACA	TLR4	Cloning
TLR4-I R	ATAGGC CGGCCTTCCTTCCTGCCTCTAGCCC	TLR4	Cloning
TLR4-II F	ATATCTAGACCCGGAGGCCAT TATGCTAT	TLR4	Cloning
TLR4-II R	ATAGGCCGGCCCAATTTGATGAGTT TAGACATAGTCAC	TLR4	Cloning
TLR4-III F	ATATCTAGAATATCAATTATGTCTG AATGAAGCTAT	TLR4	Cloning
TLR4-III R	ATAGGCCGGCCAGAGAACTCATCTC AAACAGCC	TLR4	Cloning
NRP1 T7 F	AGGAAGAGAGTTGGGTTTTAGGTTA ATGTAGGTTG	NRP1	Sequenom
NRP1 T7 R	CAGTAATACGACTCACTATAGGGAG AAGGCTACTATCCAACAAAACCCCA AATAAT	NRP1	Sequenom
NRXN2 T7 F	AGGAAGAGAGGTTTAAGGAATTGGT TGGTAGGAAT	NRXN2	Sequenom
NRXN2 T7 R	CAGTAATACGACTCACTATAGGGAG AAGGCTATATAAACCCCTCCACCTT ATACCC	NRXN2	Sequenom
miR-29b-2 T7 F 5´_T7 F	AGGAAGAGAGTTTGATGGGATTAAA TTTTGGAATA	miR-29b-2	Sequenom
miR-29b-2 T7 R 5´_T7 F	CAGTAATACGACTCACTATAGGGAG AAGGCTAAAAAAACAAAACACCC ACATACAC	miR-29b-2	Sequenom

5. RESULTS

5.1. Genome-wide promoter analysis of histone modifications (Ref. I)

5.1.1. Genome-wide mRNA expression profiles

As monocyte-derived DCs are the most common type of DCs used in immunotherapeutic approaches (Melief, 2008; Tyagi *et al.*, 2009), we used *in vitro* differentiated human blood monocyte-derived DCs and macrophages in our analyses (Sallusto and Lanzavecchia, 1994). First, an Illumina Human-6 v2 BeadChip array was carried out to obtain detailed mRNA transcription profiling. Overall, the gene expression profiles of monocytes, macrophages and DCs were similar. However, we found a significantly higher correlation between the expression profiles of macrophages and DCs than between the profiles of monocytes and macrophages or monocytes and DCs. A similar number of genes were upregulated in macrophages and DCs (1663 and 1630 genes; approximately 8%) (Figure 5) and approximately the same proportion were downregulated during macrophage (1952; 9%) and DC (1654; 8%) differentiation. Most of the upregulated (1037) and downregulated (1328) genes in macrophage and DC populations overlapped, indicating a similarity between the two cell populations.

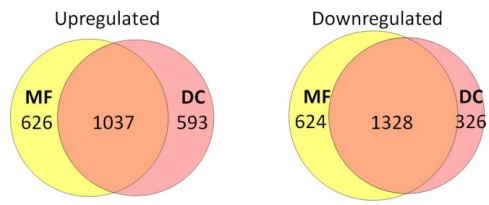

Figure 5. Upregulated and downregulated genes in macrophages and DCs.

According to the gene expression array, there are specific markers and cytokines that are differentially expressed in the cell populations studied. For example, the monocyte population had very high expression of CD14, CCR2, CSF1R and SELL/CD62L mRNA, which were all downregulated after the differentiation to macrophages or DCs (Table 4). In contrast, two marker genes specific to the human $CD16^{+}$ monocyte subpopulation, FCGR3A/CD16A and FCGR3B/CD16B, were expressed at low levels. In both macrophage and DC populations, we observed highly increased expression of surface markers TM7SF4/DC-STAMP and TREM2 and the chemokines CCL3 and CCL22. Despite large similarities between the expression profiles of macrophage and DC populations, there were many genes that were differentially expressed in these cell types. In DCs, several additional surface markers were strongly upregulated, such as CD209/DC-SIGN, SLAM and CD1 family genes, as well as the chemokines CCL2, CCL13, CCL17, CCL23 and CCL26.

The expression data showed that macrophages and DCs share a similar global mRNA expression pattern that is different from their monocyte precursors.

Table 4. Selected marker and cytokine gene expression signals and their differential scores in monocytes and after differentiation to macrophages and dendritic cells

Gene	Monocyte	Macrophage		Dendritic cell	
	Signal	Signal	Score*	Signal	Score
CD1A	226	160	-4	7934	48
CD1B	34	794	9	11970	103
CD1C	308	105	-16	4012	39
CD4	1191	494	-26	452	-30
CD14	13904	4056	-41	122	-77
CD40	57	624	28	762	85
CCL2	19	1542	6	1022	54
CCL3	72	6355	49	3436	99
CCL13	7	462	6	21325	103
CCL17	1	65	21	19817	75
CCL22	15	7026	124	14991	103
CCL23	1	406	16	2200	18
CCL26	14	13	0	2295	26
CCR2	912	1	-26	2	-26
CSF1R	11566	3911	-26	6423	-11
LY9/SLAMF3	13	113	42	635	52
SLAMF7	63	851	58	1226	76
SLAMF8	52	2745	68	2182	98
SLAMF9	11	469	108	217	55
SELL/CD62L	2807	10	-77	7	-77
DC-SIGN/CD209	46	798	28	8191	102
DC-STAMP/TM7SF4	1	8783	125	3809	103
TREM2	1	1810	123	2834	103

*The Illumina BeadStudio differential expression score indicate a significant difference between expression levels. A differential score >13 correspond to a statistically significant change with a P-value less than 0.05. Positive values correspond to the upregulation of expression and negative values to downregulation.

5.1.2. Histone modification patterns

To explain the different gene expression patterns in monocytes, macrophages and DCs, we decided to investigate histone modifications and their correlation with gene expression profiles. The influence of histone modifications on gene expression profiles has been shown in different studies (Roh *et al.*, 2006; Lehtonen *et al.*, 2007; Araki *et al.*, 2009; Wei *et al.*, 2009; Karlic *et al.*, 2010). We used a high-resolution promoter microarray screen with immunoprecipitated chromatin (ChIP-chip). In our study we used antibodies to different modifications of histone H3; H3K4me3 and AcH3, as active chromatin marks, and to H3K27me3, as an inactive chromatin mark. The immunoprecipitated material

was hybridised to the NimbleGen promoter array covering 19222 gene promoters in the human genome, which overlap with 17289 genes on the Illumina Human-6 v2 BeadChip expression array.

The most prevalent mark in the studied cell populations was H3K4me3, which was present on 45% of monocyte genes, 43% of macrophage genes and 37% of DC genes (Figure 6A). The AcH3 mark was found in the three cell subsets with nearly equal frequency and was present on 30–32% of the gene promoters. The H3K27me3 mark had the lowest frequency of the studied modifications; it was present on 23–27% of promoters. Notably, more than one third of gene promoters (36 to 39%) lacked the histone modifications studied here. As expected, the most prevalent combination of marks was the double H3K4me3/AcH3 (25–28%), whereas the combination of H3K4me3 and H3K27me3 was present on only 5–6% of gene promoters (Figure 6A). A considerable proportion of genes (16–19%) were positive for the H3K27me3 modification alone.

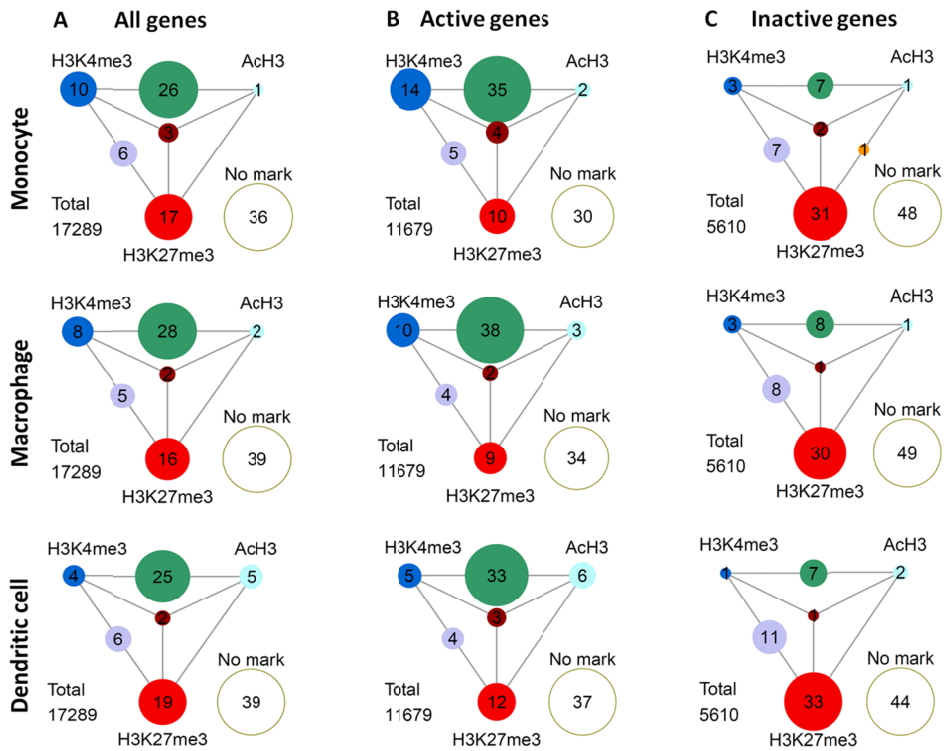

Figure 6. Frequencies of the H3K4me3, AcH3 and H3K27me3 marks and their combinations in (A) all genes (B), transcriptionally active genes and (C) inactive gene promoters. Frequencies of the modifications are shown as percentages in scaled circles positioned in the outer corners of the triangles. Frequencies of co-occurring modifications are shown between them. "No mark" represents a lack of all the histone modifications that were analysed in this study.

The H3K4me3 modification often overlaps with the AcH3 mark on active genes, but in combination with H3K27me3, it marks genes poised for either activation or repression (Bernstein *et al.*, 2007). Therefore, we analysed the presence of histone marks among a subset of transcriptionally active genes (Figure 6B). Similar to the data that includes all of the genes in the genome, the most common combination among expressed genes was the H3K4me3/AcH3 modification, which was present in 33–38% of promoters. The double modification of H3K4me3 and H3K27me3, marking the so-called bivalent poised genes, was present in 4–5% of genes; however, a mark for silenced genes, H3K27me3 alone, was clearly decreased (9–12%) in the expressed genes. We noted that during the differentiation of monocytes to DCs, the proportion of genes with H3K4me3 alone decreased from 14% to 5%, in contrast to the AcH3 mark, which slightly increased from 2% to 6% (Figure 6B). These changes, although to a lesser extent, also occurred in macrophage differentiation; H3K4me3 decreased to 10% and AcH3 increased to 3%. Modifications on transcriptionally inactive genes were clearly different; the main modification on these gene promoters was H3K27me3 alone (30–33%) or in combination with H3K4me3 (7–11%). Only 7% to 8% of genes had the combination of the H3K4me3 and AcH3 marks (Figure 6C).

The most prevalent histone mark in monocytes, monocyte-derived macrophages and dendritic cells is H3K4me3, followed by AcH3 and then by H3K27me3. The most common combination was the H3K4me3/AcH3 modification. In addition, histone modification patterns can be correlated with gene activity; the most common combination among active genes was the H3K4me3/AcH3 modification and among inactive genes H3K27me3 alone.

5.1.3. Histone marks in differentiation

As histone modifications are highly dynamic, we analysed their dynamics during the differentiation process from monocytes to macrophages and DCs. The mark characteristic for active genes, the double H3K4me3/AcH3, often persisted on genes throughout the differentiation process (Figure 7). However, we noted a striking decrease of H3K4me3 and AcH3 modifications when they occurred alone. The H3K4me3 mark was lost or turned into a double mark (by an additional acetylation) in 72% or 86% of corresponding genes in macrophages and DCs, respectively. Similarly, 84–85% of genes with the AcH3 mark alone lost this modification status during differentiation from monocytes. In contrast, the H3K27me3 modification alone was relatively stable, although less so when it occurred in combination with H3K4me3 (Figure 7).

These results demonstrate the dynamic nature of histone modifications during differentiation and show that presence of the H3K4me3 and AcH3 modifications alone leads to either a loss of these individual marks or a gain of the H3K4me3/AcH3 mark.

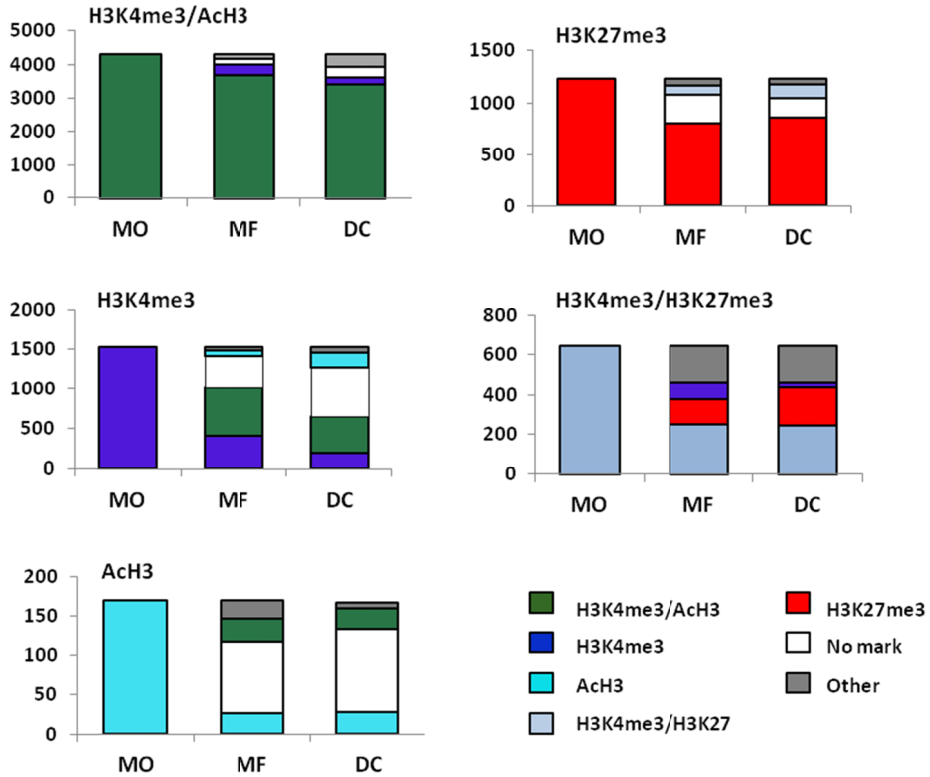

Figure 7. Dynamics of histone modifications and the combinations of modifications observed. Gene sets with indicated histone modifications present in monocytes (MO) were analysed further in macrophages (MFs) and DCs. The scale indicates the number of genes in each set in the monocyte population.

5.1.4. Correlation of histone modifications and gene expression

To study the correlation of histone modifications and gene expression profiles, we studied the promoters of several well-established marker genes, many of which are associated with macrophage and DC differentiation (Figure 8A). For example, the CD14 and CD4 genes had the double H3K4me3/AcH3 mark on their promoters in monocytes, where they are expressed at high levels, but they lost these marks during differentiation. CD8A and CD8B genes were not expressed in any of the cells studied and were marked by the repressive H3K27me3 modification. The gene encoding costimulatory ligand CD86 is relatively highly expressed in all three cell types and has the double H3K4me3/AcH3 mark but CD80 gene, which is expressed at lower level, was marked by AcH3 only. The highly expressed integrin marker ITGAM/CD11b had the H3K4me3 modification in monocytes and macrophages and acquired an AcH3 mark in DCs. The expression of ITGAL/CD11a decreased during diffe-

rentiation and lost its H3K4me3 and AcH3 marks in DCs. Active H3K4me3/ AcH3 chromatin marks were also present on the integrin ITGAX/CD11c and SIRPA genes.

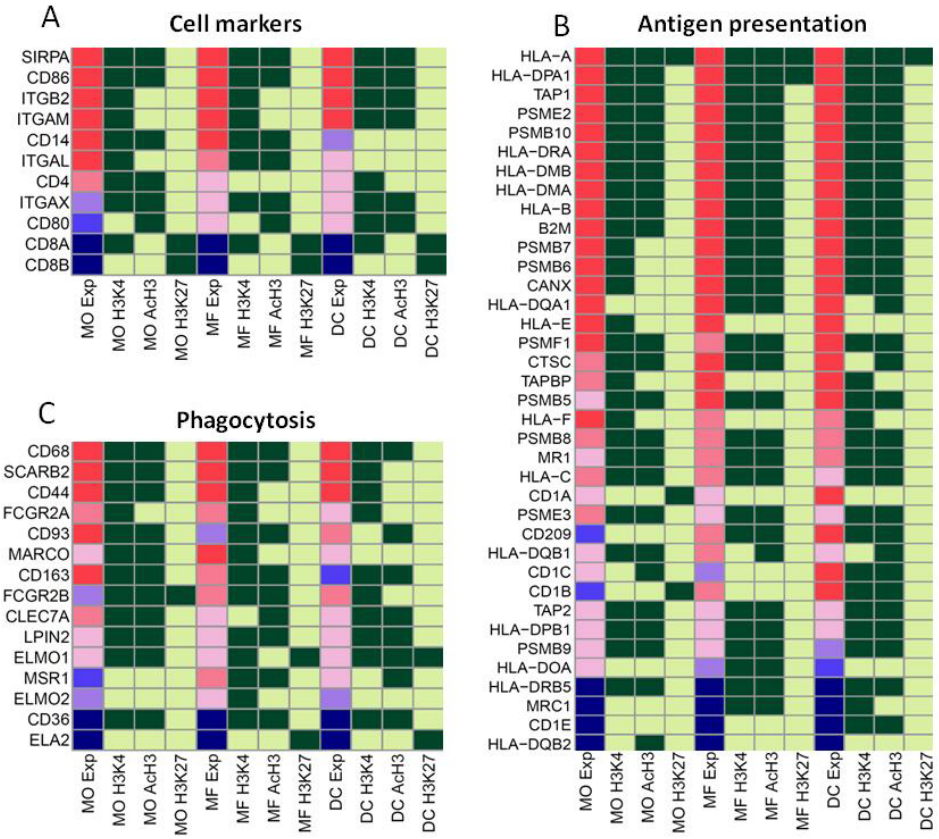

Figure 8. Comparison of gene expression levels and H3K4me3, AcH3 and H3K27me3 modifications among functional gene groups in monocytes, macrophages and DCs. Histone modifications and their correlation with expression levels in (A) individual genes of cell markers and genes involved in (B) antigen presentation or (C) phagocytosis. Colour scale indicates the expression level from no expression (blue) to high expression (red). Dark green represents presence of the modification and light green represents absence.

Phagocytosis and antigen presentation are the two most central functions of monocytes and monocyte-derived cells. Therefore, we studied the histone modifications of genes involved in these two key categories of antigen-presenting cells, in correlation with their gene expression levels. Out of 15 genes associated with phagocytic function, the double H3K4me3/AcH3 mark was present on 11 promoters in monocytes, 8 promoters in macrophages and 6 promoters in DC populations (Figure 8C). Most of the 37 genes associated with antigen presentation, including human leukocyte antigen (HLA) class I and class II, β2-microglobulin (B2M), proteasome-associated and peptide transporter (TAP1 and TAP2), had the double H3K4me3/AcH3 mark on their promoters (Figure 8B). These data are in agreement with the general functionality of antigen-presenting cells that is monocytes and macrophages are more active in phagocytic activities, whereas macrophages and DCs are more efficient at antigen presentation.

These data provide evidence that histone modifications correlate with gene activity and, more specifically, with the general functionality of the cells.

5.2. MicroRNA expression profiles (Ref. II)

5.2.1. miRNA profiles

The role of miRNAs in regulating immune cell differentiation and function has been described previously. To describe the expression and roles of recently described miRNAs, we differentiated human blood monocytes into DCs and macrophages and stimulated them, on day 6, with LPS, as a TLR4 ligand, and curdlan, as a Dectin-1 (CLEC7A) ligand. Total RNA was analysed on Illumina miRNA arrays containing probes for 858 human mature miRNAs, based on miRBase 12.0 (Griffiths-Jones *et al.*, 2008). Altogether, 307 to 380 miRNAs were expressed; 39 miRNAs were upregulated and 10 were downregulated in immature or mature DCs and macrophages (Figure 9). The three most highly upregulated miRNAs in DCs and macrophages were miR-99b, miR-212 and miR-511. Some miRNAs were specific to DCs; for example miR-193b, miR-125b and miR-99a, whereas others were more specific to macrophages; for example, miR-139-5p, miR-1 and miR-218. Changes in miRNA expression emerged mainly during differentiation and not in the stimulation process. After stimulation, a relatively small increase in expression was detected for 16 miRNAs. No miRNAs specific to either LPS or curdlan stimulation were detected.

Our data describes updated miRNA expression profiles of human monocytes, monocyte-derived macrophages and DCs, including also miRNAs that were not reported in these cells earlier.

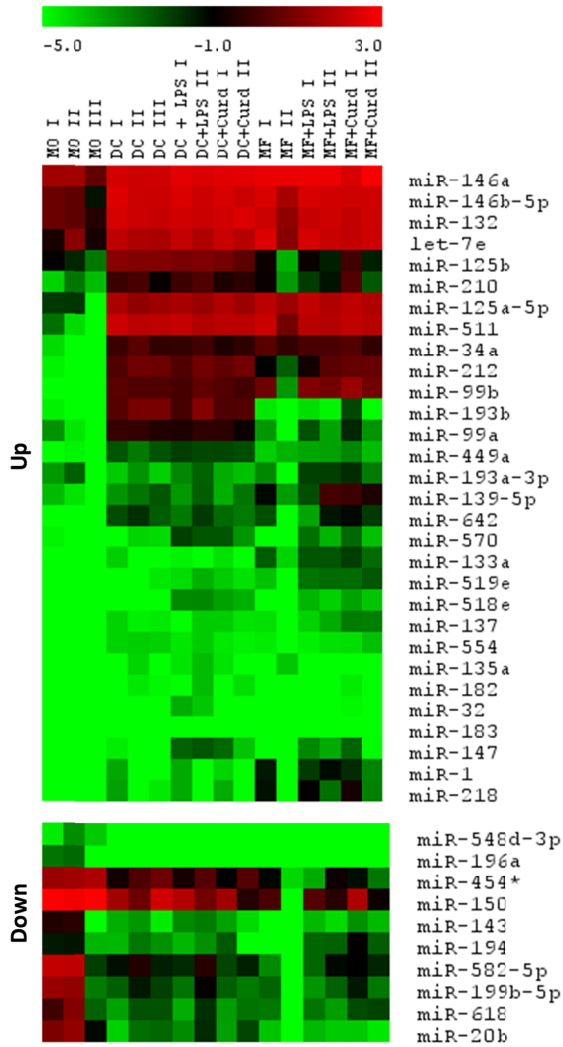

Figure 9. Monocytes (MOs), dendritic cells (DCs), and macrophages (MFs) have specific miRNA expression profiles. Heatmaps of miRNAs that are up- or downregulated (differential $p>0.05$) in DCs and/or macrophages compared to their levels in monocytes. miRNAs shown have an average expression level of over 100 in at least one condition. Each column represents expression levels of miRNA (shown right) in each sample (shown above). Log2 expression values for each miRNA are mean-centred across all the analysed miRNA expression values. The colour scale from green (lower) to red (higher) represents deviation from the mean (black).

5.2.2. Inhibition of miRNAs results in delayed differentiation phenotype of DCs

To further prove that miRNAs are essential for DC differentiation, we carried out a siRNA knockdown of DICER1, a protein that is indispensable for miRNA processing (Perron and Provost, 2009) (Figure 4). The knockdown efficiency of DICER1 was approximately 50% (Figure 10A), and knockdown resulted in a lower protein level of DC-SIGN and a higher level of CD14 (Figure 10B), which is indicative of a delayed differentiation phenotype in DCs. To further show the effect of DICER1 knockdown on DC differentiation, the DC-SIGN/CD14 ratios were calculated and found to be significantly different (Figure 10C).

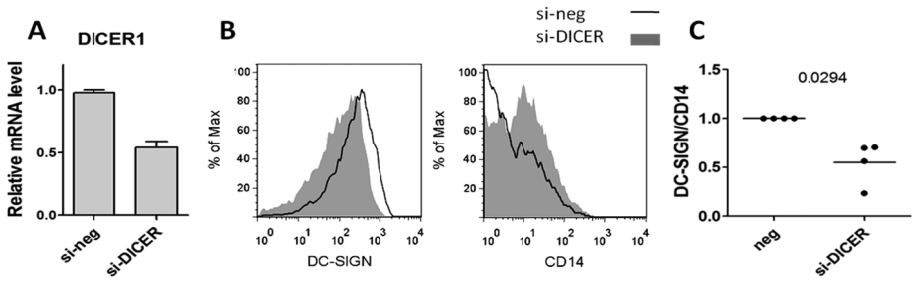

Figure 10. Reduced expression of DICER1 results in the downregulation of the DC-specific surface marker, DC-SIGN. (A) Inhibition of DICER1 (si-DICER) is shown as an average mRNA expression level with S.E. of three different treatments normalised to an average of the negative controls (si-neg, which equals 1). (B) A histogram representing the geometric mean fluorescent intensities of the indicated surface molecules shown as a percentage of maximum in DICER1 and siRNA treated cells (filled grey) relative to control treated cells (black line). (C) Ratios of DC-SIGN and CD14 mean fluorescence intensity values were calculated and are shown relative to the control transfections (neg, adjusted to 1); data from four different donors are plotted and the p-value was calculated using the Mann-Whitney test.

Next, we investigated the impact of specific miRNAs on DC differentiation. We performed *in vitro* DC differentiation in the presence of sequence specific miRNA inhibitors of DC- and macrophage-specific miRNAs, miR-511 and miR-99b, and of a DC-specific miRNA, miR-193b (Figure 9). These miRNAs were selected for further analyses, because they had high expression values that were remarkably changed in the differentiation process. The transfection of miRNA inhibitors resulted in a 50–90% reduction in expression of specific miRNA (Figure 11A), which led to reduced DC-SIGN protein levels, especially in case of miR-511 and miR-99b (Figure 11B). The specific miRNA inhibitors did not influence the levels of two surface markers, CD14 and HLA-DR.

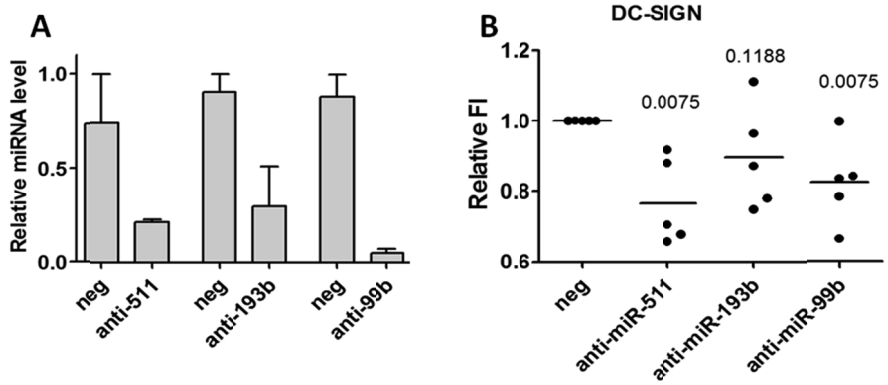

Figure 11. Reduced expression of specific miRNAs results in down-regulation of the DC-specific surface marker, DC-SIGN. (A) Inhibition of individual miRNAs is shown as the average miRNA expression level with S.E. of three different experiments normalised to the average levels of control transfections (neg, adjusted to 1). (B) FACS analyses of DCs treated with indicated miRNA inhibitors. Geometric mean fluorescence intensities were normalised to control transfected cells (neg, adjusted to 1) and are presented as relative fluorescence intensities (FI); data from five different donors are plotted and p-values were calculated using the Mann-Whitney test.

Data from the DICER1 knockdown and miRNA inhibition experiments showed that miRNAs, particularly miR-511 and miR-99b, impact DC-SIGN expression and thus are likely to affect DC differentiation.

5.2.3. Potential target genes of selected miRNAs

To determine the importance of selected miRNAs, we searched for the targets of miR-511, miR-99b and miR-193b using TargetScan. We selected the top third of the TargetScan predicted targets (context score <-0.2) and searched the list for immunologically important genes present in the Immport Database (https://www.immport.org) (4274 genes). In addition, we compiled a second list of potential targets using 5 different algorithms; Targetscan 5.1 (Friedman *et al.*, 2009), Miranda (John *et al.*, 2004), DIANA-microT (Maragkakis *et al.*, 2009), Pictar (Krek *et al.*, 2005) and rna22 (Miranda *et al.*, 2006). The putative targets were ranked by their position in the input lists and the top 500 genes were used in subsequent analysis. Genes with low expression were excluded. The remaining lists, which contained 135–247 targets for each studied miRNA, were analysed using the g:GOSt tool at the g:Profiler website (http://biit.cs.ut.ee/gprofiler/); g:GOSt retrieves most significant Gene Ontology (GO) terms, together with KEGG and REACTOME pathways data (Reimand *et al.*, 2007). Based in these analyses, miR-511 showed the greatest potential to target several pathways important in DC and macrophage differentiation, maturation and function (Table 5).

Table 5. Dendritic cell and macrophage-specific miRNAs potentially target genes from functional pathways or groups important for the differentiation or immune functions

miRNA	Nr of targets	Functional group and nr of genes		ID	Putative targets in functional group	Significance[b]	Method[a]
miR-511	135	Toll-like receptor signaling pathway	107	KEGG:04620	TLR4 STAT1 CD80 MAP3K7IP2 CD86 IRAK1 MAP3K7 TIRAP	2.56e-05	T
		Myeloid cell differentiation	93	GO:0030099	BCL6 IRF4 PPARG JAK2 SMAD5 TIRAP	4.11e-05	T
		JAK-STAT cascade	47	GO:0007259	NLK STAT1 SOCS2 JAK2 STAT5A SOCS6 STAT4	2.84e-08	T
		Regulation of interleukin-2 production	28	GO:0032663	IRF4 CD80 CD86 STAT5A MAP3K7	1.16e-06	T
		Cell adhesion	860	GO:0007155	ALCAM ERBB2IP ENTPD1 CD36 CDH2 COL8A2 NRP1 OLR1 LPP ITGA4 CCR1 CD93 ITGB1 RAPH1 ROCK1 CD44 CD9 CD84 VCL	5.61e-06	T
		Vesicle-mediated transport	570	GO:0016192	ZFYVE16 RAB22A CD36 FNBP1L MRC1 MRC1L1 ARFGEF1 RAB6A PICALM CD93 RAB2A PRKCI AP1S2 RIMS3	3.39e-05	T
		Pathways in cancer	334	KEGG:05200	BID STAT1 EP300 TGFBR1 HDAC2 PPARG ITGB1 STAT5A PTEN CBL IGF1R VHL	3.36e-04	T
		Cell migration	282	GO:0016477	BTG1 CDH2 TGFBR1 NRP1 ITGA4 ITGB1 IL16 SRF ROCK1 PTEN CD44 VHL	5.10e-07	T
		Regulation of transcription from RNA polymerase II promoter	732	GO:0006357	HIPK2 TIAL1 EP300 RYBP BCL6 AHR YY1 HDAC2 IRF4 PPARG STAT5A SRF SMAD5 IRF2 VHL LITAF	3.73e-05	T
	193	Negative regulation of gene expression	497	GO:0010629	ATBF1 CBX1 DEDD DEDD2 GABPA NAB2 ORC2L POU2F1 RUNX2 RYBP SATB2 SIN3A ZNF281 TARBP2 TGIF1 TNRC6B	2.29e-05	C
miR-193b	234/ 247	Chemokine signaling pathway	189	KEGG:04062	CRK CRKL KRAS SOS2	1.56e-06	T
		Chronic myeloid leukemia	76	KEGG:05220	CRKL CCND1 BCL2L1 RUNX1 CRK KRAS	5.23e-04	C
miR-99b	225	Beta-catenin binding	27	GO:0008013	NUMB DVL3 SMAD7 C5ORF22 AXIN1	8.49e-06	C
		Pathways in cancer	334	KEGG:05200	AXIN1 BID CBL CDK6 DVL3 FZD1 HSP90B1 IGF1R IKBKG ITGB1 STAT5B	7.03e-04	C

[a] The target lists used for analysis were generated based on the top third of Targetscan (T) or by compiling predictions from five different algorithms; Targetscan 5.1, Miranda, DIANA-microT, Pictar and rna22 (C).

[b] The p-value form Fisher exact test showing the significance of the overlap between the target list and indicated functional category.

Among the Targetscan predictions, TLR4 was one of the most highly scored (3 target sites, context score –0.89) miR-511 targets with expression in DCs. Therefore, we studied whether TLR4 mRNA and protein levels are influenced by miR-511. We followed miR-511 and TLR4 expression during the DC differentiation, where the miR-511 level increased during differentiation (Figure 12A). The total amount of TLR4 mRNA was relatively constant during the differentiation (Figure 12B), but the protein level increased gradually to 3.4 times compared to monocytes (Figure 12C).

To study the impact of multiple miR-511 sites on TLR4 expression, we inhibited miR-511 and measured the amount of TLR4 mRNA and protein in DC differentiation. The TLR4 protein levels were approximately 2-fold higher in control cells compared with miR-511 inhibited samples (Figure 12D), correlating with miR-511 accumulation in the cells (Figure 12A).

These data showed that there is correlation between the expression of miR-511 and an enhanced level of the TLR4 protein, suggesting that miR-511 is a positive regulator of TLR4 protein translation in monocyte-derived DCs.

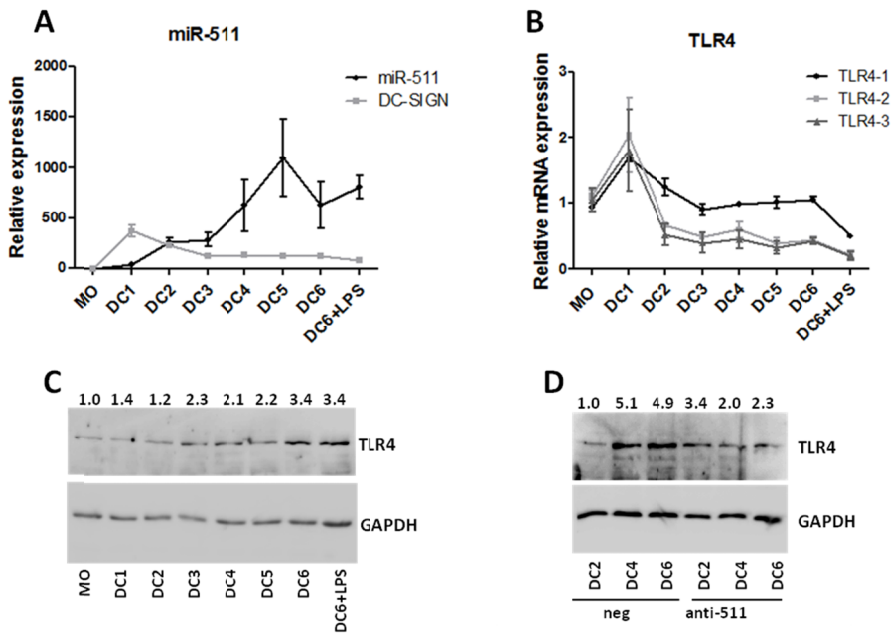

Figure 12. Expression of miR-511 correlates with an enhanced level of the TLR4 protein in differentiating DCs. (A) The RNA expression levels of miR-511, DC-SIGN, and (B) TLR4 transcripts in differentiating DCs. RT-PCRs of specific genes are normalised to either HPRT or let-7a and the expression levels in MOs (adjusted to 1). (C) Western blot analysis of TLR4 in differentiating DCs and (D) in the presence of the miR-511 inhibitor (anti-511) or the control inhibitor (neg) are normalised to GAPDH; the numbers indicate the fold difference compared to the MOs (adjusted to 1, C) or to transfected control cells on day 2 (DC2, neg, D).

5.2.4. The impact of miR-511 on TLR4

To further investigate the positive effect of miR-511 on TLR4 protein level, we looked whether TLR4 is directly targeted by miR-511 in LUC reporter assays. As TLR4 contains polyadenylation signals between each potential miR-511 target sequence, three different fragments of TLR4 3'UTR were inserted downstream of the LUC coding region in the vector and were transfected alongside either pre-miR-511 or control pre-miRNA into the HEK293 cells. In previous studies, it was shown that miRNAs upregulate translation of target mRNA when the cell cycle is arrested (Vasudevan *et al.*, 2007; Vasudevan *et al.*, 2008). As human blood monocytes do not proliferate during differentiation and we observed a positive correlation between miR-511 expression and the TLR4 protein level in differentiating DCs, we studied the cells in normal conditions and in contact-inhibited conditions, where cells are arrested in the G0 phase (Vasudevan *et al.*, 2008). In normal growth conditions, expression of LUC constructs with 3'UTR fragments containing the first and second predicted TLR4 target sites of miR-511 (TLR4 I and TLR4 II) were downregulated in the presence of miR-511 (Figure 13A). LUC expression levels of TLR4 I and TLR4 II were higher in contact-inhibited cells in the presence of transfected miR-511; and for the TLR4 III construct, LUC expression was significantly enhanced when compared to the control cells (Figure 13B).

These data indicate that miR-511, depending on the target mRNA 3'UTR and the conditions, can either up- or downregulate the expression of target genes.

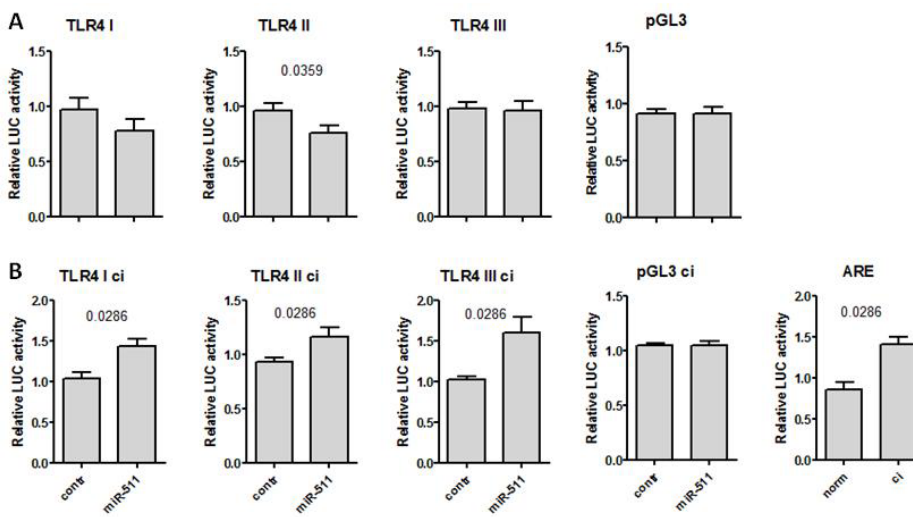

Figure 13. Determining the influence of miR-511 on expression of its proposed target using the LUC reporter assay. (A) The LUC activity measured in normal conditions after 48 h and (B) in contact-inhibited conditions after 96 hours following transfection of the LUC reporters and either pre-miR-511 or the control pre-miRNA. LUC activity of the ARE reporter was used as a positive control and pGL3 as a negative control. The data are displayed as the means with S.E.M. of at least eight different transfections.

5.3. Monocyte DNA methylation profile in ageing (Ref. III)

A diverse range of age-associated changes has been reported in human innate immune cells, which are important in increased susceptibility to infectious diseases and inflammatory pathology (Rymkiewicz *et al.*, 2012; Solana *et al.*, 2012). It is therefore important to study epigenetic changes occurring in these cells during ageing. To describe age-related DNA methylation profiles, we studied monocytes from the peripheral blood of eight young (age range 22–25 years, mean 23.75 years; 4 females and 4 males) and eight elderly healthy volunteers (age range 77–78 years, mean 77.13 years; 4 females and 4 males). A whole genome methylation analysis was performed using the Infinium HumanMethylation450 BeadChip (Illumina Inc.). Methylation at each CpG site is estimated by the Beta (β) value, ranging from 0 to 1, defined as the proportion of the methylated signal to the total signal and calculated from the normalised intensity values.

Table 6. Differentially methylated sites in young versus old monocyte cell populations

Direction of methylation	Target ID	β-difference*	Adjusted p-value	Gene
Hypo-methylated	cg10501210	**-0.38**	**0.002884**	***miR-29b-2*****
	cg24892069	**-0.30**	**0.00312**	**NRP1**
	cg27209729	**-0.30**	**0.020571**	**NRXN2**
	cg11807280	-0.27	0.013523	
	cg08128734	-0.27	0.015965	RASSF5
	cg11693709	-0.26	0.024853	PAK6
	cg18826637	-0.25	0.022927	
	cg00329615	-0.25	0.012874	IGSF11
	cg00740914	-0.25	0.008061	
	cg03873281	-0.25	0.005647	PDLIM4
	cg13039251	-0.23	0.008793	PDZD2
	cg07583137	-0.23	0.007904	CHMP4C
	cg12317815	-0.22	0.014773	ASPA
	cg06781608	-0.22	0.030063	PTPRN2
	cg13001142	-0.22	0.011913	STXBP5
	cg16932827	-0.21	0.015062	
	cg19344626	-0.21	0.010547	NWD1
	cg14295611	-0.21	0.04857	
	cg14614643	-0.21	0.008462	
	cg03915012	-0.21	0.026415	GAK
	cg03473532	-0.20	0.035003	MKLN1
Hyper-methylated	cg19907915	0.21	0.014934	IGSF9B
	cg20665157	0.22	0.008629	CADPS2
	cg21184711	0.23	0.024671	CADPS2
	cg04875128	0.30	0.017558	OTUD7A
	cg02978201	0.47	0.015062	PRM1

The three replicated CpG loci are shown in bold.

* β-difference is the difference of β-values. A negative β-difference indicates hypomethylation in the elderly and a positive β-difference indicates hypermethylation in the elderly. Only CpG sites with FDR-adjusted p-values of less than 0.05 were considered to be differentially methylated.

**approximately 1 kb upstream

Altogether, we found 368 CpG sites that were significantly differentially methylated ($p<0.05$), from which 26 CpG sites had an absolute β value difference greater than or equal to 0.2 between the young and old individuals (Table 6). Most of the CpG sites, a total of 21 positions, were hypomethylated in the elderly individuals; only five positions were hypermethylated in these individuals. Decreased methylation during the ageing process has been previously described in a study of PBMCs (Johansson *et al.*, 2013). The most significantly altered sites mapped within the *NRP1, NRXN1, RASSF5, OTUD7A* and *PRM1* genes. The loci that did not reach the 0.2 β-difference threshold but were significantly different ($p<0.05$) included two *ELOVL2* sites, cg16867657 and cg24724428 (both with β-diff. of 0.17); two *FHL2* sites, cg22454769 and cg24079702 (β-diff. of 0.15 and 0.14, respectively); and a *PENK* site, cg16419235 (β-diff. of 0.08); all these sites are associated with increased methylation in the peripheral blood mononuclear cells of older individuals (Garagnani *et al.*, 2012; Johansson *et al.*, 2013).

To validate our results, we focused our investigation on the three differentially methylated CpG sites with the highest hypomethylation values, cg24892069, cg27209729 and cg10501210. cg24892069, which had a very low standard deviation in both age groups (young STDEV: 0.05; old STDEV: 0.06), is located in the gene body of the neuropilin 1 (*NRP1*) gene. We replicated the array results of the three differentially methylated loci using the EpiTYPER assay (Sequenom Inc.) with a separate set of young and old samples. To our analyses, we added two sex-matched age groups consisting of 10 young (age range 24–28 years, mean 26.4 years; 5 men and 5 females) and 10 elderly (age range 76–84 years, mean 79.4 years; 5 men and 5 females) samples. Using the EpiTYPER assay, we found hypomethylation of the NRP1-associated cg24892069 site in the monocytes of the older individuals, similar to the results from the HumanMethylation450 BeadChip analysis (Figure 14A). We also analysed the methylation differences in men and women separately and we observed a significant difference in both gender groups (both $p<0.0001$) (Figure 14A). To explore this region further, we selected another CpG site, cg24892069-40bp, which was located 40 bp upstream of the cg24892069 site in the genomic sequence; this site was not included on the methylation BeadChip. We found that the cg24892069-40bp site had a statistically significant methylation difference between the studied age groups ($p<0.0001$) that was observed in both sexes ($p<0.0001$) (Figure 14B). The similar DNA methylation pattern of the two CpG sites in close proximity is most likely the result of a shared, differentially methylated, region that is modified from the nearby methyltransferase binding site. We also found significant differences between the age groups at the cg27209729 and cg10501210 sites, located in the *NRXN2* gene and upstream of the miR-29b-2 gene, respectively (Figure 14C&D). Both of these CpG sites had statistically significant methylation differences in the combined study group ($p<0.0001$) and in the male and female study group ($p<0.001$ and $p<0.01$ in NRXN2 and $p<0.0001$ in miR-29b-2) (Figure 14C&D).

In summary, we demonstrated differentially methylated DNA patterns in monocytes of young and elderly individuals. In elderly most of the altered CpG sites were hypomethylated and three most hypomethylated CpG sites located in or near relevant genomic loci.

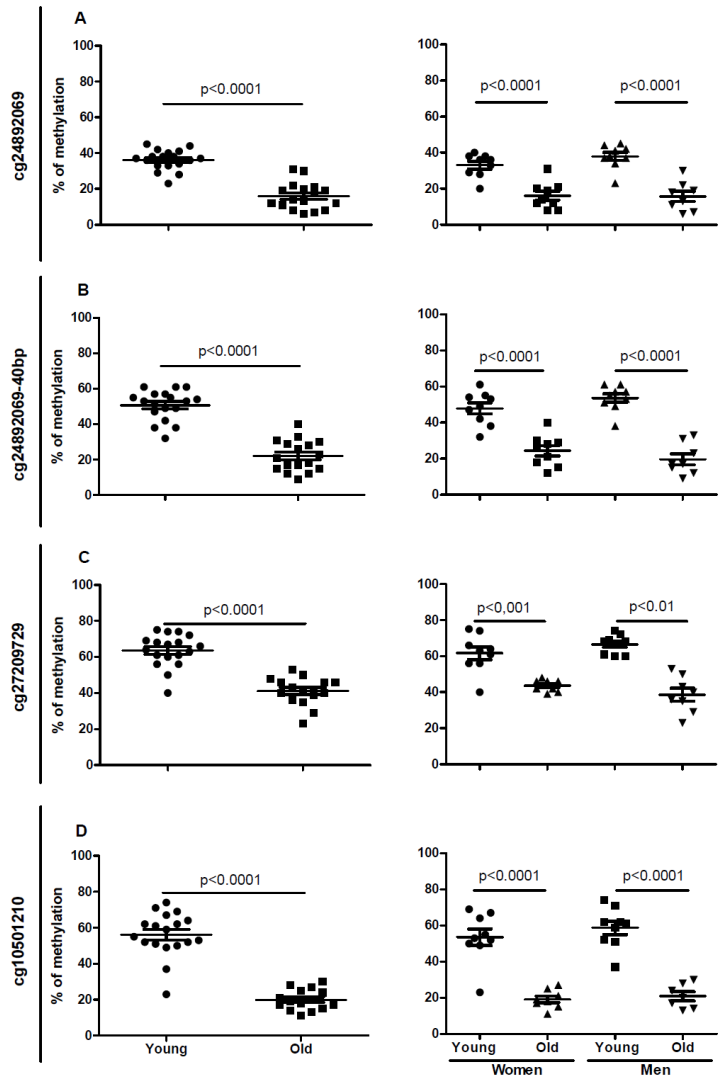

Figure 14. The percentage of CpG site methylation that differs between young and elderly individuals (left panel) and between young and elderly men and women (right panel). (A) cg24892069 (*NRP1*), (B) cg24892069-40bp (*NRP1*), (C) cg27209729 (*NRXN2*) and (D) cg10501210 (miR-29b-2). The mean (±S.E.M.) methylation difference measured in young and old individuals was measured using Sequenom's EPITYPER assay.

6. DISCUSSION

6.1. Histone modification differences in monocytes, monocyte-derived macrophages and dendritic cells (Ref. I)

Increasing evidence shows that epigenetic modifications can influence gene expression profiles without affecting the DNA sequence. Recent studies have demonstrated that epigenetic mechanisms are involved in the inheritance of gene expression patterns (Sharma *et al.*, 2010) and in mechanisms of cellular differentiation (Wei *et al.*, 2009). In this study, we examined three aspects of the monocyte and monocyte-derived macrophage and DC system, histone modifications, microRNAs and also DNA methylation changes that occur during ageing.

Our genome-wide correlation of mRNA expression profiles with three major histone modifications was the first large scale study of gene expression associations with histone modifications in monocytes, macrophages and DCs; our derived expression profile adds to previous studies in other immune cells (Roh *et al.*, 2006; Lehtonen *et al.*, 2007; Araki *et al.*, 2009; Wei *et al.*, 2009; Ghisletti *et al.*, 2010). The gene expression profiles of monocytes and monocyte-derived DCs have since been described in several other studies that considered different aspects that affect these cells; for example, health and disease, stimulation, differentiation and cell type (Robbins *et al.*, 2008; Bullwinkel *et al.*, 2011; Frankenberger *et al.*, 2012; Haniffa *et al.*, 2012; Lundberg *et al.*, 2013). Our results showed that the overall gene expression profiles of studied monocytes, monocyte-derived macrophages and DCs were similar but that there were some important differences that help to define the cell types. We observed greater differences between monocytes and DCs and between monocytes and macrophages than between DCs and macrophages. This is in concordance with the fact that although both DCs and macrophages have gone through separate differentiation processes, they have developed from the same cell type.

We analysed whether observed differences in gene expression profiles between monocytes and monocyte-derived cells could be the consequence of divergent epigenetic backgrounds. First, we investigated the histone modifications in promoter regions, as it has been shown that they can regulate gene expression (Kouzarides, 2007). Histone marks H3K4me3 and H3K27me3 are usually enriched on active and inactive chromatin regions, respectively (Azuara, 2006; Jiang *et al.*, 2011). The most predominant histone mark in our study was H3K4me3, and it was commonly coupled with AcH3. H3K4me3 was also the most frequently found promoter modification indentified in genome-wide studies from embryonic stem cells, $CD4^+$ and $CD8^+$ T cells (Roh *et al.*, 2006; Guenther *et al.*, 2007; Zhao *et al.*, 2007; Araki *et al.*, 2009; Wei *et al.*, 2009; Tian *et al.*, 2011).

When studying the dynamics of histone modifications, we noticed a gradual decrease of the H3K4me3 mark in active genes during the differentiation of monocytes to macrophages and DCs. This suggests that a subset of genes lose their H3K4me3 mark during differentiation and that histone modifications may be highly dynamic during differentiation. Others have reported that there is a change towards more inactive chromatin when monocytes are differentiated into DCs *in vitro* in the presence of Toll-like receptor ligands (Huang *et al.*, 2012). We found that the H3K4me3 and AcH3 marks are unstable when they occur alone but are stable when in combination of in gene promoters. The overall decrease of the H3K4me3 mark and the increase of genes with the H3K27me3 mark in the DC population suggest that the *in vitro* differentiation induces a global shift towards less active chromatin. Therefore, monocyte differentiation to macrophages and DCs likely requires a different pattern of histone modifications to regulate gene expression.

In monocyte-derived DC differentiation, the role of histone modifications has been shown in the regulation of the DC-SIGN gene (Bullwinkel *et al.*, 2011). In addition, many other important immunorelated genes, including monocyte chemotactic protein-1 (MCP-1), TNF-α, IL-12 and IL-4, are regulated by changes in histone modifications (Fields *et al.*, 2002; Barthel and Goldfeld, 2003; Boekhoudt *et al.*, 2003; Wen *et al.*, 2008). At the single gene level, we found many differences in histone modification profiles that correlate with transcriptional changes. We detected a strong correlation between the H3K4me3 mark and the transcriptional activity of a gene. As expected, the expression levels of many marker genes correlated with the presence of active histone modifications. Similar to marker genes, genes associated with antigen presentation activity and phagocytosis had high levels of expression and had either the H3K4me3 or the AcH3 mark.

6.2. miR-511 affects the differentiation of DC and regulates positively its putative target TLR4 (Ref. II)

To further study differences between monocytes, monocyte-derived macrophages and DCs, we analysed the microRNA expression profiles of these cells. The importance of miRNAs in the immune system has been described in several different studies: miR-146 regulates the inflammatory responses in many different cell types through the suppression of genes in the NF-κB pathway (Taganov *et al.*, 2006; De Benedetto *et al.*, 2009; Jurkin *et al.*, 2010); miR-155 has a pro-inflammatory role in mouse DCs, macrophages, B and T cells (O'Connell and Keegan, 2006; O'Connell *et al.*, 2007; Rodriguez *et al.*, 2007); miR-34 and miR-21 function in human monocyte-derived DCs by targeting the mRNAs encoding Jagged1 and WNT1 (Hashimi *et al.*, 2009); and miR-21 functions in mouse macrophages by negatively regulating TLR4 (Sheedy *et al.*, 2010). In addition, several other miRNAs are involved in regulation of components of the TLR signalling system (O'Neill *et al.*, 2011). In our study, we

have described updated miRNA expression profiles of human blood monocyte-derived immature and mature DCs and macrophages. Consistent with previous studies (Landgraf *et al.*, 2007; Lehtonen *et al.*, 2007; Ceppi *et al.*, 2009; Hashimi *et al.*, 2009; Jin *et al.*, 2010), we found that these cell types have distinct miRNA expression profiles and we detected several novel strongly up-regulated miRNAs, including miR-511.

DC-SIGN is a marker involved in DC trafficking and T-cell contact establishment (Banchereau *et al.*, 2000; Geijtenbeek *et al.*, 2000), whereas CD14 is important in monocyte function and mediates the innate immune response to bacterial lipopolysaccharides and thereby intracellular effects (Wright *et al.*, 1990). In the process of monocyte differentiation into DCs, CD14 expression is replaced by DC-SIGN expression and the variation of this replacement indicates a deviation in the differentiation process (Mirghomizadeh *et al.*, 2009); this has been shown in the case of miR-99b, miR-21, miR-34a and miR-155 (Hashimi *et al.*, 2009; Martinez-Nunez *et al.*, 2009; Cekaite *et al.*, 2010). In addition, miR-146a is known to inhibit the maturation of monocyte-derived DC differentiation (Du *et al.*, 2012). In our study, the knockdown of DICER1 with siRNAs resulted in lower expression of DC-SIGN and a higher level of monocyte marker CD14, confirming that miRNAs are needed for proper differentiation of DCs. Similarly, the specific inhibition of miR-511 and miR-99b resulted in a reduction of DC-SIGN, indicating that these two miRNAs influence DC differentiation. In the Illumina miRNA array analyses, only one strand of miR-511 (miR-511-5p) was included. In a study of mouse macrophages, it was shown using sensistive reporter vectors that miR-511-3p is the biologically bioactive strand (Chang *et al.*, 2012; Squadrito *et al.*, 2012). As miR-511-3p was not present in our study, we cannot assess its differential expression in human cells. The miRNA strand selection mechanisms are still unclear but some studies have proposed that it may depend on cell type, species and the sequence context (Biasiolo *et al.*, 2011; Kuchenbauer *et al.*, 2011).

In humans and in mice, the miR-511 gene is encoded in the mannose receptor gene MRC1, which is mainly expressed in DCs, macrophages and subsets of vascular and lymphatic endothelial cells (Taylor *et al.*, 2005; Kerrigan and Brown, 2009). It is thought that miR-511 and MRC1 may be co-ordinately regulated, and studies show that the functional activities are correlated (Squadrito *et al.*, 2012). In the computational prediction of miRNA targets, we found that genes from ontology groups and pathways with immune functions are overrepresented in the list of miRNA-511 potential targets. In this study, we examined the possible impact of miR-511 on its proposed target TLR4. In contrast to the expected result, we found that knockdown of miR-511 enhanced TLR4 protein levels in differentiating DCs. In cell cycle arrest at the G0 phase miRNAs can enhance the target protein level (Vasudevan *et al.*, 2007; Vasudevan *et al.*, 2008). Although human monocytes can differentiate into DCs, they do not proliferate and are cell cycle arrested. The alternative possibility is that miR-511 competes with degradation factor, as has been shown for miR-466, which replaces tristetraprolin (TTP) protein and therefore upregulates

the mRNA and protein level of IL-10 (Ma *et al.*, 2010). In some cases, the mechanism of expression enhancement is not clear, such as for miR-155, which has a stabilising influence on TNF-α mRNA in macrophages (Tili *et al.*, 2009; Bala *et al.*, 2011). The influence of miR-511 on TLR4 protein expression seems to be direct, as the presence of miR-511 in HEK293 cells reduced Luciferase levels for TLR4-I and TLR4-II in normal conditions and enhanced the LUC level for TLR4-III in contact inhibited cells.

6.3. Distinct age-related methylation patterns in human monocytes (Ref. III)

We also studied changes in DNA methylation that occur in monocyte cells during ageing as ageing affects many components of the immune system, including the innate and adaptive immune responses. A diverse range of age-associated changes has been reported in human innate immune cells, which are important during the early response to pathogens. Although the number of monocytes does not change significantly during ageing, several functional age-related changes have been reported, such as the altered expression of cytokines, defective Toll-like receptor signalling and a decreased capacity for phagocytosis. Changes in DNA methylation patterns occur gradually throughout an individual's lifespan (Bell *et al.*, 2012; Garagnani *et al.*, 2012) and may result in the age-related phenotypes of a specific set of genes (Bell *et al.*, 2012). Correlation between gene expression and methylation has been shown in studies of lymphoblastoid cell lines and monocytes that incorporated 77 and 1264 subjects, respectively (Bell *et al.*, 2011; Liu *et al.*, 2013). The same methylation patterns evident in ageing also appear in cancer development (Esteller, 2008).

In our study of DNA methylation in ageing, we found 368 CpG sites that were significantly differentially methylated, of which 26 CpG sites had absolute β value differences greater than or equal to 0.2 in the young and old individuals; most of the CpG sites were hypomethylated in the elderly individuals. Decreased methylation during the ageing process has been previously described in a study of PBMCs (Johansson *et al.*, 2013). However, PBMCs are a mixture of different cell types, and the observed changes in DNA methylation may result from age-related changes in cell composition. It has been shown that DNA methylation depends on the cell composition (Reinius *et al.*, 2012; Liu *et al.*, 2013). From the 26 CpG sites, the most significantly changed loci mapped within the *NRP1, NRXN1, RASSF5, OTUD7A* and *PRM1* genes. The significantly different loci included sites are associated with increased methylation in peripheral blood mononuclear cells from older individuals; *ELOVL2* sites cg16867657 and cg2472442; *FHL2* sites cg22454769 and cg24079702 and *PENK* site cg16419235 (Garagnani *et al.*, 2012; Johansson *et al.*, 2013). We validated our results on the three differentially methylated CpG sites with the highest hypomethylation values, cg24892069, cg27209729 and cg10501210. These three sites are located in or near immunologically important genes,

cg24892069b in the *NRP1* gene body, cg27209729 in the *NRXN2* gene body, and cg10501210 ~1000 bp upstream of the miR-29b-2 gene. NRP1 is a cell surface receptor with functional roles in several biological processes, including angiogenesis, immune response, the regulation of vascular permeability, development of the nervous system (Miao *et al.*, 2000; Geretti *et al.*, 2007). NRP1 has also been associated with increased tumour angiogenesis and cancer progression (Bielenberg *et al.*, 2006; Bagri *et al.*, 2009). In our previous mRNA expression study, NRP1 was expressed at low levels in monocytes and demonstrated a significantly increased expression in monocyte-derived DCs and macrophages (Paragraph 0). NRP1 is also expressed in regulatory T cells (Bruder *et al.*, 2004) and is needed for prolonged cellular contact between regulatory T-cells and DCs (Sarris *et al.*, 2008). Another CpG site, cg27209729, is located in the body of the neurexin 2 (*NRXN2*) gene. NRXN2 is a member of the neurexin family, which affects synaptic plasticity and cognitive functioning (Rozic *et al.*, 2012), and has been linked to autism spectrum disorders and schizophrenia (Gauthier *et al.*, 2011). The third CpG site, cg10501210, is located ~1,000 bp upstream of the miR-29b-2 gene. miR-29b-2 belongs to the miR-29 family, which is important in thymic involution (Papadopoulou *et al.*, 2012), T cell polarisation (Ma *et al.*, 2011) and oncogenesis (Santanam *et al.*, 2010; Papadopoulou *et al.*, 2012).

DNA methylation in gene promoters is usually associated with transcriptional silencing (Thomson *et al.*, 2010), while methylation in the gene body is associated with transcriptional activation (Portela and Esteller, 2010). Many studies have shown that men a have higher global DNA methylation level than women (El-Maarri *et al.*, 2007; Zhu *et al.*, 2010; El-Maarri *et al.*, 2011; Zhang *et al.*, 2011), but we could not detect this difference in our study of CpG sites. Global DNA methylation can also be influenced by demographic, environmental and behavioural risk factors; for example, ethnicity (Axume *et al.*, 2007; Terry *et al.*, 2008), air pollution (Baccarelli *et al.*, 2009; Tarantini *et al.*, 2009), smoking (Philibert *et al.*, 2009; Breitling *et al.*, 2011), alcohol (Bonsch *et al.*, 2004; Bleich *et al.*, 2006; Hamid *et al.*, 2009) and dietary preferences (Kok *et al.*, 2007; Heijmans *et al.*, 2008). Despite these reports, there are large scale studies showing that basic epigenetic features are common across sex and ethnicities (Liu *et al.*, 2013), suggesting that sample size and composition can significantly affect reported correlations.

6.4. Closing remarks

In this thesis, we provide a large-scale epigenetic characterisation of changes that occur in the differentiation of monocyte-derived macrophages and DCs. We described distinct gene expression, histone modification and microRNA profiles in monocytes, monocyte-derived macrophages and DCs. Gene expression profiles correlated with histone modification patterns. A novel macrophage and DC specific miRNA, miR-511, which has strong immunoregulatory potential, was

described. In addition, we found several differentially methylated CpG sites in the monocytes of elderly individuals.

Our findings form a good basis for further epigenetic studies in monocytes and monocyte-derived cells and aid in the understanding of the novel mechanisms of gene regulation that have roles in differentiation. Our results may be useful to better understand the role of epigenetics in ageing and to provide new approaches to control inflammatory processes.

7. CONCLUSIONS

1. Monocytes, macrophages and dendritic cells have different gene expression and histone modification patterns. In the studied cells, the most prevalent histone mark is H3K4me3, but there were changes in the overall histone mark pattrens during the differentiation process. Histone marks can be correlated with gene transcriptional activity and functional properties.
2. We have determined updated miRNA expression profiles for human blood monocytes, macrophages and dendritic cells and have found miR-511 to be a highly expressed macrophage and dendritic cell specific miRNA with strong immunoregulatory potential. In addition, our data highlight that the cellular environment can modulate the influence of miRNAs.
3. Purified monocytes from young and elderly individuals have differentially methylated DNA patterns; most of the altered CpG sites were hypo-methylated in the elderly. We determined the three most frequently hypo-methylated CpG sites located in or near relevant genomic loci.

SUMMARY IN ESTONIAN

Monotsüütide, monotsüütidest pärinevate makrofaagide ja dendriitrakkude epigeneetilised profiilid

Inimese immuunsüsteem koosneb kaasasündinud ning omandatud immuunsusest, millest esimene tagab kiire, kuid mitte nii spetsiifilise reaktsiooni patogeenide vastu, ning teine väga spetsiifilise immuunvastuse ning immunoloogilise mälu. Sillana kahe erineva immuunsüsteemi osa vahel toimivad dendriitrakud, mis on peamised antigeeni esitlevad rakud immuunsüsteemis. Dendriitrakud tunnevad patogeenid ära spetsiifiliste ohuretseptorite abil, esitavad antigeene T rakkudele ja seeläbi vallandavad omandatud immuunvastuse ning vahendavad erinevate immuunrakkude ja antikehade abil organismi puhastamist patogeenidest. Koostöös monotsüütide, makrofaagide ning teiste immuunrakkudega tagavad dendriitrakud organismi homöostaasi.

Normaalsetes tingimustes ei läbi dendriitrakud ja makrofaagid diferentseerumisel monotsüüdi etappi, kuid põletikulistes tingimustes toimub dendriitrakkude ja makrofaagide arvu kiire kasv just monotsüütide kaudu, mis värvatakse kiiresti põletikukoldesse. Dendriitrakkude uurimist raskendab asjaolu, et nende kontsentratsioon inimese perifeerses veres on väga madal (< 1 % mononukleaarsetest rakkudest). *In vitro* tingimustes saab dendriitrakke ja makrofaage diferentseerida monotsüütidest, kasutades granulotsüüdi makrofaagi kolooniat stimuleerivat faktorit (*granulocyte macrophage colony-stimulating factor* - GM-CSF) ja interleukiin 4 (IL-4). Selliseid rakke kasutatakse laialdaselt erinevates uuringutes, kuna neid on suhteliselt lihtne suurtes hulkades toota.

Epigeneetika on teadus, mis üritab selgitada, kuidas piiratud arv geene suudab määratleda diferentseerumist erinevateks rakutüüpideks ning kuidas fenotüüp pärandatakse ühelt tütarrakult teisele, sealjuures ilma DNA järjestust muutmata. Epigeneetiliste modifikatsioonide tähtsus geeniekspressiooni regulatsioonis on viimasel ajal väga palju tähelepanu pälvinud valdkond. Epigeneetiliste modifikatsioonide rolli tähtsust on näidatud mitmete erinevate protsessides, nagu rakutsükli regulatsioon, organismi areng ja vananemine ning X kromosoomi inkativatsioon. Käesolev uurimistöö uurib monotsüüte ning monotsüütidest diferentseeritud makrofaage ja dendriitrakke kolmest erinevast aspektist: histooni modifikatsioonide seos geeniekspressiooniga, mikroRNAde (miRNAd) ekspressiooni mõju nimetatud rakkudes ning DNA metülatsiooni monotsüütides vananemisel.

Käesolev ülegenoomi töö on oluline lisa eelnevalt avaldatud mRNA ja miRNA ekspressiooni ja histooni modifikatsioone käsitletavatele uuringutele immuunsüsteemi rakkudes. Uurimaks ekspressioonimustrite ja epigeneetiliste modifikatsioonide vahelisi seoseid, analüüsisime geenide promootorite regioonides kolme kõige levinumat histooni modifikatsiooni, millest H3K4m3 ja AcH3 tähistavad aktiivset ja H3K27me3 inaktiivset geeni. Kõige levinum uuritud modifikatsioonidest oli aktiivne märgis H3K4me3, mis tihtipeale esines koos märgisega AcH3. Täheldasime, et histooni modifikatsioonid üksinda ei ole

väga stabiilsed ning kaotavad olemasoleva või omandavad uue modifikatsiooni makrofaagide ja dendriitrakkude diferentseerumise käigus. Uurides eraldi aktiivseid ja inaktiivseid geene, nägime suurepärast korrelatsiooni geeni aktiivuse ja histooni märgiste vahel, kus inaktiivsed geenid olid tähistatud pigem märgisega H3K27me3 ning aktiivsed geenid märgistega H3K4me3 ja AcH3. Kuna fagotsütoos ja antigeeni esitlemine on monotsüütide ja monotsüütidest diferentseeritud rakkude peamised funktsioonid, siis uurisime ka geene, mis on seotud nende võtmefunktioonidega. Ka siin nägime väga head korrelatsiooni nimetatud funktsioonide, geeni aktiivuse ja histooni modifikatsioonide vahel.

miRNAd on väikesed mittekodeerivad RNA molekulid, mis reguleerivad märklaudgeeni ekspressiooni taset rakus. miRNAde rolli olulisust on näidatud mitmetes erinevates bioloogilistes protsessides, sealhulgas ka immuunsüsteemi funktsioneerimisel. Kooskõlas eelnevate uuringutega kirjeldasime nii ebaküpsete kui ka küpsete dendriitrakkude ja makrofaagide miRNA ekspressiooni profiilid ning leidsime mitmeid uusi ülesreguleeritud miRNAsid, sealhulgas varem kirjeldamata miR-511. Kasutades miRNA protsessinguks vajaliku faktori DICER1 ja spetsiifiliste miRNAde vaigistamist, näitasime, et miRNA-511 ning ka miR-99b mõjutavad dendriitrakkude diferentseerumist. miR-511 potentsiaalsete märklaudade hulka kuuluvad mitmed immunoloogiliste funktsioonidega geenid. Uurisime ühte parima skooriga miR-511 võimaliku märklaua TLR4 mRNA ekspressiooni ja valgu taset ning leidsime, et mittejagunevates dendriitrakkudes miR-511 inhibitsiooni tulemusena tema märklaudgeeni TLR4 ekspressiooni tase suureneb.

Edasi vaatasime monotsüütide rakupopulatsioonis DNA metülatsiooni noortel ja vanadel indiviididel. Monotsüütide arv vananemise käigus küll oluliselt ei muutu, kuid erinevusi on täheldatud nende funktsioonides. DNA metülatsiooni muutused toimuvad järk-järgult kogu inimese eluea jooksul ning aitavad kaasa teatud geenide vanusest sõltuva fenotüübi tekkele. Leidsime oma uuringus 368 CpG saiti, mis olid statistiliselt erinevalt metüleeritud, kusjuures 26 CpG saiti olid sellised, mille β väärtuse erinevus oli suurem või võrdne 0,2-ga noorte ja vanade populatsiooni vahel. Kooskõlas eelnevalt avaldatud DNA metülatsiooni uuringuga perifeerse vere mononukleaarsetes rakkudes, nägime enamusel vanusegruppide vahel olulisi erinevusi antud aladel hüpometülatsiooni vanades indiviidides. Kõige enam hüpometüleeritud CpG aladel teostasime ka tulemuste valideerimise, kus nägime suuremat metülatsiooni erinevust nii naiste kui meeste populatsioonis.

Kokkuvõtteks tõestavad saadud tulemused, et monotsüütide diferentseerimisel makrofaagideks ja dendriitrakkudeks leiavad aset mitmed epigeneetilised muutused. Kirjeldasime uuritud rakkude erinevat geenikspressiooni, histooni modifikatsioone ning miRNA profiilid. Nägime suurepärast korrelatsiooni geenikspressiooni ning histooni modifikatsioonide vahel ning kirjeldasime uue dendriitraku ja makrofaagi spetsiifilise miRNA, miR-511. Samuti leidsime mitmeid erinevalt metüleeritud CpG alasid noortes ja vanades indiviidides. Kokkuvõttes on käesolev töö erinevate epigeneetiliste muutuste põhjalik kirjeldus, mille alusel on võimalik avastada uusi geeniregulatsiooni mehhanisme ning

nende osa makrofaagide ja dendriitrakkude diferentseerumises. Lisaks näitavad meie tulemused, et vananemisel leiavad aset epigeneetilised muutused monotsüütides, mis võivad aidata kaasa uute põletikulisi protsesse kontrollivate ravimeetodite väljatöötamisel tulevikus.

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PUBLICATIONS

CURRICULUM VITAE

Name: Liina Tserel
Date of birth: 21.01.1984, Valga, Estonia
Citizenship: Estonian
Address: Molecular Pathology, Institute of Biomedicine and Translational Medicine, University of Tartu, Ravila 19–1036, 50411, Tartu; Estonia
Telefon: +372 737 4052
E-mail: liina.tserel@ut.ee

Education:

1990–1999	Valga Secondary School
1999–2002	Valga Gymnasium – *silver medal*
2002–2005	University of Tartu, Bachelor's degree in Natural Sciences, Gene Technology
2005–2007	University of Tartu, *magister scientiarum*, Biomedicine
2008–...	University of Tartu, PhD student

Professional employment:

2005–2007	Quattromed AS; technician
2006–2007	University of Tartu, Faculty of Medicine, Department of Pathological Anatomy and Forensic Medicine, Chair of Pathological Anatomy; technician
2007–2012	University of Tartu, Faculty of Medicine, Institute of General and Molecular Pathology, Molecular pathology study group; laboratory specialist
2012–...	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine (until May 2013 Institute of General and Molecular Pathology), Molecular pathology study group; research fellow of molecular immunology

Teadusorganisatsiooniline ja -administratiivne tegevus:

2010–...	Member of Estonian Society for Allergology and Immunology
2009–...	Member of Estonian Biochemical Sociaty
2007–...	Member of Estonian Human Genetics Association

Publications:

1. Tserel, Liina; Limbach, Maia; Saare, Mario; Kisand, Kai; Metspalu, Andres; Milani, Lili; Peterson, Pärt (2014). CpG sites associated with NRP1, NRXN2 and miR-29b-2 are hypomethylated in monocytes during ageing. Immun Ageing. 2014 Jan 9;11(1):1.
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Inventions:

Invention: MicroRNA miR-511 as diagnostic marker and gene therapy target in cancer and immune system related diseases; Owner: University of Tartu; Authors: Ana Rebane, Liina Tserel, Toomas Runnel, Kai Kisand, Maire Pihlap; Priority number: P201100037; Priority date: 16.05.2011

ELULOOKIRJELDUS

Nimi: Liina Tserel
Sünniaeg: 21.01.1984, Valga, Eesti
Kodakondus: Eesti
Aadress: Molekulaarpatoloogia, Bio- ja siirdemeditsiini instituut, Tartu Ülikool, Ravila 19–1036, 50411, Tartu; Eesti
Telefon: +372 737 4052
E-post: liina.tserel@ut.ee

Haridus:

1990–1999	Valga II Põhikool
1999–2002	Valga Gümnaasium- hõbemedal
2002–2005	Tartu Ülikool, loodusteaduste bakalaureusekraad, geenitehnoloogia
2005–2007	Tartu Ülikool, *magister scientiarum,* biomeditsiin
2008–...	Tartu Ülikool, arstiteaduse doktoriõpe

Teenistuskäik:

2005–2007	Quattromed AS; Laborant
2006–2007	Tartu Ülikool, Arstiteaduskond, Patoloogilise anatoomia ja kohtuarstiteaduse instituut, Patoloogilise anatoomia õppetool; laborant
2007–2012	Tartu Ülikool, Arstiteaduskond, Üld- ja molekulaarpatoloogia instituut, Molekulaarpatoloogia uurimisgrupp; laborispetsialist
2012–...	Tartu Ülikool, Arstiteaduskond, Bio- ja siirdemeditsiini instituut (kuni mai 2013 Üld- ja molekulaarpatoloogia instituut); molekulaarse immunoloogia teadur

Teadusorganisatsiooniline ja -administratiivne tegevus:

2010–...	Eesti Immunoloogide ja Allergoloogide Seltsi liige
2009–...	Eesti Biokeemia Seltsi liige
2007–...	Eesti Inimesegeneetika Ühingu liige

Publikatsioonid:

1. Tserel, Liina; Limbach, Maia; Saare, Mario; Kisand, Kai; Metspalu, Andres; Milani, Lili; Peterson, Pärt (2014). CpG sites associated with NRP1, NRXN2 and miR-29b-2 are hypomethylated in monocytes during ageing. Immun Ageing. 2014 Jan 9;11(1):1.
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